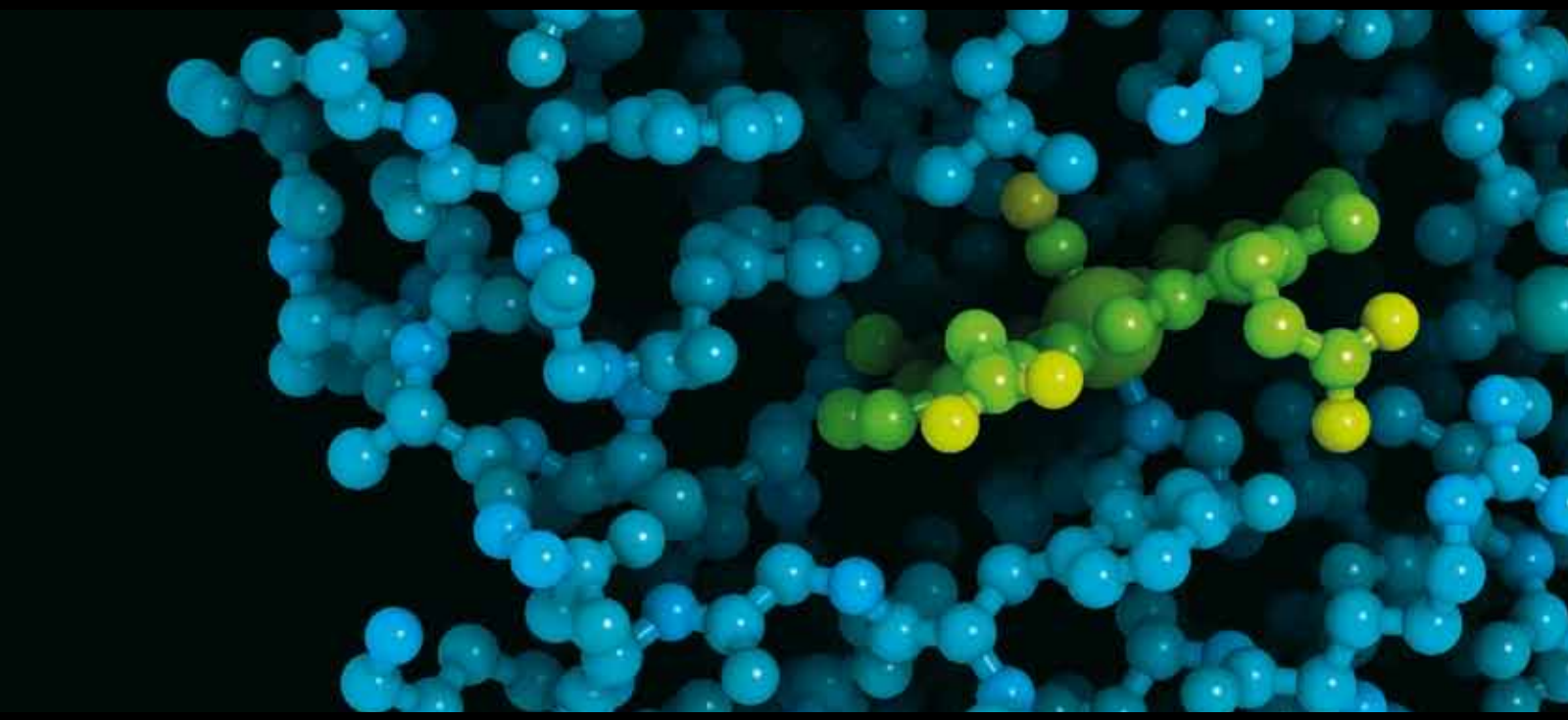
A Molecular, Genetic, and Diagnostic Spotlight on Fanconi Anemia

Guest Editors: Laura E. Hays, Stefan Meyer, and Henri J. van de Vrugt



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Editorial Board

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Editorial

A Molecular, Genetic, and Diagnostic Spotlight on Fanconi Anemia

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Eighty-five years ago Dr. Guido Fanconi described a family with three brothers with microcephaly, hyperpigmentation of the skin, hypoplasia of the testes, and who developed a lethal anemia between the ages of 5 and 7 [1, 2]. Since 1931, patients with this distinctive combination of clinical features have been classified as having Fanconi anemia (FA). To date, diagnosis of this disease, which as we know can manifest with variable clinical presentation, is based on increased chromosomal breakage in response to DNA crosslinking agents.

In addition to anemia, FA patients are highly predisposed to leukemia, head and neck squamous cell carcinoma, and gynecological cancers [3]. FA is caused by biallelic or X chromosome-linked mutations in one of 15 different genes, eight of whose gene products (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) form a nuclear core complex that must be intact to ubiquitinate FANCD2 and FANCI. The ubiquinated FANCD2/FANCI heterodimer then functionally interacts with downstream FA proteins (FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, FANCO/RAD51C, and FANCP/SLX4) to mediate DNA damage responses [4].

This special issue provides a wide range of scientific papers and reviews that examine in detail techniques for diagnosis of FA, molecular and genetic mechanisms of FA protein function, comprehensive details of the varied clinical manifestations, new model systems for study of this disease, and potential new therapeutics. Three papers describe FA relevant aspects in FA diagnoses using chromosomal breakage analysis and mutation detection by multiplex ligation-dependent probe amplification (MLPA) and PCR-based Sanger sequencing, or next generation sequencing. The strategy to detect mutations and the genetic counseling of involved families should be adapted when there is evidence for a founder effect, which results in a high prevalence of a common mutation in a specific population. In this special issue, Y. de Vries et al. provide evidence that FA-C patients with the *FANCC* c.67delG mutation in the Dutch and Canadian Manitoba Mennonite populations originate from a common founder.

One impediment to FA research has been the relative lack of a murine model that truly recapitulates the severity of FA hematopoietic defects. In the data reported by J. H. E. Verhagen-Oldenampsen et al., Ercc1-deficient mice were used as model for FA-like bone marrow failure. Although no *ERCC1* mutations have been detected in FA patients, ERCC1 interacts with FANCP/SLX4 and functions in interstrand crosslink repair [5], the critical type of DNA damage recognized and repaired by the FA/BRCA pathway [6] (Figure 1).

Another group of papers in this issue provides detailed and thorough scientific reviews of the varied clinical and molecular aspects of this disease, new model systems, and potential future treatments. First, the review by M. D. Milsom et al. details FA-associated defects in hematopoietic

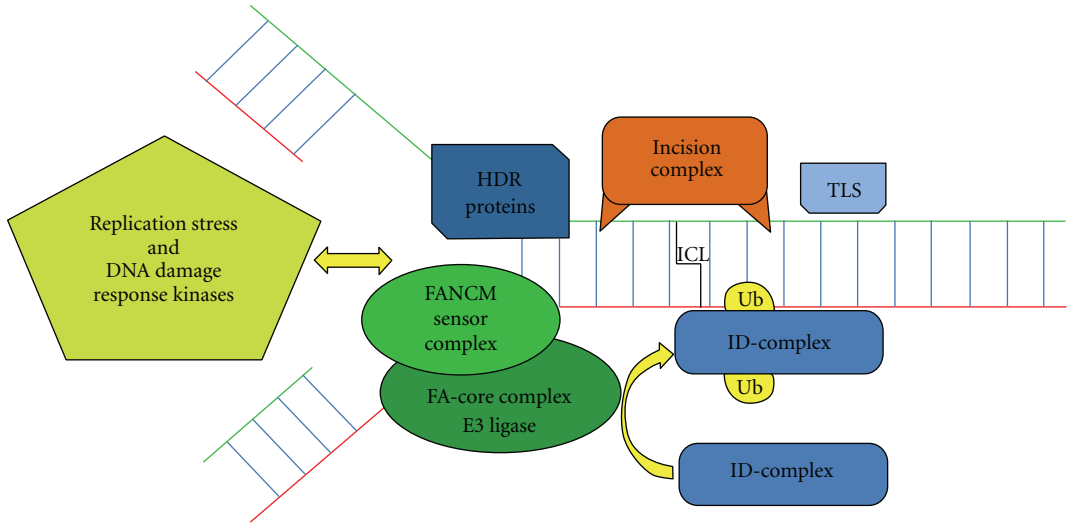

Figure 1: Schematic overview of the FA pathway. The FA pathway operates to maintain genomic stability in response to stalled replication forks, particularly in the context of interstrand DNA crosslinks (ICLs) that covalently link the two strands of the DNA helix. Replication stalling is detected by the FANCM sensor complex and triggers the activation of the Rad3-related protein kinase ATR, resulting in the phosphorylation of several FA proteins. FANCM becomes a docking platform for the other FA core complex proteins including the FANCL E3 ubiquitin ligase. The core complex activates the two members of the ID complex, FANCD2 and FANCI, by monoubiquitination (Ub) to promote ICL repair. The incision complex includes FANCP/SLX and ERCC1 and is essential to resolve the crosslink. Repair and replication fork restart is further accomplished using translesion synthesis (TLS) and homology-directed repair (HDR) which involves BRCA2/FANCD1, PALB2/FANCN, and RAD51C/FANCO.

stem cell biology and resultant bone marrow failure. FA chromosomal aberrations associated with clonal evolution and leukemic transformation are the focus of the review by S. Meyer et al. T. Kaddar and M. Carreau review aspects of FA protein function that have been placed in the shadow by the recent focus on the role of the FA pathway in DNA repair. The paper by C. Hodson and H. Walden comprehensively reviews aspects of protein interactions and function in the FA core complex, while the review by M.R. Jones and A.M. Rose focuses on utilization of a relatively new model system for FA, the worm *C. elegans*. They examine the functions of DOG1, a functional ortholog of FANCJ, and FANCD2 in interstrand crosslink repair. To conclude the special issue, Jenkins et al. summarize the efforts that have been made to generate and utilize FA pathway inhibitors as novel anticancer therapies.

The great progress in understanding FA on a molecular, cellular and clinical level illustrated in this special issue has made a big difference to people affected by FA, but also an enormous contribution to hematology, cancer, and developmental biology research. This has been achieved to a large extent through close interactions between scientists, clinicians and patients, which provide a resourceful platform for not only exchange and stimulation, but also a constant reminder of the goals of this research—aiming to understand biology in order to make the journey of families with FA a more hopeful one.

FA Patient Support Groups by Bob Dalgleish, Fanconi Hope, UK. FA patients, although relatively few in number in each country, are fortunate in having a number of strong family support groups that coordinate their activities on an international basis to ensure a coherent approach to supporting research. The longest established group, the United States-based Fanconi Anemia Research Fund (FARF: http://www.fanconi.org.uk/) has been responsible for sponsoring a significant amount of research for over 20 years. Also in existence over a similar period, the German group Deutsche Fanconi-Anämie-Hilfe e.V. (Fanconi Anemia Aid Association: http://www.fanconi.de/), has been actively involved on an international scale in long-term research programs. The more recently formed UK-based organization, Fanconi Hope (http://www.fanconi.org.uk/) has been supporting research through the auspices of the FARF Scientific Advisory Board, for UK research projects selected by FARF.

Fanconi Hope and FARF also sponsor an International FA Gene Therapy Working Group, now in its third year, which aims to accelerate the transition from research to gene therapy trials for FA patients by coordinating activities on an international scale. FA patients, their families and friends are in large part responsible for raising the funds for this research. This is of great benefit to those affected as this allows them to believe they can make a difference, if not to the current generation of FA patients then at least as a legacy to the benefit of the next generation.

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Research Article

Diagnosis of Fanconi Anemia: Mutation Analysis by Multiplex Ligation-Dependent Probe Amplification and PCR-Based Sanger Sequencing

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Fanconi anemia (FA) is a rare inherited disease characterized by developmental defects, short stature, bone marrow failure, and a high risk of malignancies. FA is heterogeneous: 15 genetic subtypes have been distinguished so far. A clinical diagnosis of FA needs to be confirmed by testing cells for sensitivity to cross-linking agents in a chromosomal breakage test. As a second step, DNA testing can be employed to elucidate the genetic subtype of the patient and to identify the familial mutations. This knowledge allows preimplantation genetic diagnosis (PGD) and enables prenatal DNA testing in future pregnancies. Although simultaneous testing of all FA genes by next generation sequencing will be possible in the near future, this technique will not be available immediately for all laboratories. In addition, in populations with strong founder mutations, a limited test using Sanger sequencing and MLPA will be a cost-effective alternative. We describe a strategy and optimized conditions for the screening of *FANCA, FANCB, FANCC, FANCE, FANCF,* and *FANCG* and present the results obtained in a cohort of 54 patients referred to our diagnostic service since 2008. In addition, the follow up with respect to genetic counseling and carrier screening in the families is discussed.

1. Introduction

Fanconi anemia (FA) is a rare inherited syndrome with diverse clinical symptoms including developmental defects, short stature, bone marrow failure, and a high risk of malignancies. Fifteen genetic subtypes have been distinguished: FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, -N, -O, and -P. [1–4]. The majority of patients (~85%) belong to the subtypes A (~60%), C (~10–15%), or G (~10%), while a minority (~15%) is distributed over the remaining 12 subtypes, with relative prevalences between <1 and 5%. These percentages may differ considerably within certain ethnic groups, due to founder effects. All subtypes seem to fit within a "classical" FA phenotype, except for D1 and N (mutated in *BRCA2/FANCD1* and *PALB2/FANCN*), which are associated with a distinct, more severe, syndromic association. The mode of inheritance for all subtypes is autosomal recessive, except for FA-B, which is X-linked. These two different modes of inheritance have important consequences for the counseling of FA families. The relative prevalence of FA-B amongst unselected FA patients is estimated at 1.6% [5]. For all genetic subtypes disease genes have been identified (Table 1). Many mutations found in the various subtypes are private, but recurrent mutations are known, particularly in specific ethnic backgrounds (Table 2).

Most FA genes encode orphan proteins with no known molecular function. At least eight FA proteins (FANCA, -B, -C, -E, -F, -G, -L, and -M) assemble into a nuclear multiprotein core complex, which is required to activate FANCD2 and FANCI by monoubiquitination [6]. FANCL, which carries a RING finger domain, is supposed to represent the ubiquitin E3 ligase in this activation [7]. FANCM probably acts as a sensor of DNA damage and recruits the FA core complex to the site of damage, but FANCM also interacts with other proteins including Blm [6]. Monoubiquitination of FANCD2 and FANCI directs these proteins to areas of damaged chromatin where they interact with other proteins, resulting in repair of the damage [6]. The remaining FA

Table 1: Fanconi anemia complementation groups, genes, and proteins.

Group	Gene symbol(s)[a]	Cytogenetic location	Protein (amino acids)	Domain structure (references)
A	*FANCA*	16q24.3	1455	HEAT repeats [8]
B	*FANCB*	Xp22.31	859	—
C	*FANCC*	9q22.3	558	HEAT repeats [8]
D1[b]	*BRCA2*	13q12.3	3418	RAD51- and DNA-binding motifs [9]
D2	*FANCD2*	3p25.3	1451	—
E	*FANCE*	6p21.3	536	—
F	*FANCF*	11p15	374	—
G	*FANCG*	9p13	622	Tetratricopeptide repeats (TPR) [10]
I	*FANCI*	15q26.1	1328	—
J[b]	*BRIP1*	17q22	1249	DNA helicase [11, 12]
L	*FANCL*	2p16.1	375	RING finger motif (E3 ligase) [7, 8]
M	*FANCM*	14q21.3	2014	DNA helicase, nuclease [13]
N[b]	*PALB2*	16p12.1	1186	—
O[b]	*RAD51C*	17q25.1	376	—
P[b]	*SLX4*	16p13.3	1834	Endonuclease scaffold [3, 4]

[a] For gene nomenclature see http://www.genenames.org/.
[b] The proteins defective in groups D1, J, N, O, and P (boldface) act downstream or independent of the monoubiquitination of FANCD2; all other FA proteins act upstream of this process.

Table 2: Major recurrent mutations in FA.

Gene	Mutation*	Geographic/ethnic background	Comment	References
FANCA	c.3788_3790del (p.Phe1263del)	European, Brazilian	Relatively mild	[14, 15]
	c.1115_1118delTTGG (p.Val372fs)	European	Relatively mild	[16]
	Exon 12–17del Exon 12–31del	South-African	Relatively common in Afrikaners	[17]
	c.295C>T (p.Gln99X)	Spanish Gypsy population	Worldwide highest prevalence of mutant *FANCA* allele	[18]
FANCC	c.711+4A>T (originally reported as IVS4+4A>T)	Homozygous in 80% of Ashkenazi Jewish FA; relatively common in Japan.	Severe phenotype in Jews, milder in Japanese.	[19–22]
	c.67delG (originally reported as 322delG)	Homozygous in approx. 50% of Dutch FA patients	Like other exon 1 mutations, relatively mild phenotype.	[19, 23–25]
FANCD2	c.1948-16T>G	Turkish	Founder mutation	[26]
FANCG	c.313G>T (p.Glu105X)	European	44% of mutated *FANCG* alleles in Germany.	[27]
	c.1077-2A>G	Portuguese/Brazilian	Founder mutation	[27, 28]
	c.1480+1G>C	French-Canadian	Founder mutation	[28]
	c.307+1G>C	Japanese	Founder mutation	[28, 29]
	c.1794_1803del (p.Trp599fs)	European		[28]
	c.637_643del (p.Tyr213fs)	Sub-Saharan Africa	82% of all black FA patients	[30]
FANCJ	c.2392C>T (p.Arg798X)		Found in ca. 50% of FA-J patients of diverse ancestry; ancient mutation or hot spot.	[11, 12]

Nucleotide numbering based on ATG = +1.
Published sequence variations in FA genes, with their descriptions conforming to the current nomenclature rules, are listed at http://www.rockefeller.edu/fanconi/.

proteins function downstream of or parallel to the FANCD2 activation step [6]. The exact nature of the DNA damage response, which when defective causes FA, remains to be defined. FANCJ/BRIP1 and FANCM possess DNA helicase motifs, which strongly suggests that the FA pathway acts through a direct interaction with DNA, presumably to resolve or remodel blocked DNA replication forks resulting from DNA interstrand cross-link damage [6]. This idea is strengthened by the recent extension of the FA pathway with SLX4, a scaffold protein for structure-specific endonucleases involved in unhooking the DNA cross-link [3, 4].

2. Laboratory Diagnostics in FA

Cells derived from FA patients are—by definition—hypersensitive to chromosomal breakage induced by DNA cross-linking agents such as mitomycin C (MMC) or diepoxybutane (DEB) [31]. This cellular phenotype is ascertained using stimulated blood T lymphocytes. The indications for FA laboratory testing are rather broad [32]. As a consequence, in only a small proportion of patients (about 10%) the chromosomal breakage test is positive, and an FA diagnosis is established. Since mutation testing by Sanger sequencing and MLPA is rather laborious, time consuming and therefore expensive, a positive chromosomal breakage test is a prerequisite for starting mutation screening. Confirmation of the FA diagnosis at the DNA level is important in patients in whom the chromosomal breakage test was inconclusive. Furthermore, knowledge about the FA subtype is relevant for the treatment and prognosis of the patients. In addition, identification of mutations allows carrier testing in the family and will enable prenatal DNA testing and preimplantation genetic diagnosis (PGD) in future pregnancies. Finally, this information can be used to rule out FA in potential donors for bone marrow transplantation.

Although simultaneous testing of all FA genes by next generation sequencing will be possible in the near future, this technique will not be available immediately for all laboratories worldwide. In addition, in populations with strong founder mutations, a limited test using Sanger sequencing and MLPA will be a cost-effective alternative [33]. The strategy outlined below has been developed at our DNA diagnostics laboratory to provide a molecular diagnosis of FA. It is recognized that mutations in *FANCA* account for 60–70% of all FA cases and that about 15–20% of the mutations in this gene are large deletions [33, 34]. Therefore, DNA testing usually starts with a screen for deletions in *FANCA*. However, depending on the circumstances strategies may differ from case to case.

2.1. Materials. Genomic DNA (from e.g., leukocytes or fibroblasts derived from the proband or the parents) is adequate for most mutation screening assays. Screening on cDNA is more efficient but has several drawbacks: for high-quality cDNA, growing cells (stimulated leukocytes, lymphoblastoid cell lines, or fibroblasts) are necessary. In addition, common alternative splice variants will hamper the evaluation of DNA sequences. Therefore, screening on gDNA is the preferred method for mutation screening. However, during the diagnostic process, growing cells from the proband will be helpful in a couple of situations. Growing cells are indispensable for studying the effect of unclassified variants on splicing or to verify the disease gene by functional complementation of the cellular phenotype with a construct expressing a wild type copy of the suspected gene [35–37]. Finally, if no mutations can be de detected, growing cells can be used to reconfirm the diagnosis FA by checking MMC sensitivity in cell growth or G2-arrest assays [38, 39].

2.2. Mutation Screening Strategy

2.2.1. Hints from Ethnic Background or Phenotype. Information on the ethnic background of the proband may provide a clue for a specific pathogenic mutation that most likely causes the disease, such as c.711 + 4A>T (IVS4 + 4A>T) in *FANCC,* a mutation present in homozygous state in 80% of all FA cases of Ashkenazi Jewish ancestry, and c.295C >T in *FANCA*, which was present homozygously in all 40 FA cases of Spanish Gypsy ancestry so far investigated. More examples of recurrent mutations are shown in Table 3. The distinct clinical phenotype of D1 and N patients (severely affected, often combined with leukemia or solid tumors below the age of 5 years) may provide a clue to favor *BRCA2/FANCD1* and *PALB2/FANCN* as the first gene to be screened [40–44]. This is especially worthwhile if confirmed by the cellular phenotype: in contrast to cells from all other known FA subtypes, cells from D1, N and O patients are unable to form RAD51 foci upon exposure to X rays or MMC [43–45].

2.2.2. No Clues Available

(1) In the absence of any clue to the disease gene, mutation screening starts with a search for deletions in *FANCA*, as this type of mutation accounts for 40% of all mutant *FANCA* alleles. The quantitative multiplex ligation-dependent probe amplification (MLPA) method [46] is used for this initial screen, which identifies *FANCA* as the most likely disease gene in 1 out of 4 patients by the detection of a—usually hemizygous-deletion. In parallel, the *FANCA* gene is completely sequenced. The combination of these two approaches identifies 60–70% of all FA patients as FA-A.

(2) Next, *FANCC, -E, -F*, and *-G* are screened by DNA sequencing.

(3) Only if the proband is a male, *FANCB* is screened by MLPA and DNA sequencing,

In Table 4, optimized conditions are provided for the PCR amplification of *FANCA, -C, -E, -F, -G, and -B*. Most PCRs can be performed under standard conditions. The PCR primers have M13 extensions which allow sequencing of all fragments with universal sequencing primers. MLPA was performed according to the instructions of the supplier. Detailed information about the sequences of the MLPA probes is available from the website of the

Table 3: Mutations detected in a cohort of 54 patients by screening *FANCA, FANCC, FANCE, FANCF* and *FANCG*.

			Allele 1			Allele 2		
	Country of origin[1]	Gene	DNA change	Effect	Number of database entries	DNA change	Effect	Number of database entries
1	ES	FANCA	ex16_17del	del	12x	c.1115_1118del	p.Val372fs	62x
2	PT	FANCA	c.718C>T	p.Gln240X	2x	c.2870G>A	W957X	1x
3	NL	FANCA	ex15del	del	3x	ex15del	del	3x
4	NL	FANCA	c.3788_3790del	p.Phe1263del	215x	c.3788_3790del	p.Phe1263del	215x
5	CA	FANCA	c.718C>T	pGlnx240X	2x	c.1085T>C	p.Leu362Pro	novel
6	PT	FANCA	c.3788_3790del	p.Phe1263del	215x	c.4130C>G	p.Ser1377X	1x
7	IE	FANCA	c.2812_2830dup	p.Asp944fs	3x	c.2812_2830dup	p.Asp944fs	3x
8	AU	FANCA	c.2303T>C	p.Leu768Pro	5x	c.2303T>C	p.Leu768Pro	5x
9	NL	FANCA	c.862G>T	p.Glu288X	1x	c.862G>T	p.Glu288X	1x
10	NL	FANCA	ex11_33del	del	1x	c.2121delC	p.Asn707fs	novel
11	DK	FANCA	ex1_8del	del	1x	c.3788_3790del	p.Phe1263del	215x
12	UK	FANCA	c.337_338del	p.Ala114fs	1x	c.3349A>G	p.Arg1117Gly	2x
13	UK	FANCA	c.3568C>T	p.Gln1190X	novel	c.3568C>T	p.Gln1190X	novel
14	NL	FANCA	c.487delC	p.Arg163fs	1x	c.2851C>T	p.Arg951Trp	11x
15	SE	FANCA	c.88delG	p.Val30fs	novel	c.100A>T	p.Lys34X	2x
16	NL	FANCA	c.862G>T	p.Glu288X	9x	c.1771C>T	p.Arg591X	9x
17	PT	FANCA	c.1709_1715+4del	p.Glu570fs	novel	c.3430C>T	p.Arg1144Trp	novel
18	NO	FANCA	c.100A>T	p.Lys34X	2x	c.1378C>T	p.Arg460X	novel
19	PT	FANCA	ex15_17del	del	2x	ex15_17del	del	2x
20	NL	FANCA	c.2982-192A>G	splice[2]	novel	ex7_31del	del	
21	AU	FANCA	c.427-8_427-5del	splice	novel	c.1771C>T	p.Arg591X	9x
22	AU	FANCA	c.3491C>T	p.Pro1164Leu	novel	c.3491C>T	p.Pro1164Leu	novel
23	CA	FANCA	ex4_29del	del	novel	ex31del	del	6x
24	NL	FANCA	c.3391A>G	p.Thr1131Ala	15x	c.3391A>G	p.Thr1131Ala	15x
25	GR	FANCA	c.2T>C	p.Met1?	1x	c.3788_3790del	p.Phe1263del	215x
26	IE	FANCA	c.851dup	p.Val285fs	novel	c.2534T>C	p.Leu845Pro	4x
27	NL	FANCA	c.2852G>A	p.Arg951Gln	6x	c.3624C>T	p.= (splice)	2x
28	AU	FANCA	c.331_334dup	p.Leu112fs	novel	ex22_29del	del	novel
29	NL	FANCA	c.862G>T	p.Glu288X	9x	c.3920delA	p.Gln1307fs	2x
30	IR	FANCA	ex21del	del	novel	ex21del	del	novel
31	SE	FANCA	ex1_12del	del	novel	ex22_29del	del	novel
32	NL	FANCB	c.755_767del	p.Leu252fs	novel	—	—	—
33	NL	FANCC	c.67delG	p.Asp23fs	50x	c.553C>T	p.Arg185X	14x
34	NL	FANCC	c.67delG	p.Asp23fs	50x	c.67delG	p.Asp23fs	50x
35	CA	FANCC	c.67delG	p.Asp23fs	50x	c.553C>T	p.Arg185X	14x
36	NL	FANCC	c.67delG	p.Asp23fs	50x	c.1155-1G>C	splice	novel
37	NL	FANCC	c.67delG	p.Asp23fs	50x	c.67delG	p.Asp23fs	50x
38	NL	FANCC	c.67delG	p.Asp23fs	50x	c.467delC	p.Ser156fs	novel
39	PT	FANCE	c.1111C>T	p.Arg371Trp	6x	c.1111C>T	p.Arg371Trp	6x
40	UK	FANCF	c.496C>T	p.Gln166X	4x	c.496C>T	p.Gln166X	4x
41	UK	FANCG	c.307+2delT	splice	novel	c.307+2delT	splice	novel
42	UK	FANCG	c.1471_1473delinsG	p.Lys491fs	novel	c.1471_1473delinsG	p.Lys491fs	novel
43	NL	FANCG	c.65G>C	p.Arg22Pro	6x	c.65G>C;	p.Arg22Pro	6x
44	IR	FANCG	c.307+1G>C	splice	21x	c.307+1G>C	splice	21x
45	NL	FANCG	c.85-1G>A	splice	novel	c.85-1G>A	splice	novel

[1] Country of origins: AU: Australia; CA: Canada; DK: Denmark; ES: Spain; GR: Greece; IE: Ireland; IR: Iran; NL: Netherlands; PT: Portugal; SE: Sweden; UK: United Kingdom

Number of database entries refers to the FA database at: http://www.rockefeller.edu/fanconi/.

The pathogenic state of novel missense mutations is based upon *in silico* prediction algorithms (SIFT, POLYPHEN2, Align GVGD), the presence of a second clearly pathogenic mutation in the same gene and segregation in the family.

[2] Effect c.2982-192A>G: by studying cDNA it was shown that the mutation created a new splice donor site resulting in an aberrant mRNA.

Table 4: Primers and conditions for PCR on genomic DNA of the coding sequence plus intron/exon boundaries of *FANCA, FANCC, FANCE, FANCF, FANCG,* and *FANCB*.

(a)

FANCA		
Primer name	Sequence (5′ > 3′)	Product length (bp)
FANCA_ex1F	gtaaaacgacggccag GCGCCTCCCCCAGGACCAACA	362
FANCA_ex1R	caggaaacagctatga AGGCTCTGGCGGGAAGGGATCGG	
FANCA_ex2F	gtaaaacgacggccag CTCTTCGGGAGGGTGTCGCTGGT	328
FANCA_ex2R	caggaaacagctatga CTCTTCGGGAGGGTGTCGCTGGT	
FANCA_ex3F	gtaaaacgacggccag GCCTGGCCTGGAGCTTGAAT	392
FANCA_ex3R	caggaaacagctatga CGCAGGTTGAATCAGACGCTGTT	
FANCA_ex4F	gtaaaacgacggccag TAAGGCATTTTAAACAGCAAGTC	430
FANCA_ex4R	caggaaacagctatga TGCCAATAAATACTGAGCAAACT	
FANCA_ex5F	gtaaaacgacggccag AGTATTGTTTCAGGTAATTTGTT	356
FANCA_ex5R	caggaaacagctatga TGAAGGTACTTCTTTCCAATCCA	
FANCA_ex6F	gtaaaacgacggccag AGATGTGTTTCAGGCTCTAAGTT	402
FANCA_ex6R	caggaaacagctatga GCAATGCAATCTAGTCTAGTACA	
FANCA_ex7F	gtaaaacgacggccag TGGGATTTAGTTGAGCCTTACGTCTGC	421
FANCA_ex7R	caggaaacagctatgaAAGGTGAATGGAAACACTTAAACTCATGTCA	
FANCA_ex8F	gtaaaacgacggccag GTGGTCAGGTGAGCAGTAACTTC	401
FANCA_ex8R	caggaaacagctatga TAAATAGGTACAAACAGCACGTT	
FANCA_ex9F	gtaaaacgacggccag TTCTCTTGTGTGATGCAGGTATC	332
FANCA_ex9R	caggaaacagctatga TGACCCACAGATTCATGAGGTAT	
FANCA_ex10F	gtaaaacgacggccag TTTTGATTAAGGCCTACAGATTG	406
FANCA_ex10R	caggaaacagctatga CCTCCTCCTCACGCACGTTATCG	
FANCA_ex11F	gtaaaacgacggccag TTTCAAGTCTGTGGTTATAGTGG	410
FANCA_ex11R	caggaaacagctatga AGACGTAAAAGAGGTCCTAGAAT	
FANCA_ex12F	gtaaaacgacggccag CTGTGGGGCTGGTCCTTAACAAA	236
FANCA_ex12R	caggaaacagctatga AGGCAGCATGACAAGTACTAGGC	
FANCA_ex13F	gtaaaacgacggccag ACATTGGTTTGCTTGGATATTGA	377
FANCA_ex13R	caggaaacagctatga CTGACAAAGAATGTTCCATCGAC	
FANCA_ex14F	gtaaaacgacggccag TGCTGTAATTGCTGTGTAGTCTT	411
FANCA_ex14R	caggaaacagctatga ACTCACATGACAGAGAATCAGGT	
FANCA_ex15F	gtaaaacgacggccag ACTACAGCAGCCGCCCGGACACT	430
FANCA_ex15R	caggaaacagctatga GCAGATCTGCAGGAGGCTCTTGG	
FANCA_ex16F	gtaaaacgacggccag TCCCAGGCAGTTCCCAGACTAAC	312
FANCA_ex16R	caggaaacagctatga AGCTGATGACAAATCCTCGTAGA	
FANCA_ex17F	gtaaaacgacggccag ACCGCTCCCTCCTCACAGACTAC	334
FANCA_ex17R	caggaaacagctatga AAGGCTGAAAAACTCAACTCAAG	
FANCA_ex18F	gtaaaacgacggccag GCGCACAGCATGTGGGCCTTTAC	397
FANCA_ex18R	caggaaacagctatga GCAGCTGCTAGAGGCCTTTTCGG	
FANCA_ex19F	gtaaaacgacggccag GTGCACAAGAAGACTTCATAATG	284
FANCA_ex19R	caggaaacagctatga AGTCCTTGCTTTCTACACAACTG	
FANCA_ex20F	gtaaaacgacggccag CTTCTCTGTGTTGCAGCATATTC	298
FANCA_ex20R	caggaaacagctatga AGAAGAAACCTGGAAGTAGTCAT	
FANCA_ex21F	gtaaaacgacggccag ATAATAGATTTGGGGATTGTAAT	255
FANCA_ex21R	caggaaacagctatga CAACAGACACTCAAGGTTAGGAA	
FANCA_ex22F	gtaaaacgacggccag TGCAGTGAAGAGTCCTGTTGAGT	305
FANCA_ex22R	caggaaacagctatga ACACACCAGCCTGATGTCACTAT	
FANCA_ex23F	gtaaaacgacggccag CAGTCAGCAGGATCCGTGGAATC	416

(a) Continued.

FANCA		
Primer name	Sequence (5′ > 3′)	Product length (bp)
FANCA_ex23R	caggaaacagctatga GGCCCTGGAACATCTGATACGAC	
FANCA_ex24F	gtaaaacgacggccag CCTTCCTGCTGCTCCCGTCC	229
FANCA_ex24R	caggaaacagctatga CAGACTTGGCCCAGCAAGAG	
FANCA_ex25F	gtaaaacgacggccag CCGCTGGTGGTTGGATTAGCTGT	296
FANCA_ex25R	caggaaacagctatga TTTCCAGGGCACTGAAGACGAAT	
FANCA_ex26F	gtaaaacgacggccag AGCTTGGAAGAGGGCAGTCTGCT	347
FANCA_ex26R	caggaaacagctatga CTCTTCTAATTTTATCAAACGAG	
FANCA_ex27F	gtaaaacgacggccag AGACTGTCTCACAACAAACGAAC	356
FANCA_ex27R	caggaaacagctatga CGGTCCGAAAGCTGCGTAAAC	
FANCA_ex28F	gtaaaacgacggccag GTTGATGGTCTGTTTCCACCTGA	401
FANCA_ex28R	caggaaacagctatga GAAGGAACGGTCACCTACGTGCT	
FANCA_ex29F	gtaaaacgacggccag GACATGGAGGACTGCGTATGAGA	411
FANCA_ex29R	caggaaacagctatga GTGGCTGTGATGACTGGAACGTG	
FANCA_ex30F	gtaaaacgacggccag CCCGAGCCGCCAGTCTCAACCCA	411
FANCA_ex30R	caggaaacagctatga AAAGGCAGACCCACCCTAAGCTA	
FANCA_ex31F	gtaaaacgacggccag GATAAGCCTCCTTGGTCATGGTA	406
FANCA_ex31R	caggaaacagctatga TGGCAATAAATATCTTAATAGCA	
FANCA_ex32F	gtaaaacgacggccag TTCCTGTGCCAGCATACTGCTCT	359
FANCA_ex32R	caggaaacagctatga GGGTGGGGACACACAGACAAGTA	
FANCA_ex33F	gtaaaacgacggccag TGGGTTTCAGGGTGGTGGTTGCT	356
FANCA_ex33R	caggaaacagctatga GAACCCTTTCCTCAGTAATTCAC	
FANCA_ex34F	gtaaaacgacggccag CGCCCAGGGAAGCCGTTAAGTTT	333
FANCA_ex34R	caggaaacagctatga GCGTTCTGAGAAGGCCACGAGAG	
FANCA_ex35F	gtaaaacgacggccag TTCCTTCACTCTACTAGTTGTGG	311
FANCA_ex35R	caggaaacagctatga TGAGATGGTAACACCCGTGATGG	
FANCA_ex36F	gtaaaacgacggccag CCATCTCAGCCACCCTCATCTGT	350
FANCA_ex36R	caggaaacagctatga AGGCGCCCACCACCACGAGAACT	
FANCA_ex37F	gtaaaacgacggccag GACTTGGTTTCTATGGCGTGGTT	310
FANCA_ex37R	caggaaacagctatga CCCAGAGAAATAGCACTGATTGA	
FANCA_ex38F	gtaaaacgacggccag GTTTTCTAAGATCCACTTAAAGG	362
FANCA_ex38R	caggaaacagctatga CTCACTCACACTTCCGCAAACAC	
FANCA_ex39F	gtaaaacgacggccag CTGTCCAGAGGCCCAGTATTACC	387
FANCA_ex39R	caggaaacagctatga AGGAGGGCTCGTTCTTAACCATT	
FANCA_ex40F	gtaaaacgacggccag GGTGTCCCCAGCACTGATAATAG	353
FANCA_ex40R	caggaaacagctatga AGACATAGTGACAAATGGCTACA	
FANCA_ex41F	gtaaaacgacggccag CCCTTGGCATCACCTGCTACCTT	403
FANCA_ex41R	caggaaacagctatga AACAGGCAAACTCACAGGTTAGA	
FANCA_ex42F	gtaaaacgacggccag ACCAGCCCTGTTTCTGTATGTCT	248
FANCA_ex42R	caggaaacagctatga ACATGGCCCAGGCAGCTGTCAAT	
FANCA_ex43F	gtaaaacgacggccag TGTGGGGGACATGAGAATTGACA	378
FANCA_ex43R	caggaaacagctatga GTAATCCACTTTTTAGTGCAACA	
FANCAIVS10F	gtaaaacgacggccag TTTACATGTGCATCAGTTAGCTT	184
FANCAIVS10R	caggaaacagctatga CATGAAGACACAGAAAAAGTAGGT	

(b)

FANCC		
Primer name	Sequence (5′ > 3′)	Product length (bp)
FANCC_ex1F	gtaaaacgacggccag ACCATTTCCTTCAGTGCTGGACA	378
FANCC_ex1R	caggaaacagctatga CCATCGGCACTTCAGTCAATACC	

(b) Continued.

FANCC		
Primer name	Sequence (5′ > 3′)	Product length (bp)
FANCC_ex2F	gtaaaacgacggccag CTAAACAAGAAGCATTCACGTTC	303
FANCC_ex2R	caggaaacagctatga GGAGAAAGGTTCATAATGTAAGC	
FANCC_ex3F	gtaaaacgacggccag TCAGCAGAAAGAGAATGTGCAAA	405
FANCC_ex3R	caggaaacagctatga AACATCATAGAACTGGATTCCAC	
FANCC_ex4F	gtaaaacgacggccag TGTACATAAAAGGCACTTGCATT	380
FANCC_ex4R	caggaaacagctatga TCCCATCTCACATTTCTTCCGTA	
FANCC_ex5F	gtaaaacgacggccag AGAACTGATGTAATCCTGTTTGC	367
FANCC_ex5R	caggaaacagctatga TTACTGCTCTGTGAGAGTTGAGA	
FANCC_ex6F	gtaaaacgacggccag GTCTTTGACCTTTTTAGCATGAA	387
FANCC_ex6R	caggaaacagctatga AACGTTTGGACACTGCTGTCGTA	
FANCC_ex7F	gtaaaacgacggccag ATTAGTGATTGCATTTTGAACTT	422
FANCC_ex7R	caggaaacagctatga CAAAAATAAAATGTAAATACACG	
FANCC_ex8F	gtaaaacgacggccag CTCCTTTGGCTGATAATAGCAAG	336
FANCC_ex8R	caggaaacagctatga CTGATTTTTGAGTTTTTACCTCT	
FANCC_ex9F	gtaaaacgacggccag ATACTGCTGAAGCTTATGGCACA	400
FANCC_ex9R	caggaaacagctatga TAACCTTTGTTGGGGCACTCATT	
FANCC_ex10F	gtaaaacgacggccag TATGAGGTTATTGGGAGCTTATT	382
FANCC_ex10R	caggaaacagctatga CTGTCTCCCTCATGCTGTAGATA	
FANCC_ex11F	gtaaaacgacggccag GAACCAGAAGTAAAGGGCGTCTC	416
FANCC_ex11R	caggaaacagctatga CTGACCTGCTCCAAGCCATCCGT	
FANCC_ex12F	gtaaaacgacggccag AAGTACAATTTAAGCCAACCGTT	451
FANCC_ex12R	caggaaacagctatga AGGTTGCCATGACATATGCCATC	
FANCC_ex13F	gtaaaacgacggccag CCTCTCTCAGGGGCCAGTGCTTA	435
FANCC_ex13R	caggaaacagctatga AGACCCTCGGACAGGTAACCCAC	
FANCC_ex14F	gtaaaacgacggccag ACTTGCTATGCTAATCACCTTGC	437
FANCC_ex14R	caggaaacagctatga AATGCGTGGCCACAGGTCATCAC	

(c)

FANCE		
Primer name	Sequence (5′ > 3′)	Product length (bp)
FANCE_ex1F	gtaaaacgacggccag CGCCTCCCTCCTTCCCTTTC	540
FANCE_ex1R	caggaaacagctatga CCCGCCTCCCATACCTGCTAA	
FANCE_ex2aF	gtaaaacgacggccag GCTCTGCCCAGTCTGCCTTGTGC	469
FANCE_ex2aR	caggaaacagctatga CTCTGAGTCCTTTCTGCGTTTCC	
FANCE_ex2bF	gtaaaacgacggccag GCCAGAGACAGCTCCAAAGTCTA	479
FANCE_ex2bR	caggaaacagctatga CAGCCTTCCCCATGGATAAAGCC	
FANCE_ex3F	gtaaaacgacggccag GCCTCTTGACTTTCTTGAATCAT	352
FANCE_ex3R	caggaaacagctatga ACTGTCCTCAGACCTTTACTCCA	
FANCE_ex4F	gtaaaacgacggccag TTGAACCAAGTGTAGACTTACCA	436
FANCE_ex4R	caggaaacagctatga GGGAAGGAACCAAGGGCTAAAAG	
FANCE_ex5F	gtaaaacgacggccag GTATCTTTTAGCCCTTGGTTCCT	431
FANCE_ex5R	caggaaacagctatga GAATCCCCTCTCTCAAGTACCAC	
FANCE_ex6F	gtaaaacgacggccag TTTCCTTTGTAACATGTATCATC	433
FANCE_ex6R	caggaaacagctatga AGCAGAAAGCAGGGAGGCGGTAA	
FANCE_ex7F	gtaaaacgacggccag ACAGGCTGGGCATTCTGTTACCG	425
FANCE_ex7R	caggaaacagctatga AGTGAGACACAAGGATCCCCTAA	
FANCE_ex8F	gtaaaacgacggccag TTGGAGCAGCAGATAGATACTCA	380
FANCE_ex8R	caggaaacagctatga AGAGGTGGAGCTGAAGTGACCAT	
FANCE_ex9F	gtaaaacgacggccag GTTACCTGCCCAGGGTCACCTAG	388
FANCE_ex9R	caggaaacagctatga CTGGCCAGCACTCAGGGTTTTAT	

(c) Continued.

FANCE		
Primer name	Sequence (5′ > 3′)	Product length (bp)
FANCE_ex10F FANCE_ex10R	gtaaaacgacggccag TGGCCTCCTCTCTCCTCAATAGA caggaaacagctatga AACAGGGAGGCAGTTGCAATCTG	369

(d)

FANCF		
Primer name	Sequence (5′ > 3′)	Product length (bp)
FANCF_ex1aF FANCF_ex1aR	gtaaaacgacggccag TTTCGCGGATGTTCCAATCAGTA caggaaacagctatga CTGCACCAGGTGGTAACGAGCTG	449
FANCF_ex1bF FANCF_ex1bR	gtaaaacgacggccag AGTGGAGGCAAGAGGGCGGCTTT caggaaacagctatga GCTATCACCTTCAGGAAGTTGTT	456
FANCF_ex1cF FANCF_ex1cR	gtaaaacgacggccag CCCAAATCTCCAGGAGGACTCTC caggaaacagctatga TTTCTGAAGGTCATAGTGCAAAC	444
FANCF_ex1dF FANCF_ex1dR	gtaaaacgacggccag GCTTTTGACTTTAGTGACTAGCC caggaaacagctatga ATTTGGTGAGAACATTGTAATTT	456

(e)

FANCG		
Primer name	Sequence (5′ > 3′)	Product length (bp)
FANCG_ex1F FANCG_ex1R	gtaaaacgacggccag AGCCTGGGCGGGTGGATTGGGAC caggaaacagctatga TCATTTCTGGCTCTTTGGTCAAG	389
FANCG_ex2F FANCG_ex2R	gtaaaacgacggccag CAGGCCAAGGTAACACGGTTGCT caggaaacagctatga CCAGTCTCCTCTGTGCCTTAAAC	460
FANCG_ex3F FANCG_ex3R	gtaaaacgacggccag TATTGTAGCTGTTTTGGTTGGAG caggaaacagctatga GGTGACAGATGTTGTTTATCCTC	362
FANCG_ex4F FANCG_ex4R	gtaaaacgacggccag GGAGATGGAGGATGAGGTGCTAC caggaaacagctatga CGACCACCAACCCAGCCGCCTGT	411
FANCG_ex5F FANCG_ex5R	gtaaaacgacggccag AGATGGAGATAGGAGAAGACGAG caggaaacagctatga GCTTCATGAAGGCTGCTTAGTGC	454
FANCG_ex6F FANCG_ex6R	gtaaaacgacggccag CAGTTCCATGGGCTTCTTAGACC caggaaacagctatga TCAGGGCTGCAACCAAGTACAAC	393
FANCG_ex7F FANCG_ex7R	gtaaaacgacggccag GCACTGGGGTCCTGTCACCGTAA caggaaacagctatga ATAATCTTTGGGAGCCATACTTC	418
FANCG_ex8F FANCG_ex8R	gtaaaacgacggccag GCTTGTGATGGGGTGACTTGACT caggaaacagctatga AGTTCAGGTCTAGAAGCAAGGTA	438
FANCG_ex9F FANCG_ex9R	gtaaaacgacggccag CCTCCTCAGGGCCCATGAACATC caggaaacagctatga GCAGTGTCTTGAAAGGCATGAGC	400
FANCG_ex10F FANCG_ex10R	gtaaaacgacggccag CAGGACTCTGCATGGTACCAG caggaaacagctatga CCAATCAGAAAATCATCCCTC	460
FANCG_ex11F FANCG_ex11R	gtaaaacgacggccag AGCTCCATGTTCACCTACTTACC caggaaacagctatga CAGTGCCGCATCTGACTTACATC	397
FANCG_ex12F FANCG_ex12R	gtaaaacgacggccag AGGATTTGGGGTTTTGGTGACTG caggaaacagctatga AACTCTTGGGAGCCCTGCATACA	445
FANCG_ex13F FANCG_ex13R	gtaaaacgacggccag CCGCTTCCATATGTGAGTGTAGG caggaaacagctatgaC CACAATAGGTCCAAGGACTCTA	340
FANCG_ex14F FANCG_ex14R	gtaaaacgacggccag CCAAACTAAGGGGTCACATGAAG caggaaacagctatga GATGGTGAAGCAGAAAGCCCTCC	405

(f)

FANCB		
Primer name	Sequence (5′ > 3′)	Product length (bp)
FANCB_ex3AF FANCB_ex3AR	gtaaaacgacggccag GATATGGTTATTTGAATTCTTAGCAcaggaaacagctatga GCCATCCTTCATCTCATAGCCTAGT	721

(f) Continued.

FANCB		
Primer name	Sequence (5′ > 3′)	Product length (bp)
FANCB_ex3BF FANCB_ex3BR	gtaaaacgacggccag ATTAACCTCCCTTACATTGTGATAGcaggaaacagctatga CAATAAGACTCCAGAATGAACTCTA	811
FANCB_ex4F FANCB_ex4R	gtaaaacgacggccag TTTACAAATGACAACTACATGAcaggaaacagctatga TTAAGTATAAAACCACCAATAT	391
FANCB_ex5F FANCB_ex5R	gtaaaacgacggccag ACTGCATCTGGCCTATAGTTcaggaaacagctatga AATACCATTTTTACCCAAGC	411
FANCB_ex6F FANCB_ex6R	gtaaaacgacggccag GTATTTCCTGAATTATTGGTATGTC caggaaacagctatga CATAAAAGTCCACCATTATAACCTC	395
FANCB_ex7F FANCB_ex7R	gtaaaacgacggccag TGTTTGGGCCATAAGCCCTA caggaaacagctatga TTCTGGAGCATCAAGACAGT	355
FANCB_ex8F FANCB_ex8R	gtaaaacgacggccag GTTGTTTGTATGACATTTAATCATC caggaaacagctatga ATCATTAAACTCTGCCCATTATCAG	636
FANCB_ex9F FANCB_ex9R	gtaaaacgacggccag AGGTAATTTTGTTGGCACTT caggaaacagctatga ATGCGTTCATTCATGCTAGG	531
FANCB_ex10F FANCB_ex10R	gtaaaacgacggccag AATTGGTTCTGTTTATCATTATGGT caggaaacagctatga CTACTACAGTAAGCCTCGGTGTTTA	686

PCR conditions:
PCR was performed in Applied Biosystems PE9700 system using 96-well plates. PCR reactions (final volume 25 μl) contained 0.5 units Platinum Taq polymerase (Invitrogen), 1,5 mM $MgCl_2$, 0.2 mM NTPs (Invitrogen), and 10 pmol primer.
For the large majority of amplicons, standard PCR conditions were used: preheat 95°C, 5 min, denaturation 95°C, 30 sec, annealing 60°C, 30 sec., elongation 72°C, 1 min, number of cycles: 33.
Fragments with a different annealing temperature were *FANCA* exons 5, 7, 13, 21, 26, 31, and 38, *FANCC* exon 7, *FANCF* fragment 1d and *FANCE* exon 1 : 55°C; *FANCA* exon 1 : 64°C. For *FANCE* exon 1 the PCR mix was supplemented with 10% DMSO.
For *FANCB* different PCR conditions were used: preheat 95°C, 5 min, denaturation 95°C, 1 min, annealing 50°C, 1 min., elongation 72°C, 1 min., number of cycles 30. For *FANCB* exon 7 and 9 the annealing temperature was 55°C. For sequencing of exon 7 forward, a special sequencing primer was used: 5′-TTTTTAGAAGGAATGTCTTG-3′.
FA gene specific part of the primer is indicated in capitals. Primers are extended with M13 sequence (indicated in normal letter type), which is used for the sequencing reaction.

supplier (www.mlpa.com). In a well-equipped laboratory with sufficient dedicated personal, testing of *FANCA, -C, -E, -F, -G and -B* can be completed within 1-2 weeks.

After screening *FANCA, -C, -E, -F, -G, and –B,* a molecular diagnosis is obtained for ∼85% of the patients [34]. In our cohort of 54 patients, referred to our diagnostic service since 2008, mutations were detected in 45 patients (83%). *FANCA* mutations were found in 31 of the patients (57%), *FANCC* mutations in 6 patients (11%), and *FANCG* mutations in 5 patients (9%). *FANCB*, *FANCE,* and *FANCF* mutations were found in single families (Table 3). In the small group of patients without mutations no complementation analysis or FANCD2 western blotting was performed. Therefore, we do not know if we missed *FANCA, -C, -E, -F, -G, and -B* mutations in these patients or that these patients have mutations in other FA genes. Table 3 does not include prenatal cases, because prenatal testing is only offered in couples in which the FA-causing mutations are already established. Testing was offered as a diagnostic service for which a fee was charged.

For the patients negative for *FANCA, -C, -E, -F, -G, and -B* mutations, next generation sequencing can be used to analyze all other FA genes. If this technique is not available, further analysis will depend on the availability of growing cells from the proband. In that case a western blot should reveal whether both FANCD2 isoforms are present at normal levels.

(1) If both FANCD2 bands are absent or very weak, *FANCD2* is sequenced. Because of the presence of *FANCD2* pseudogene sequences in the genome, this testing must be performed on cDNA or gDNA using specially designed primers [26].

(2) If only the short isoform of FANCD2 is present, *FANCL* and *FANCM* are sequenced. If no mutations are found, the patient may be mutated in *FANCI* or in another unidentified FA gene acting upstream of FANCD2.

(3) If both isoforms are present, and if the clinical phenotype is compatible with FA-D1 or FA-N, *BRCA2/FANCD1* and *PALB2/FANCN* are screened by MLPA and DNA sequencing.

(4) If negative, *BRIP1/FANCJ*, *PALB2/FANCN*, *RAD51C/FANCO,* and *SLX4/FANCP* are sequenced.

(5) If negative again, the patient should be screened for mutations in *NBS1*, *ESCO2* and *DDX11* to test for Nijmegen breakage syndrome, Roberts syndrome and Warsaw Breakage syndrome, respectively [47, 48]. The latter two syndromes can also be

excluded by analyzing metaphase spreads for sister chromatid cohesion defects. If again negative, the patient is likely to be mutated in a novel FA gene acting downstream of FANCD2 ubiquitination.

3. Notes

3.1. Mutation Screening in Mosaic Patients. If an available lymphoblastoid cell line from an FA patient is phenotypically normal due to genetic reversion at the disease locus, mutation screening is still possible in the reverted cell line, since at least one mutation will be present [49–51]. The second mutation may be identified through investigating the parents.

3.2. Unclassified Variants. Missense mutations or *in-frame* deletions or insertions should be judged using *in silico* prediction algorithms (SIFT, POLYPHEN2, Align GVGD). Alternatively, they can be tested for pathogenicity in a cellular transfection assay to check the ability of the variant gene product to complement the cellular FA defect in a deficient cell line (see e.g., [10, 35, 52]). Generally, these tests are only feasible in a setting where a diagnostic laboratory is equipped with a research laboratory with all necessary technology.

3.3. Functional Assignment to Genetic Subtypes. Retroviral constructs have been used to identify the FA subtype by functional complementation, as an intermediate step before a mutation screen is undertaken [36]. Although knowing the disease gene facilitates mutation screening, retroviral transduction has some drawbacks in comparison to direct mutation screening: (i) growing, MMC-sensitive cells either from a cell line or fresh blood sample are required, which are not always easy to obtain; (ii) overexpression of some FA proteins (e.g., FANCM and FANCP) may be toxic for cells; (iii) novel genetic subtypes that emerge after all known groups have been excluded and cannot be readily distinguished from false negatives, that is, transductions that for some unknown reason have failed to cause complementation; (iv) the method requires relatively advanced laboratory facilities and technology. However, functional assignment of complementation group can rapidly be provided by laboratories with capability for this type of analysis [37], which has greatly facilitated reliable genotyping for over 95% of FA patients for which viral constructs are available.

3.4. Genetic Counseling. All patients with a diagnosis of FA confirmed by mutation analysis should be referred for genetic counselling, together with their parents and siblings. Mutation testing should be performed in all sibs regardless of any clinical symptoms. A complete pedigree, including a cancer history anamnesis, should be prepared. Mutation carriers might be at increased cancer risk (see Section 3.7) whose aspect should be included in the counseling (see Section 3.7).

FA patients themselves usually have decreased fertility. Women usually have late menarche, irregular menses, and early menopause. However, pregnancies in women with FA have been described, and therefore women should be adequately informed about the risks for their offspring, which is mainly related to an increase in pregnancy-related complications [53].

Sibs of the parents of an FA patient often request carrier screening to assess their risk of getting a child with FA. If a sib appears to be carrier, this risk is still minimal because of the very low carrier frequency in the population. In the US the carrier frequency has been estimated to be about 1 in 181 [54]. The risk of a proven carrier to get a child with FA is therefore about 1 in 724. However, in small communities or in consanguineous couples this risk is much higher, and mutation screening in spouses of proven carriers may be indicated.

3.5. Prenatal Diagnosis. Prenatal diagnosis of FA is relatively straightforward after the pathogenic mutations in a given family have been identified. Fetal cells can be obtained by chorionic villus sampling (CVS) during weeks 10–12 of the pregnancy or by amniocentesis, which is performed between weeks 14 and 16. However, CVS may be preferred as the diagnosis will be known at an earlier stage. If the mutation is not known, a chromosomal breakage test on fetal material may be performed [55], but this test may be considered less reliable than screening for mutations in the fetal material. Alternatively, flow cytometric testing of MMC sensitivity in amniotic cell cultures might be an option; however this technique is only available in a limited number of specialized laboratories [56]. Occasionally, FA may be suspected by fetal ultrasound imaging and confirmed by parental carrier testing when the family is not yet known to carry a risk for FA [57].

3.6. Genotype-Phenotype Correlation. FA is considered as one disease, and the question may be raised whether all fifteen genetic subtypes equally conform to the clinical FA phenotype. Genotype-phenotype correlation studies comparing the 3 most common groups A, C, and G indicated modest phenotypic differences, which were rather correlated with the relative severity of the mutations [23]. However, bias due to the ethnic distribution of the studied population is very well possible. Other studies reported significant differences between FA-A/G versus FA-C [58]. Cases in group FA-D1 (mutated in BRCA2) and FA-N (mutated in *PALB2*) present with a distinct, relatively severe, phenotype that is characterized by the development of leukemia at very young age (median 2.2 years) and by pediatric cancers such as nephroblastoma (Wilms tumor) or medulloblastoma [40–44]. The observations that one of the pathogenic mutations in *BRCA2* in FA-D1 patients is hypomorphic and that mice with biallelic null alleles in *Brca2* are embryonic lethals suggest that the BRCA2 protein serves a function that is essential for survival.

Different mutations in the same gene may be associated with divergent phenotypes, as illustrated by the two *FANCC* mutations, c.711+4A>T and c.67delG. The former (splice-site) mutation is associated with a relatively severe phenotype in Ashkenazi Jewish people [19] although the associated phenotype was reportedly less severe in patients of Japanese

ancestry [20]. The carrier frequency for this mutation in the Ashkenazi population is relatively high (1 in 87), which has led to the recommendation of carrier detection to prevent disease [59]. In the Netherlands more than 50% of FA cases are homozygous for the *FANCC* frameshift mutation c.67delG. The phenotype associated with this mutation, like other exon 1 mutations, seems relatively mild, as these patients rarely have skeletal abnormalities and show a relatively late age of onset of their marrow failure [24]. Awareness of such genetically determined phenotypic differences may help in clinical decision making, including the counselling of patients and families.

3.7. Cancer Risk in Heterozygous Mutation Carriers. An important issue is whether FA mutation carriers are at increased risk to develop cancer or other types of disease. Overall, there is no increased risk for cancer among FA heterozygotes [60, 61]. However, the situation is different in some of the less prevalent FA subtypes. The FA-D1 subtype is caused by mutations in *BRCA2* [62] which is a well-known breast and ovarian cancer predisposition gene [63]. In FA-D1 one of the mutations will be hypomorphic because biallelic "severe" mutations are supposed to be lethal [26]. Therefore, one of the parents of a FA-D1 patient will be a heterozygous carrier of a "severe" inactivating *BRCA2* mutation and may thus have an increased risk for breast cancer and other BRCA2-associated cancers. Whether the parent with the hypomorphic mutation is also at increased risk is unknown: in breast cancer families these hypomorphic mutations are considered as variants with unknown clinical significance. Two other genes involved in FA and related to breast or ovarian cancer predisposition are *PALB2/FANCN* [64, 65] and *RAD51C/FANCO* [66]. Although cancer patients have been identified with germ-line mutations in these genes, an accurate estimate of the relative cancer risk for mutation carriers is still lacking.

Another special case is represented by female *FANCB* mutation carriers, who are supposed to consist of 50% FA-like cells due to silenced expression of the wild type *FANCB* allele by the random process of X inactivation that occurs during early embryonic development. Nevertheless, in the few female *FANCB* mutation carriers studied so far, inactivation appeared strongly skewed towards the mutated allele [67]. This suggests that FA cells have a poor chance to survive next to unaffected cells in the same tissue, and these FA cells may therefore not give an increased cancer risk. However, the data are scarce at present so that no firm conclusions can be drawn regarding the cancer risk of female *FANCB* mutation carriers [60].

Conflict of Interests

The authors do not declare any conflict of interests related to this study.

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Review Article

Fanconi Anemia Proteins and Their Interacting Partners: A Molecular Puzzle

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In recent years, Fanconi anemia (FA) has been the subject of intense investigations, primarily in the DNA repair research field. Many discoveries have led to the notion of a canonical pathway, termed the FA pathway, where all FA proteins function sequentially in different protein complexes to repair DNA cross-link damages. Although a detailed architecture of this DNA cross-link repair pathway is emerging, the question of how a defective DNA cross-link repair process translates into the disease phenotype is unresolved. Other areas of research including oxidative metabolism, cell cycle progression, apoptosis, and transcriptional regulation have been studied in the context of FA, and some of these areas were investigated before the fervent enthusiasm in the DNA repair field. These other molecular mechanisms may also play an important role in the pathogenesis of this disease. In addition, several FA-interacting proteins have been identified with roles in these "other" nonrepair molecular functions. Thus, the goal of this paper is to revisit old ideas and to discuss protein-protein interactions related to other FA-related molecular functions to try to give the reader a wider perspective of the FA molecular puzzle.

1. The FA Clinical Phenotype

Fanconi anemia (FA) is a complex disease that is considered a congenital form of aplastic anemia. The genetic mode of transmission is both autosomal and X-linked, and a growing number of identified genes are distributed among the various chromosomes. The common clinical manifestation in most patients with FA, which may occur in all FA patients eventually, is life-threatening bone marrow failure (BMF) [1, 2]. FA is also associated with diverse birth defects and a predisposition to malignancies. FA-associated congenital malformations can affect many organ systems including the central nervous system, the gastrointestinal system, and the skeletal system [3–8]. Other findings in patients with FA include short stature, skin pigmentation abnormalities, and small facial features. In addition, more than 70% of patients with FA show endocrine dysfunctions including deficiencies in growth hormone and thyroid hormone as well as diabetes [9, 10]. All of these disease manifestations suggest a role for FA genes in mechanisms that bear on hematopoiesis, development, and neoplasia.

2. The FA Molecular Pathway

Patients with FA are classified into complementation groups (to date 14 groups from A to P have been identified), and all of these groups correspond to one of the following cloned genes: *FANCA*, *FANCB*, *FANCC*, *FANCD1/BRCA2*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ/BRIP1/BACH1*, *FANCL/PHF9, FANCM/HEF, FANCN/PALB2*, *and FANCP/SLX4* [11–27]. Approximately 85% of FA patients have a defective FANCA, FANCC or FANCG gene, while the other genes account for less than 5% of the mutations found in FA patients. To date, some patients still remain unassigned indicating the possibility of novel FA genes [28]. Mutations in the *RAD51C* gene (provisionally termed *FANCO*) have been associated with a FA-like disorder, suggesting that this gene may represent yet another FA gene [29, 30]. Patients

with mutations in one of the 15 FA and FA-like genes present clinical FA aspects to various degrees but show a common cellular phenotype: hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC), diepoxybutane, and cisplatin [28, 31]. When exposed to those agents, cells from FA patients show an abnormally prolonged cell cycle arrest in the G2/M phase, increased chromosomal aberrations, and reduced survival. These cellular features define FA, and presumably, all FA proteins cooperate in a pathway, termed the FA pathway, to maintain chromosome integrity.

In the canonical FA pathway, FA proteins are subdivided into three complexes based on protein-protein interaction studies. The first complex, known as the core complex or complex I, is composed of seven FA proteins, including FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, and FANCL [22, 27, 32–40]. The FANCM protein was also considered part of this core complex; however, further analysis revealed confounding results in FANCM-mutated cells [41]. Other proteins found in association with the core complex include Fanconi anemia-associated protein 24 (FAAP24), FAAP100, FANCM-associated histone fold protein 1 (MHF1), MHF2, and hairy enhancer of split 1 (HES1) [42–44]. All of these associated proteins are required for efficient FA pathway activation, but disease-causing mutations have yet to be found. Following DNA cross-link damage, the core complex associates with the FANCM-FAAP24 heterodimer on the chromatin [27, 39, 45, 46] and monoubiquitinates, through the activity of the FANCL E3 ubiquitin ligase, the complex II (or ID complex) components FANCD2 and FANCI [22, 47–50]. This ubiquitin tag alters the cellular distribution of complex II and promotes its association with the FA pathway complex III components FANCD1, FANCJ, FANCN and FANCP, and the FA-like disorder protein RAD51C [29, 30]. Complex III, similar to FANCM, is dispensable for the monoubiquitination of ID complex components, supporting the role of FA proteins in a linear (or canonical) response pathway. The deubiquitination of FANCD2 and FANCI by the UAF1/USP1 deubiquitinating enzyme complex appears to be required for the completion of the repair process [50–52].

3. FA Protein Cellular Localization

One intriguing aspect of the FA molecular pathway is the cellular distribution of FA proteins. Although the well-characterized function of this pathway in DNA crosslink damage occurs in the nucleus, FA core complex proteins can be found in different cellular compartments in addition to the nucleus.

The first identified FA protein, FANCC, is principally found in the cytoplasm [53–56]. It was first reported that FANCC function in DNA cross-links resistance required its cytoplasmic location and that enforced nuclear FANCC expression abolished its ability to correct FA-C cells [55, 57]. It was later found that FANCC is partially localized to the nucleus, and this FANCC nuclear localization is critical for the function of the FA core complex in DNA crosslink responses [56, 58]. These conflicting results may suggest that FANCC is required in both cellular compartments, and that regulation of its protein level in each cellular compartment is important for its function. The molecular chaperone, glucose-related protein 94 (GRP94) involved in protein quality control, protein folding, and ER stress responses [59], directly binds FANCC and regulates its intracellular levels [60]. Reducing the levels of GRP94 through the use of a specific ribozyme affects FANCC stability and renders cells hypersensitive to MMC suggesting a possible mechanism of FANCC quality control and subsequent cellular localization.

FANCA cellular localization has been extensively studied [32, 36, 37, 61–63]. FANCA possesses a bipartite nuclear localization signal (NLS) domain that is required for its nuclear shuttling [64] and contains five nuclear export signal NES domains involved in chromosome region maintenance 1 (CRM1/exportin 1)-dependent nuclear export [65]. It has been proposed that the interaction of FANCA with the sorting nexin 5 protein (SNX5) may be involved in its subcellular trafficking [66]. Other studies have reported that FA core complex proteins could be found in the cytoplasm, in an unbound form, but also in a 600 kDa complex composed of at least four FA core complex proteins (i.e., FANCA, FANCC, FANCF, and FANCG) [57, 61, 67, 68]. Other studies have suggested the formation of different cytoplasmic subcomplexes, including FANCA/FANCG [36, 38], FANCB/FANCL [23], and FANCC/FANCE [33, 69–71]. It has been suggested that these subcomplexes are probably translocated to the nucleus independently before association into a core complex through FANCF, which acts as a linker protein [33, 72].

Because the nuclear presence of both FANCC and FANCE depends on each other, an interdependence between FA proteins has been proposed. Indeed, studies on FA mutant cell lines have shown that the core complex fails to form if one protein is absent or mutated [33]. Studies of synchronized cells have shown that FA proteins shuttle between the nucleus and the cytoplasm during the cell cycle. Early in the G1 phase, FA core complex proteins are localized to the cytoplasm, at the G1-S border, they are loaded onto chromatin, and throughout mitosis they migrate to the nuclear periphery to become completely excluded from the condensed chromosomes [67, 73, 74]. The exclusion of FA proteins from condensed chromosomes occurs in the absence of DNA damage, whereas treatment with MMC results in an increased binding of core complex proteins to chromatin [67, 73, 75]. Considering these results, it is not inconceivable to surmise that the cytoplasmic forms of FA proteins or FA subcomplexes are likely to be critical for cell signaling events in normative conditions.

4. Posttranslational Modification of FA Proteins

FA proteins undergo multiple posttranslational modifications, including monoubiquitination, phosphorylation and proteolytic processing. The most widely studied modification is the monoubiquitination of FANCD2 and FANCI. Although only two of the fifteen FA proteins are monoubiquitinated, several FA proteins, including FANCA, FANCE,

FANCG, FANCD2, FANCI, and FANCM, are phosphorylated, and two FA proteins are regulated through a caspase-mediated proteolytic process. These data suggest that post-translational modifications play an important role in FA proteins activity. In this section, we will only discuss FA protein modifications other than the ubiquitination of FANCD2 and FANCI because it has been extensively discussed [47, 76, 77].

The FANCA protein was the first FA protein shown to be phosphorylated [58, 63, 78]. The FANCA phosphorylation site was first thought to be located at S1149 [79], which harbors an AKT kinase consensus sequence; however, the FANCA S1149A mutant was shown to be more efficiently phosphorylated than wildtype FANCA. The FANCA phosphorylation site was later identified by mass spectrometry as serine 1449 [80]. The phosphorylation of FANCA at S1449 is functionally important because it was found to be defective in lymphoblasts from several patients with FA, and FANCA S1449A failed to fully rescue FA-A mutant cells. A novel wortmannin-sensitive protein kinase termed FANCA-PK was first suggested as the kinase responsible for FANCA phosphorylation [81]; however, the true kinase that phosphorylates FANCA in response to DNA damage was later identified as ATR [80]. FANCA was also shown to associate with the IKK signalosome through direct interaction with IKK2, the IκB Kinase-2 [82]. Although this kinase affected the phosphorylation state of several FANCA-associated proteins, no clear, direct phosphorylation of FANCA by IKK2 has been reported. Different groups have reported that FANCG is also phosphorylated. FANCG phosphorylation occurs at serines 7, 383, and 387, with the two latter sites being cell cycle-dependent and actively phosphorylated during mitosis [83–85]. The exclusion of FA proteins from chromosomes during mitosis appears to coincide with phosphorylated FANCG. All the FANCG phosphorylation sites are functionally important because mutant forms of these FANCG serine residues compromised its ability to rescue FA-G mutant cells. The FANCC-interacting protein kinase cdc2 [86] was shown to be required for the phosphorylation of at least the S387 FANCG residue. The kinases responsible for the phosphorylation of the other FANCG target sites are unknown.

The FANCD2 protein undergoes two different posttranslational modifications. In addition to monoubiquitination on lysine 561, FANCD2 is phosphorylated on several residues by different kinases depending on the cellular signal. In response to various DNA damaging agents, including ultraviolet light, MMC, hydroxyurea, and ionizing radiation, FANCD2 is phosphorylated on T691 and S717 followed by S222 in an ATM/ATR-dependent mechanism [87, 88]. S222 phosphorylation triggers the activation of an intra-S-phase checkpoint response. In addition, the FANCD2-T691A/S717A double mutant does not complement the MMC sensitivity in FA-D2 cells, suggesting that this post-translational modification is required for FANCD2 function in the DNA damage response. Other ATM-dependent FANCD2 phosphorylation sites have been described and have been shown to be functional in *in vitro* assays, including S1401, S1404, and S1418, and only S1401 has been confirmed *in vivo*. S331 of FANCD2 has been shown to be phosphorylated by the checkpoint kinase 1 (CHK1) and is essential for MMC resistance [89]. Although FANCD2 phosphorylation is independent of other posttranslational modifications, it promotes or enhances the monoubiquitination process. For instance, ATR-mediated phosphorylation of FANCD2 is essential for its monoubiquitination in response to DNA damage as shown by absence of FANCD2 monoubiquitination in ATR-deficient cells and cells from patients with Seckel syndrome, a disease resembling FA [90]. FANCI was identified in the search for ATR-inducible phosphorylated proteins in response to ionizing radiation [50]. Three phosphorylation sites were detected in the human FANCI protein (S730, T952, and S1121), and two other sites were detected in the mouse protein (S555 and T558). FANCI phosphorylation is essential for the FA pathway activation following DNA damage as measured by FANCD2 monoubiquitination [91].

FANCE and FANCM phosphorylation has also been studied in the context of induced DNA damage. In response to DNA damage, CHK1 was shown to phosphorylate FANCE on two residues, notably T346 and S374 [92]. FANCE phosphorylation is required for MMC resistance but is dispensable for FA pathway activation as measured by FANCD2 monoubiquitination. The CHK1-induced phosphorylation of FANCE promotes its assembly with FANCD2 into nuclear foci and promotes its degradation, serving as a potential negative regulatory mechanism of the FA pathway. FANCM is a phosphoprotein that contains multiple predicted ATR phosphorylation sites and becomes hyperphosphorylated in response to DNA damage and during mitosis [27, 93]. FANCM phosphorylation occurs independently of the FA core complex activation leading to the monoubiquitination of the ID complex.

Finally, another posttranslational modification found in FANCC is caspase-mediated proteolytic processing [94]. Similar to many proteins involved in signaling mechanisms, FANCC is cleaved by a caspase during apoptosis. This proteolytic modification of FANCC is not required for its function in DNA repair or DNA damage signaling, but the cleavage of FANCC inhibits its suppressor of apoptosis function. Recently, a second FA protein, FANCD2 was shown to be regulated by a caspase-mediated proteolytic processing in response to DNA crosslink damage and the non-DNA damaging agent TNF-α [95]. Both the TNF-α and DNA crosslink-mediated disappearance of FANCD2 was blocked by caspase inhibitors but not by proteasome inhibitors, suggesting that FANCD2 is regulated through a caspase-dependent mechanism in response to cellular stress.

5. FA Proteins Partners with Roles in Oxidative Metabolism

The abnormal sensitivity of FA cells to reactive oxygen species (ROS) was first suggested in 1977 by Nordenson [96] who showed reduced chromosomal breaks in FA lymphocytes cultured in the presence of superoxide dismutase, catalase, or both enzymes. The role of oxygen on chromosomal instability in FA mutant cells was confirmed by

Table 1: List of FA protein partners.

Functional class	Specific function	Protein name	Interacts with	References
Transcription	Transcriptional repressor	FAZF	FANCC	[112, 147]
		HES1	FANCA, F, G, L	[44]
	Stress-induced chaperone	Hsp70	FANCC	[112, 140]
		GRP94	FANCC	[60, 112]
	Chromatin modifier	BRG1	FANCA	[112, 154]
Cell cycle	Serine/threonine kinase	cdc2	FANCC	[86, 112]
Cell signaling	Cytokine response	STAT1	FANCC	[133]
		IKK2	FANCA	[79, 82, 112]
	Secondary modification	Akt kinase	FANCA	[79, 112]
Oxidative metabolism	Electron transfer	RED	FANCC	[112, 114]
	Cytosolic Detoxifying enzyme	GSTP1	FANCC	[112, 113]
	Metabolism of xenobiotics	CYP2E1	FANCG	[112, 115]
	Antioxidant enzyme	PRDX3	FANCG	[68]
Transporter	Intracellular trafficking	SNX5	FANCA	[66, 112]

Joenje et al. in 1981; they showed attenuated chromosomal aberrations at low oxygen tension (5%) but aggravated chromosomal aberrations at high concentrations of oxygen [97]. Subsequently, several reports indicated that FA cells were hypersensitive to oxygen radicals showing reduced growth and blockage in the G2 phase of the cell cycle [98–101]. Increased ROS in FA leucocytes has also been reported [99]. In addition, the overexpression of detoxifying enzymes, the inhibition of enzymes involved in oxidation or the use of antioxidants in FA cells reduced the rate of spontaneous chromosomal breakage and abolished the DNA damaging effects of MMC [98, 102–106]. Other studies have established a link between an altered redox state and reduced proliferation, reduced growth, and altered cytokine responses in FA cells including hematopoietic progenitors [107–110]. Studies from FancC/Sod1 double mutant mice exhibiting defects in hematopoiesis including bone marrow hypocellularity and cytopenia, which is reminiscent of phenotypes observed in patients with FA, suggest that an abnormal redox state contribute to BMF in FA [111].

Together, these data indicate that FA proteins may be involved in responses to endogenous oxidative stress or in the regulation of the cell redox state. This hypothesis is further supported by studies showing interactions between FA proteins and proteins involved in oxygen metabolism [112]. For instance, FANCC has protein partners with roles in redox metabolisms, including glutathione *S*-transferase π I (GSTπI) and NADPH cytochrome-P450 reductase (RED) [113, 114], whereas FANCG interacts with cytochrome P450 2E1 (CYP2E1) and the mitochondrial peroxiredoxin-3 (PRDX3) [68, 112, 115]. Through these interactions, FA proteins were shown to attenuate the redox activation of xenobiotics and to prevent apoptosis. Consequently, in FA mutant cells, a loss of interaction between FA proteins and these molecular antioxidants leads to an aberrant redox metabolism that translates into ROS-mediated DNA damage and cell death (see Table 1).

6. FA Proteins Partners with Roles in Cytokine Signaling and Apoptosis

It is well established that FA mutant cells are prone to apoptosis. The FA literature is rich in reports pertaining to FA mutant cells (human bone-marrow-derived cells, lymphocytes, fibroblasts, and mouse embryonic fibroblasts) that show increased apoptosis or reduced cell growth in response to various agents including ROS inducers, DNA damaging agents, growth factor withdrawal, and cytokines. It is clear from many studies of patient-derived cells and cells from FA mouse models that FA proteins are involved in pathways that regulate cell survival or cell death [116–121]. For instance, two FA proteins, FANCC and FANCD2, are caspase targets [94, 95], and FANCC overexpression or the inhibition of its caspase-mediated cleavage prevents or delays apoptosis, even in wildtype cells supporting the idea of a cell survival function of the FA proteins [94, 119, 122]. The role of FANCC in cell survival has been linked to oxidative metabolism as described above but it may also be linked to cytokine-mediated cellular responses because many cytokine-mediated signaling events lead to apoptosis. It has been suggested that abnormal cytokine regulation may account for the progressive BMF observed in patients with FA because TNF-α overproduction and underproduction of Il-6 have been detected in the sera of patients with FA [123–125]. FA-C mutant cells and *FancC*$^{-/-}$ progenitor and stem cells are hypersensitive to the inhibitory cytokines including TNF-α and IFN-γ, and show suppressed growth and increased apoptosis at doses that do not affect normal cells [116, 122, 126, 127]. In addition, the continuous injection of low IFN-γ doses *in vivo* leads to BMF in FA

mice [128, 129], whereas TNF-α leads to clonal evolution and leukemia in this FA mouse model [130]. In support of these altered cytokine responses in FA cells, the cytokine-response genes myxovirus A (*MxA*), IFN response factor 1 (*IRF1*), $p21^{CIP/WAF}$, and IFN-stimulated gene factor 3 (*ISGF3*γ) were highly expressed in FA mutant cells without exogenous cytokine stimulation, while corrected cells suppressed this overproduction and restored their MMC resistance [116, 131, 132]. These data suggest that FA proteins, or at least FANCC, function to modulate a cytokine-mediated signal. Indeed, FANCC was shown to directly interact with signal transducer and activator of transcription 1 (STAT1), which is an IFN signal transducer [133]. FANCC functions as a control factor for STAT1 docking at the IFN-γR complex and subsequent activation of the IFN type II signaling cascade [133]. Thus, the STAT1 activation defect observed in FA-C cells results in an altered nuclear STAT1-DNA complex, which diminishes the expression of IRF1. The STAT1-FANCC interaction is also induced by other cytokines, including IFN-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and stem cell factor, whereas mutant FANCC does not associate with STAT1 in cells stimulated with these factors. FANCC seems to regulate IFNγ-inducible genes (e.g., IRF1, $p21^{WAF}$, and ISGF3γ) independently of STAT1 binding. An altered response to type I IFN was also observed in FANCC mutant and $FancC^{-/-}$ cells, as shown by the reduced phosphorylation of the Janus kinases, Jak1 and Tyk2, and the subsequently diminished phosphorylation of STAT1, STAT3, and STAT5 [134]. This altered Tyk2 response translates into reduced numbers of CD4-positive cells in $FancC^{-/-}$ mice. Because Tyk2 plays a role in the differentiation and maintenance of T helper cells, failure of FANCC to normally activate Jak/STAT signaling may result in impaired immune cell differentiation and immune defects, as reported in patients with FA [135–139].

FANCC has been shown to physically interact with Hsp70 [140]. This interaction appears to be required for protection against TNF-α and IFN-γ-induced apoptosis because reduced Hsp70 expression sensitizes normal cells to these cytokines but does not further augment the hypersensitivity to apoptosis in FA-C cells. Because Hsp70 is known to suppress the IFN-inducible double-stranded RNA (dsRNA)-dependent protein kinase (PKR) activation [141] and FA-C cells have constitutively activated PKR [142], FANCC was shown to inhibit the kinase activity of PKR through physical interaction with Hsp70 [143]. Although this activity is independent of a functional FA complex, the FA core complex protein FANCA was found to interact with IKK2 (or IKKβ) a kinase and a component of the IKK signalosome [82]. The IKK signalosome is a critical mediator of the cellular response to stressors such as dsRNA and cytokines [144, 145]. Deletion of IKK2 has been shown to affect the development of CD4-positive cells [146]. Because $FancC^{-/-}$ mice have reduced numbers of $CD4^+$ cells and two FA proteins have partners that participate in cytokine-activated signaling cascades affecting the development of these lymphocytes, we can speculate that FA proteins may act as converging key molecules.

7. FA Protein Partners with Roles in Transcription

Another FA protein role less considered is the regulation of transcription. Several FA proteins have interacting partners directly involved in transcriptional regulation. The first FA protein partner identified that acts in transcription is FAZF (FA Zinc Finger) [147]. FAZF, also known as RoG (for repressor of GATA) [148], PLZP (for PLZF-like zinc finger protein) [149] and TZFP (for testis zinc finger) [150], is a transcriptional repressor that belongs to the BTB/POZ family of proteins and is similar to the PLZF protein [147]. This family of transcriptional repressors was shown to be important for several developmental processes including tissue proliferation and differentiation and tumor formation. FAZF was identified in a yeast 2-hybrid screen with FANCC. FAZF was shown to be highly expressed in CD34-positive progenitor cells; it further increased during proliferation of these cells and decreased during their terminal differentiation [151]. FAZF acts as a negative regulator of transcription. Because a disease-causing mutation in FANCC interferes with FAZF binding [147], and $FancC^{-/-}$ hematopoietic stem/progenitor cells show increased cycling and aberrant cell cycle control [152], a plausible hypothesis is that the FANCC-FAZF interaction in hematopoietic stem/progenitor cells leads to the repression of critical target genes required for growth suppression.

A second transcriptional repressor identified as a FA-binding protein is the hairy enhancer of split 1 (HES1) [44]. HES1 is a member of a highly conserved family of hairy-related basic helix-loop-helix (bHLH)-type transcriptional repressors. HES1 was shown to interact directly with several components of the FA core complex. The FA core complex was shown to contribute to the transcriptional regulation of HES1-responsive genes, both positively (*HES1*) and negatively (cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$). Two mechanisms of regulation by FA proteins have been proposed. The first proposed mechanisms is that by interacting with HES1, FA core complex proteins antagonize HES1-mediated transcriptional repression by interfering with the assembly of the HES1/transducing-like-enhancer of split (TLE) corepressor complex at the *HES1* promoter [153]. The second proposed mechanism involves an indirect mechanism where the binding of FA proteins with HES1 influences HES1 affinity or its specificity for promoters, such as that of $p21^{cip1/waf1}$.

The brahma-related gene 1 protein (BRG1) has also been identified as a FA-binding partner through a yeast 2-hybrid screen [154]. BRG1 is the central catalytic subunit of the SWI/SNF family of ATP-dependent chromatin remodeling complexes [155]. BRG1 is a major coregulator of transcription, both in activation and repression, through chromatin modulation. Although the FANCA-BRG1 interaction has been shown in cells, the functional impact of this interaction remains unclear.

In view of these FA protein partners with roles in transcriptional regulation and the fact that the FA core complex possesses E3 ubiquitin ligase activity, it is possible that FA proteins act as transcriptional coregulators through

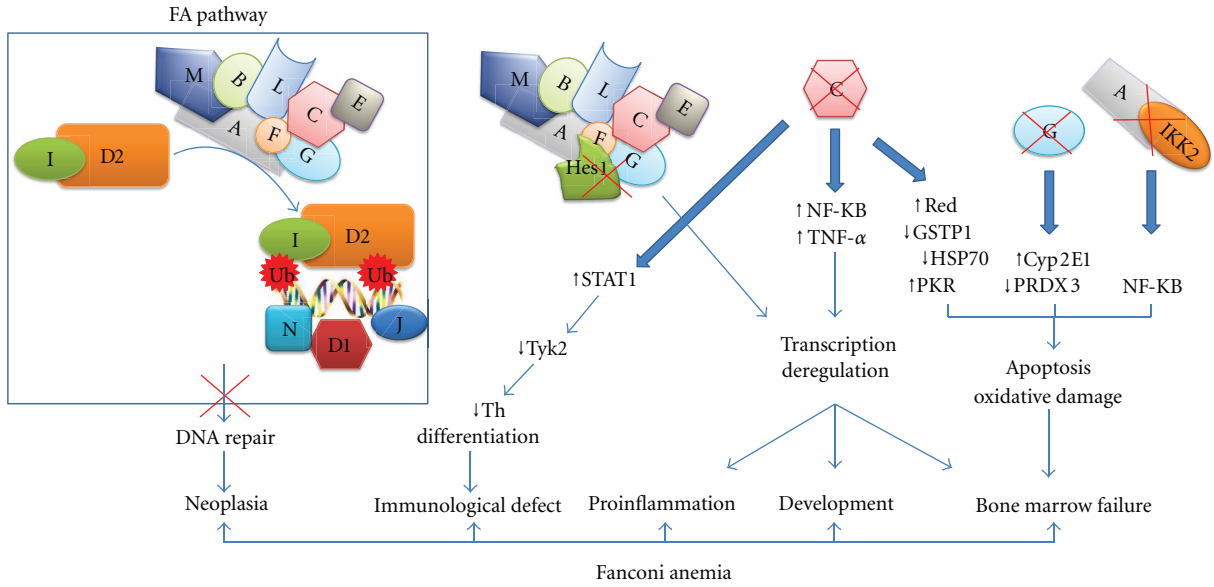

Figure 1: Putative roles of FA proteins through their interacting partners. The involvement of FA proteins with their protein partners in the different molecular mechanisms that lead to regulation of transcription, cell cycle regulation, ROS detoxification, DNA repair, and cell survival. Loss of protein interactions between FA proteins and their partners through disease causing mutations in a FA gene could lead to a defective molecular function resulting in an array of phenotypes including BMF and congenital malformations.

the posttranslational modification of these transcriptional regulators.

8. Conclusion

Since the discovery of *FANCC*, the first identified FA gene in 1992 [15], there have been significant advances in the FA molecular biology field. These advances mostly include characterization of the canonical FA pathway, which is activated in response to DNA crosslink damage. It is clear that FA proteins are required for DNA crosslink repair; however, the question of how a defective FA protein leads to BMF, and developmental abnormalities remains elusive. It is obvious that absence of a functional FA protein affects many cellular and molecular functions and leads to an array of cellular phenotypes (see Figure 1). A perplexing question is whether FA proteins interactions with their nonrepair partners act only as modifiers of the clinical manifestation of FA. Once we reconcile all the notions related to FA proteins role in these various cellular and molecular activities, we may then obtain a clearer picture of the complexities of this molecular puzzle.

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Research Article

A Dutch Fanconi Anemia *FANCC* Founder Mutation in Canadian Manitoba Mennonites

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Fanconi anemia (FA) is a recessive DNA instability disorder associated with developmental abnormalities, bone marrow failure, and a predisposition to cancer. Based on their sensitivity to DNA cross-linking agents, FA cells have been assigned to 15 complementation groups, and the associated genes have been identified. Founder mutations have been found in different FA genes in several populations. The majority of Dutch FA patients belongs to complementation group FA-C. Here, we report 15 patients of Dutch ancestry and a large Canadian Manitoba Mennonite kindred carrying the *FANCC* c.67delG mutation. Genealogical investigation into the ancestors of the Dutch patients shows that these ancestors lived in four distinct areas in The Netherlands. We also show that the Dutch and Manitoba Mennonite *FANCC* c.67delG patients share the same haplotype surrounding this mutation, indicating a common founder.

1. Introduction

Fanconi anemia is an inherited chromosomal instability disorder associated with developmental abnormalities, bone marrow failure, and a predisposition to cancer. A characteristic feature of FA cells is their hypersensitivity to DNA cross-linking agents such as diepoxybutane (DEB) and mitomycin C (MMC). This feature has been used to assign FA cells to different complementation groups. Currently, 15 different complementation groups and their associated genes have been identified. Of these, 14 have an autosomal recessive, and one has an X-linked, mode of inheritance [1–3]. The majority of FA patients belong to complementation group FA-A (65%) followed in frequency by FA-C (10%) and FA-G (10%) [4].

The incidence of FA is approximately 1 in 130,000 live births, with a carrier frequency of approximately 1 in 181 [5]. In some ethnic groups, however, the incidence is much higher due to genetic isolation and a founder effect. For example, founder mutations in the *FANCA* gene have been found in several populations including the South African Afrikaners, Spanish Gypsies, and Moroccan Israeli Jews [6–8]. Furthermore, sub-Saharan Blacks and Japanese carry founder mutations in the *FANCG* gene [9, 10]. In addition, *FANCC* c.456 + 4A > T (also known as IVS4 + 4A > T) is a previously identified founder mutation in the *FANCC* gene

in the Ashkenazi Jewish population [11, 12]. Remarkably, the *FANCC* c.456 + 4A > T mutation has a severe phenotype in Ashkenazi Jews, but a milder phenotype in Japanese FA patients, suggesting the presence of unidentified modifying factors [13]. The majority of Dutch FA patients belong to complementation group FA-C, with c.67delG (also known as 322delG) being the predominant mutation ([14], personal communication H. Joenje). In this paper, we report 15 patients of Dutch ancestry and a large Canadian Manitoba Mennonite kindred harbouring the *FANCC* c.67delG mutation. The presence of the *FANCC* c.67delG mutation in this kindred together with the fact that the Mennonites arose in The Netherlands around 1550–1600 AD suggested a common founder for the Dutch and Mennonite c.67delG mutation. We demonstrate that the Dutch and Manitoba Mennonite *FANCC* c.67delG patients do, in fact, share the same haplotype surrounding this mutation, indicating a common genetic origin.

2. Materials and Methods

2.1. Dutch Patients. All 15 patients with the *FANCC* c.67delG mutation lived in The Netherlands except VU449, VU654, and VU911 who lived in the United Kingdom, Northern France, and Canada, respectively and had Dutch grand and great grandparents. The FA diagnosis was based on clinical symptoms suggestive of FA, in combination with a positive result from a chromosomal breakage test using a standard DNA cross-linking agent. Classification as an FA-C patient was based on complementation studies or sequencing analysis.

Genomic DNA was isolated from fibroblasts, blood, or lymphoblastoid cell lines from previously diagnosed *FANCC* c.67delG patients using the Qiagen Blood mini kit (Qiagen, Venlo, The Netherlands).

2.2. Canadian Manitoba Mennonite Family. The proband of this family was diagnosed with FA at the age of five years, when she presented with bone marrow failure. At birth, there was documented intrauterine growth retardation, joint contractures, and a ventricular septal defect. At the time of presentation, the proband was also noted to be of small stature and to have a triangular facies and multiple café-au-lait macules. She died at the age of six years from complications following allogeneic bone marrow transplantation from her HLA-identical sibling. A younger sibling (VU1454) was subsequently diagnosed with FA and was found to be homozygous for the *FANCC* c.67delG mutation. She had normal growth parameters at birth and no noted FA-associated anomalies except for the development of café-au-lait macules.

A field trip was conducted to the rural community in which this family lived, and family members were given an FA information session. Forty-five members of the extended family consented to participate in this study. Clinical histories were obtained, and cheek swabs were collected for genotyping. Minors who provided assent or whose parents consented on their behalf were also included in the study.

2.3. Genotype Analysis. Patients and family members were genotyped using fluorescently labelled microsatellite markers and single-nucleotide polymorphisms (SNPs) in an 8 Mb region surrounding the *FANCC* gene. Markers used were the CA repeat markers D9S1842, D9S1781, D9S197, D9S1689, D9S1816, D9S1809, D9S1851, D9S180, and D9S176. Amplification of the CA repeat markers was performed with the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Samples were analyzed on ABI 3730 or ABI 310 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). SNPs used were rs1331216, rs2277182, and rs1016013. Amplification of the SNPs was performed with the GeneAmp PCR system 9700, and the PCR products were purified using a SAP/EXO treatment (Amersham Biosciences, Uppsala, Sweden) according to manufacturer's instructions. Sequencing was performed with the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Samples were analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Genotyping of the Canadian patient and family members for the *FANCC* c.67delG mutation was performed using standard methods (GeneDx, Inc., Gaithersburg, MD, USA). In brief, an allele-specific assay was designed to detect the presence of the single base deletion, and all samples showing the heterozygous presence of the deleted G nucleotide were sequenced bidirectionally to confirm the findings.

3. Results

Eleven of the 15 FA-C patients of Dutch ancestry analyzed in this study were homozygous for the c.67delG mutation, and 4 were compound heterozygous, each with a different second mutation. Using polymorphic microsatellite markers and SNPs, the smallest common haplotype in the 26 alleles harbouring the c.67delG mutation was determined to be 0.8 Mb, covering the region from rs1016013 until D9S1816 (Table 1(a)). A larger common haplotype of 2 Mb (D9S197-D9S1816) was found in 23 alleles. The 3 alleles responsible for the decrease of 1.2 Mb in the common haplotype belonged to 3 sibs (VU1134, VU1135, and VU1136) homozygous for the c.67delG mutation. The presence of two partially differing haplotypes in these homozygous c.67delG patients was confirmed in the parents (results not shown). The identical haplotype surrounding the c.67delG mutation indicates that all 15 FA patients have coinherited this stretch of DNA from a common ancestor. The expected size of a co-inherited stretch of DNA can be estimated by the equation 200/(number of meioses), which is based on the assumption that recombinants occurring in meioses from the first common founder on either side of the mutation are uniformly distributed over the interval of 0 to 100 Mb [15]. This indicates that the descent tree of the 15 FA patients, up to the common founder, given a shared common stretch of DNA of approximately 0.8 Mb, counts about 250 meioses. This corresponds to about 10–20 generations and a common founder living in the 16–18th centuries.

Genealogical investigations into the ancestors of these patients only retrieved 6 or 7 generations from the archives,

Table 1: The haplotype associated with the *FANCC* c.67delG mutation determined in 15 patients of Dutch ancestry (Table 1(a)) and in the affected sib of the proband, the parents, and healthy sibs of the Mennonite kindred (Table 1(b)). In Table 1(a) simplified, deducted allele lengths are shown and in Table 1(b) the observed allele lengths are shown. For comparison, patient VU1135 is included in both tables. The dark grey boxes shows the smallest common haplotype of 0.8 Mb. The light and dark grey box, together show the larger common haplotype of 2 Mb. The boxed areas indicate homozygosity for the relevant markers. At the top, the distances in Mb of the markers to the c.67delG mutation are indicated. Patients VU001 and VU002 are siblings as well as patients VU1134, VU1135, and VU1136.

(a)

	Mb	4.2	3.7	1.8	1.6	1.3	0.9	0.6	0	0.2	0.5	1.5	2.6	4.0
		D9S1842	D9S1781	D9S197	D9S1689	rs1331216	rs2277182	rs1016013	FANCC	D9S1816	D9S1809	D9S1851	D9S180	D9S176
Patients						C/T	A/G	C/T	delG					
VU001		4	1	1	4	C	G	C	delG	1	4	3	1	2
		5	1	1	4	C	G	C	delG	1	2	2	1	6
VU002		4	1	1	4	C	G	C	delG	1	4	3	1	2
		5	1	1	4	C	G	C	delG	1	2	2	1	6
VU158		6	1	1	4	C	G	C	delG	1	4	3	1	2
		7	1	1	4	C	G	C	delG	1	3	2	1	6
VU166		2	3	1	4	C	G	C	delG	1	1		1	2
		3	3	1	4	C	G	C	delG	1	1		3	3
VU239		7	2	1	4	C	G	C	delG	1	4	3	1	6
		1	6	1	5	T	A	T		1	2	6	2	2
VU449		1	4	1	4	C	G	C	delG	1	1	3	2	3
		3	2	2	2	C	A	T		2	1	3	1	3
VU654		4	1	1	4	C	G	C	delG	1	4	3	3	5
		5	1	1	4	C	G	C	delG	1	4	3	3	5
VU806		7	1	1	4	C	G	C	delG	1	3	3	3	7
		3	6	4	4	T	G	T		3	3	2	2	2
VU811		1	1	1	4	C	G	C	delG	1	4	3	1	2
		7	1	1	4	C	G	C	delG	1	4	4	3	4
VU911		5	1	1	4	C	G	C	delG	1	1	3	4	2
		7	3	1	4	C	G	C	delG	1	4	3	2	2
VU1131		3	1	1	4	C	G	C	delG	1	4	3	1	3
		7	3	1	4	C	G	C	delG	1	1	2	3	4
VU1134		7	4	1	4		G	C	delG	1	4	3	1	4
		3	5	2	3		A	C	delG	1	4	3	1	4
VU1135		7	4	1	4	C	G	C	delG	1	4	3	1	4
		3	5	2	3	T	A	C	delG	1	4	3	1	4
VU1136		7	4	1	4	C	G	C	delG	1	4	3	1	4
		3	5	2	3	T	A	C	delG	1	4	3	1	4
VU8115		5	6	1	4	C		C	delG	1	1	7	4	2
		5	4	5	3	T		T		1	5	2	2	1

(b)

	Mb	4.2	3.7	1.8	1.6	1.3	0.9	0.6	0	0.2	0.5	1.5	2.6	4.0
		D9S1842	D9S1781	D9S197	D9S1689	rs1331216	rs2277182	rs1016013	FANCC	D9S1816	D9S1809	D9S1851	D9S180	D9S176
Individual						C/T	A/G	C/T	delG					
VU1135		147	160	201	92	C	G	C	delG	151	140		220	137
		151	166	212	90	T	A	C	delG	151	140		220	137
Affected sib		150	146	201	92	C	G	C	delG	151	140		220	145
(VU1454)		150	146	201	92	C	G	C	delG	151	140		220	133
Mother		150	146	201	92	C	G	C	delG	151	140		220	145
		148	146	201	92	C	G	C	—	157	136		228	137
Father		150		201		C	G	C	delG	151			220	133
		148		211		C	G	C	—	159			220	131
sib1		150		201	92	C	G	C	delG	151	140		220	145
		148		211	92	C	G	C	—	159	126		220	131
sib2		150	146	201	92	C	G	C	delG	151	140		220	145
		148	146	211	92	C	G	C	—	159	126		220	133

and therefore it was not possible to reveal the common ancestor. However, for patient VU166 and patient VU1131, a common ancestor couple was found living around 1800 AD, and consanguinity was shown for patients VU166, VU1134, VU1135, and VU1136 (Figure 1). Our genealogical investigations also showed that most of these 6th and 7th generation ancestors lived in 4 distinct areas in The Netherlands (Figure 2), further supporting the possibility of a single common ancestor.

Forty-five individuals in the Manitoba family were genotyped for the *FANCC* c.67delG mutation. Eighteen of the 45 (40%) participants were heterozygous for the *FANCC* c.67delG mutation, and no new homozygotes were identified. The medical histories collected from kindred members did not reveal any early-onset cancer, but did document several other disorders already known to be overrepresented in the Manitoba Mennonite population, including severe combined immunodeficiency (SCID) (C.R. Greenberg, unpublished observation) and hypertrophic cardiomyopathy (HCM) [16]. Consanguinity could not be shown in five generations of ancestors. Haplotype analysis was done on the affected sib, two unaffected sibs, and the parents of the proband; it revealed the same common haplotype as found in the Dutch *FANCC* c.67delG patients (Table 1(b)).

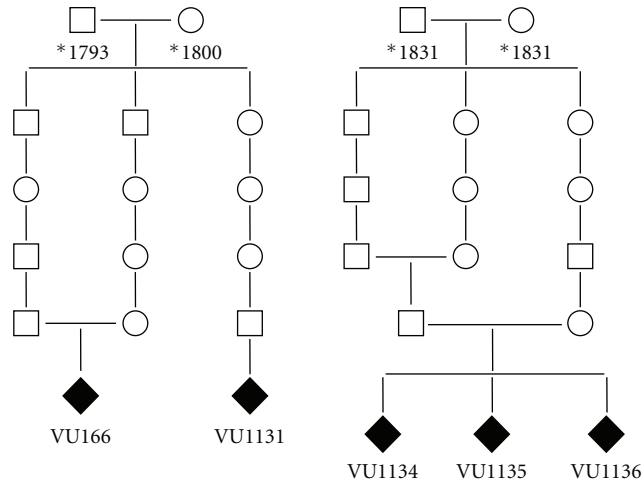

Figure 1: Pedigrees showing consanguinity for patients VU166 and VU1134/35/36. Common ancestors of patients VU166 and VU1131 were found living around 1800. *year of birth.

4. Discussion

This paper shows that c.67delG in *FANCC* is a founder mutation that has probably originated in The Netherlands. This Dutch mutation is also present in a large Mennonite kindred with members living in the province of Manitoba, Canada, and in the United States. The Mennonites are a religious denomination named after one of its founders, Menno Simons, who was born in 1496 in The Netherlands in the province of Friesland, which is the area where the ancestors of the Dutch c.67delG patient VU811 lived. They fled religious persecution over the centuries to the east and

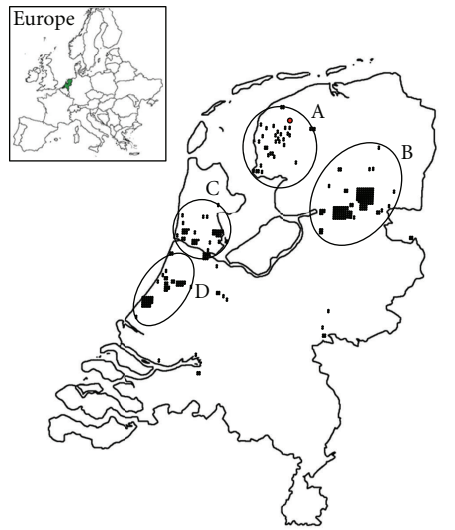

Figure 2: Domicile of the ancestors of patients. VU811 (area A), VU166, VU1131, and VU1134/35/36 (area B), VU239 (area C), and VU806 (area D). Ancestors of patients VU001 and VU002 lived in area B, C, and D. Ancestors of patient VU158 lived in areas C and D.

Russia, and from there to other parts of the world, and many settled in western Canada during the 19th century [17]. It is therefore likely that the Manitoba Mennonite FA-C patients in this study were descended from the same common ancestor as the Dutch patients in this study. This founder must have lived more than 200 years ago, since genealogical investigation could not identify the common ancestor among the ancestors living after 1800 AD.

Even though the c.67delG mutation is generally thought to be a mild mutation probably due to FAC polypeptide isoforms retaining partial function leading to a mild disease phenotype [18], clinical variability can be seen. The affected Mannitoba Mennonite siblings showed apparent divergent phenotypes, but neither had skeletal abnormalities, a characteristic of the mild disease phenotype associated with the c.67delG mutation.

Based on the high degree of consanguinity, the presence of the Dutch founder mutation, and the geographic origin being The Netherlands, we suspect that the carrier frequency for this c.67delG mutation in the North American Mennonite population is higher than expected for a rare recessive trait. An unselected and unbiased survey would be required in order to determine the actual *FANCC* carrier frequency in this Mennonite population. Several genetic disorders are well known to be overrepresented in this population, but FA has not previously been included in this list. Both HCM and 17 alpha-hydroxylase deficiency have previously been shown to be caused by Dutch founder mutations [19–21] among the Mennonites. Since present generations continue to marry according to traditional custom, we may see an increased frequency of FA-C patients in future generations. Therefore, Mennonite communities should be offered comprehensive genetic counselling and carrier testing for FA.

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Research Article

Diagnosis of Fanconi Anemia: Mutation Analysis by Next-Generation Sequencing

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Fanconi anemia (FA) is a rare genetic instability syndrome characterized by developmental defects, bone marrow failure, and a high cancer risk. Fifteen genetic subtypes have been distinguished. The majority of patients (≈85%) belong to the subtypes A (≈60%), C (≈15%) or G (≈10%), while a minority (≈15%) is distributed over the remaining 12 subtypes. All subtypes seem to fit within the "classical" FA phenotype, except for D1 and N patients, who have more severe clinical symptoms. Since FA patients need special clinical management, the diagnosis should be firmly established, to exclude conditions with overlapping phenotypes. A valid FA diagnosis requires the detection of pathogenic mutations in a FA gene and/or a positive result from a chromosomal breakage test. Identification of the pathogenic mutations is also important for adequate genetic counselling and to facilitate prenatal or preimplantation genetic diagnosis. Here we describe and validate a comprehensive protocol for the molecular diagnosis of FA, based on massively parallel sequencing. We used this approach to identify *BRCA2*, *FANCD2*, *FANCI* and *FANCL* mutations in novel unclassified FA patients.

1. Introduction

Fanconi anemia (FA) is a recessive chromosomal instability syndrome with diverse clinical symptoms and a high risk for acute myeloid leukemia and squamous cell carcinoma of the head and neck region [1]. Clinical suspicion of FA is mostly based on growth retardation and congenital defects in combination with life-threatening bone marrow failure (thrombocytopenia and later pancytopenia), which usually starts between 5 and 10 years of age. However, the clinical manifestations of FA patients are highly variable, and therefore the FA diagnosis should be confirmed by a positive chromosomal breakage test and/or pathogenic mutations in one of the FA genes. Currently, mutations in 15 different genes are known to cause FA, and their gene products act in a pathway that takes care of specific problems that may arise during the process of DNA replication [2].

The conventional Sanger sequencing-based mutation screening approach for FA is time-consuming, costly, and most importantly may not detect all types of disease-causing aberrations, such as deep intronic mutations, large deletions, and amplifications. Furthermore, the existence of *FANCD2* pseudogenes obstructs the identification of pathogenic mutations in this gene when sequencing genomic DNA. Here, we demonstrate a comprehensive mutation detection approach for FA based on massively parallel sequencing (MPS) [3].

2. Methods

We designed an in-solution oligonucleotide hybridization capture kit (SureSelect, Agilent) targeting the open reading frames of all FA genes, except for regions that contain

repetitive and low complexity DNA sequences as assessed by RepeatMasker (http://www.repeatmasker.org/). All exons-, 3′-, and 5′-UTR-regions, and exon-intron boundaries were targeted by this approach (the oligonucleotide locations, in a .BED format, are available upon request). In addition, a number of other genes involved in cancer predisposition and routinely screened in our diagnostic lab were included in the enrichment kit. We used the Illumina GAIIx platform for sequencing.

To assess the performance of the custom target kit and the massively parallel sequencing method, we selected FA samples with a spectrum of different types of known variations (Table 1). The pathogenic mutations in these samples have previously been identified either by Sanger sequencing or by multiplex ligation-dependent probe amplification (MLPA) [4]. One of the samples included in the study was from a carrier of a *BRCA1* mutation. Unique barcode sequences were used for 11 DNA libraries to allow distinction between the samples that were run on a single Illumina flow-cell lane. In addition, to evaluate the sensitivity of the approach, two DNA samples were pooled before library preparation to mimic a mosaic condition.

An in-house variation detection pipeline, including a novel tool for large deletion detection, was used to score for relevant mutations.

2.1. Library Preparation. For each sample, 1.5 μg of DNA was resuspended in 75 μL of TE buffer in a Covaris microTube, and subsequently sheared in a Covaris S220 (Covaris, Inc. MS, USA) using the following settings: duty cycle = 10%, Intensity = 5, cycles per burst = 200, time = 360, Set mode = frequency sweeping, and temperature = 4°C. Fragmented DNA containing overhangs is converted into blunt ends using T4 DNA polymerase and Klenow enzyme (New England Biolabs) by incubation for 30 minutes at 20°C. The DNA sample is then purified with QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions and eluted in 32 μL of Qiagen elution buffer. Next, the 3′ ends of the fragmented DNA are adenylated using Klenow exonuclease (New England Biolabs) by incubating for 30 minutes at 37°C. The DNA sample is then purified with MinElute PCR purification kit (Qiagen) following the manufacturer's instructions and eluted in 10 μL of Qiagen elution buffer. Next, Illumina-specific index paired-end adapters (Illumina) are ligated to the 5′ and 3′ ends of DNA fragments by incubation with DNA ligase (New England Biolabs) for 15 minutes at 20°C. The adapter-ligated DNA fragments are purified with MinElute PCR purification kit, and 1 μL is used to assess proper adapter ligation by a control PCR using InPE 1.0, InPE 2.0, and a random index primer with the following PCR conditions: 30 sec at 98°C, 18 cycles of 10 sec at 98°C, 30 sec at 65°C, 30 sec at 72°C, and a final step of 5 minutes at 72°C. The quality and quantity of the library is evaluated with the Agilent 2100 Bioanalyzer on a DNA 1000 chip following the manufacturer's instructions.

For the FA gene enrichment, we used 500 ng of adapter-ligated library following the manufacturer's instructions. Briefly, DNA libraries are incubated with the biotinylated RNA custom SureSelect library "baits" for 16 hours at 60°C. Next, DNA library that hybridized to the baits is captured using magnetic beads (Dynabeads, Invitrogen), washed, and eluted in elution buffer. Primers containing unique barcode sequences are used to amplify captured libraries, and equimolar pooling is performed after quantification on a bioanalyzer. The eleven pooled DNA libraries were then sequenced on a single flow cell lane of an Illumina GAIIx using a 72 cycle multiplex paired-end sequence protocol.

Table 1: Selected FA samples with mutations previously identified by Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA), used for validation of the next-generation sequencing approach.

Sample (affected gene)	Allele 1	Allele 2	Reference
Sam 1 (*BRCA2*)	c.9253dupA	—	BIC DB#
Sam 2 (*FANCA*)	c.3558insG	—	[4]
Sam 3 (*FANCG*)	c.271–272del	c.620delT	[4]
Sam 4a (*FANCA*)	c.1464C>G	c.2632G>C	[4]
Sam 4b (*FANCA*)	Ex 15–23 del	—	[4]
Sam 5 (*BRCA1*)	c.2694dupA	—	BIC DB#
Sam 6 (*FANCC*)	c.376–377del	c.844-1G>C	[4]
Sam 7 (*FANCA*)	Ex 1–20 del	c.893+920 C>A	[5]
Sam 8 (*FANCB*)	c.811insT	absent	[4]
Sam 9 (*FANCE*)	c.91C>T	c.91C>T	[4]
Sam 10 (*PALB2*)	Ex 1–10 del	c.1802T>A	[6]
Sam 11 (*FANCI*)	c.2509G>T	NF*	[4]

#Breast cancer information core database available at http://www.research.nhgri.nih.gov/bic/.
*NF: not found.

3. Results

Sequence data from DNA libraries of eleven carriers of mutations in the FA genes: *FANCA (4), FANCB (1), FANCC (1), FANCD1 (1), FANCE (1), FANCG (1), FANCI (1), FANCN (1)*, and one individual carrying a mutation in *BRCA1,* were generated from an Illumina GAIIx sequencer. An average of 2.8 million unique reads were obtained per library resulting in a median sequence depth of about 100 fold, with an average enrichment efficiency of >75% (Figure 1). Several types of disease-causing genetic aberrations were present in the assayed DNA samples including single nucleotide substitutions, small deletions (1–8 nucleotides), and large deletions (multiple exons). We developed a variation detection pipeline detecting all these types of aberrations.

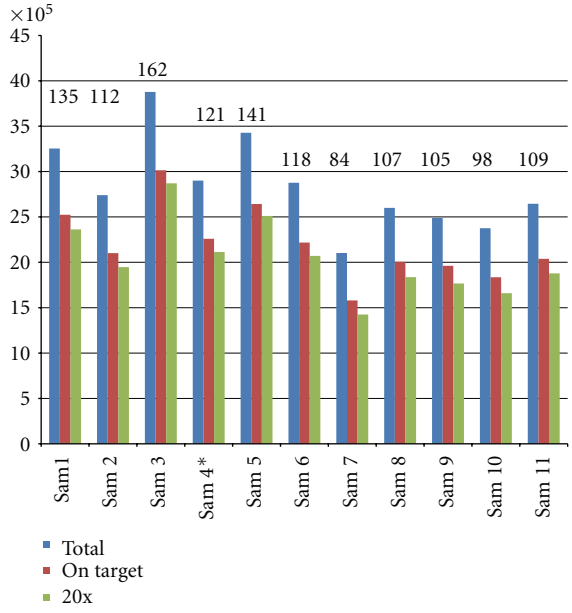

Figure 1: Specifications of 11 DNA libraries pooled and sequenced on one flow cell lane of an Illumina GAIIx. Numbers on top of the bars indicate the average sequencing depth obtained for the individual samples. Blue bars indicate total number of unique reads, red bars indicate reads that fall on target of the bait design, green bars indicate reads that fall in target regions covered with at least 20x depth. Sample 4 is a mix of genomic DNA from two individuals 4a and 4b.

3.1. Detection of Single Nucleotide Variations (SNVs) and Small Insertions/Deletions (Indels). The data analysis pipeline for the detection of SNVs and small indels that was developed is comprised of freely available tools on the web. An initial quality check of the sequence reads is followed by mapping the paired-end reads with the Burrows-Wheeler Aligner (BWA) [14] to the National Center for Biotechnology Information (NCBI) hg19 build reference genome. Subsequently, SNPs and small indels are called using Samtools [15] and Varscan [16]. The resulting list of variations is annotated with Annovar [17] that utilizes information from external databases to generate context around the mutations, such as amino acid change consequence, location to canonical splice site regions, and information about reference to dbSNP and frequencies if available. Finally, a manual filtering step is executed to prioritize relevant mutations. Low-frequency frameshifts and truncating mutations are considered pathogenic. Unreported nonsynonymous amino acid variations are analyzed *in silico* by the pathogenicity predicting programs, Align-GVGD, Polyphen-2 and SIFT [7–9] to help assess the damaging effect.

The variation detection frequency for all samples was set at a minimum of 31% of the reads covering the aberrations except for sample 4 (pre-library DNA pooling of samples 4a and 4b), for which it was set at 12%. The total number of variations detected in the samples and the subsequent reduction in number of variations through filtering is depicted in Table 2.

Using the variation detection pipeline and filtering procedure 12 SNVs out of the total of 13 were detected. The variation that escaped the initial filtering procedure resided deep in the intronic region; 920 nucleotide upstream of the exon start site. However, the variation was present in the initial variation list.

3.2. Detection of Large Insertions/Deletions. Large deletions are detected by analyzing local read depth. Firstly, a reference local read depth is established by binning read counts in a preset sliding window using data from all pooled samples. The local read depth is also determined for each sample separately, using the same preset sliding window used for the reference. A Log2 ratio is calculated for each window by dividing the local read depth of the sample by the reference. Normalization is performed through a mean shift to zero. The copy number data is projected on the open reading frame (ORF) of the gene and also projected on an exon scale, where the mean read count is aggregated on a per exon basis.

The large *FANCA* and *PALB2* deletions, previously identified by MLPA (Table 1), were readily detected using the MPS data. The DNA from sample 4b that contained a heterozygous exon 15 to 23 deletion in *FANCA* was pooled with sample 4a before library preparation. As expected, the read depth Log2 ratio as compared to the reference was approximately −0.5, which corresponds with a loss of one allele in a background of four copies. Interestingly, in sample 11 only one pathogenic nucleotide substitution in *FANCI* has previously been identified while the other mutation remained undetected. Here we show the deletion of the last exon (exon 38) of the gene as detected by our large indel analysis tool (Figure 2(d)). We confirmed the deletion by PCR and Sanger sequencing by using a SNP in the last unaffected exon 37 and two sets of primers amplifying up- and downstream of the breakpoint (Figure 3).

3.3. Identification of Pathogenic Mutations in Unclassified FA Patients. To investigate if our next-generation sequencing method also identifies mutations in unclassified FA patients, we investigated five patients that were sent in for FA mutation screening, without prior knowledge about their gene defect. Aberrations in the *FANC-A*, *-C*, *-E*, *-F*, and *-G* genes were already excluded in a routine diagnostics analysis by MLPA and Sanger sequencing. In two patients we identified known compound heterozygous pathogenic mutations in *BRCA2* and *FANCD2*. In two other patients, novel homozygous mutations were detected. In one patient (Unc4) a dinucleotide insertion/duplication in exon 9 of *FANCL* was found, which results in a frameshift and a stop codon in exon 14. The other patient (Unc3) showed a missense mutation in exon 28 of *FANCI* that changed codon 954 from a cysteine to a tyrosine. The affected amino acid is highly conserved, up to fruit fly, suggesting that it may have an important function. Moreover, *in silico* analysis by SIFT [9] and Polyphen 2 [8] predicted the amino acid change to be damaging. All the identified variations were confirmed by Sanger sequencing, and the novel variations had a proper segregation within the family.

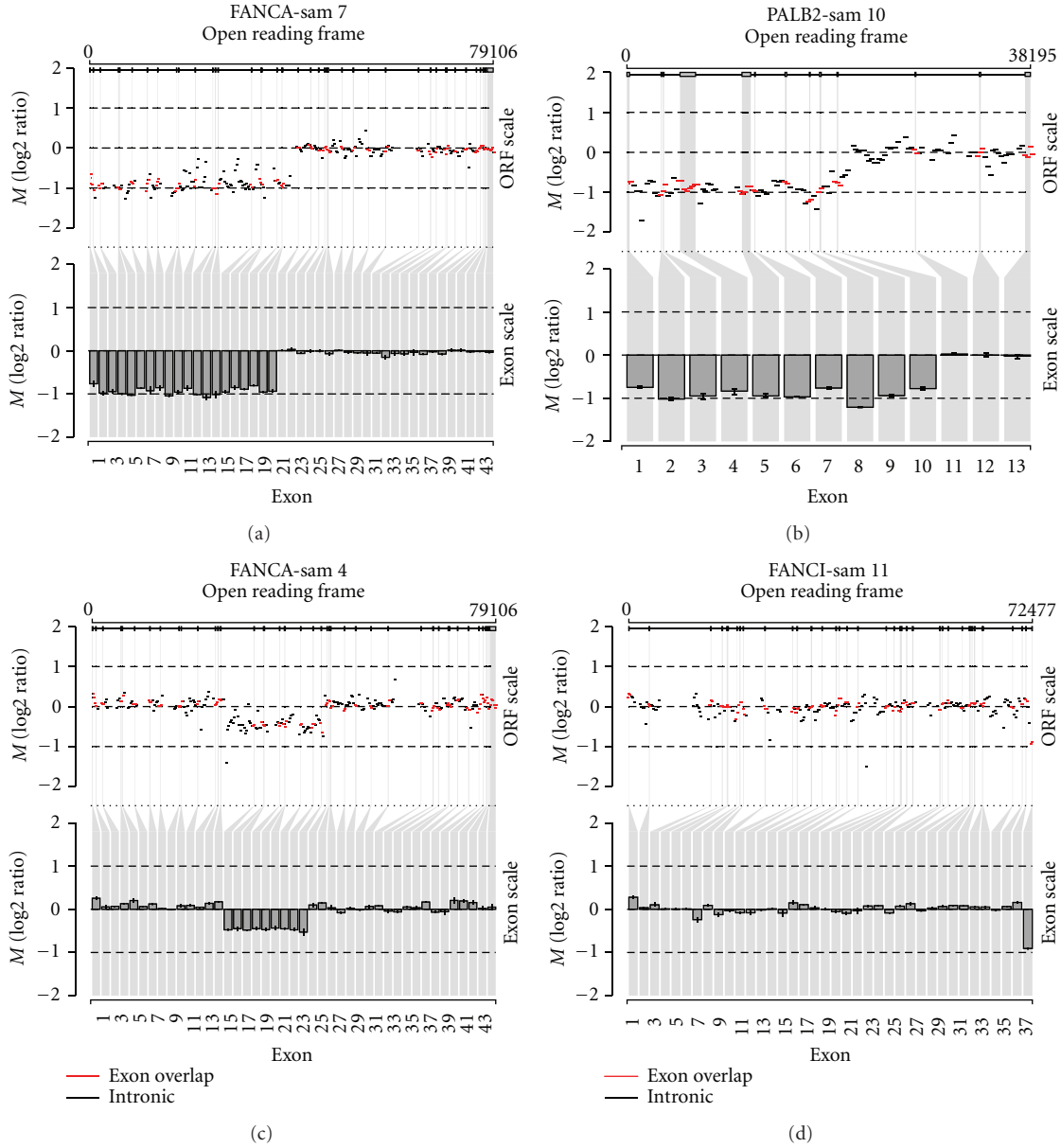

Figure 2: Large detection using next-generation sequence data. Copy number data are projected on an open reading frame (ORF) and on an exon scale. The ORF scale (upper panel) shows log2 ratios (M) for all exons and introns. Red segments indicate an overlap with an exon and black segments indicate no overlap with exons. The exon scale (lower panel) only shows the mean log2 ratio per exon with their 25th and 75th percentile. R plots of large deletion analysis. Deletion of exons 1 to 20 of *FANCA* (a), exons 1 to 10 of *FANCN* (b), exons 15 to 23 in *FANCA* (c), and exon 38 in *FANCI* (d).

In one patient no pathogenic variants were detected, although the coding regions of all known FA genes were covered deep enough to call variants. This suggests that the patient has a defect in a new FA gene or is not a true FA patient.

4. Discussion

The mutation detection strategy described here proved to be efficient for the molecular diagnosis of FA although patients with mutations in *FANCF*, *-J*, *-M*, *-O*, and -P were not included in our study. All the FA mutations identified by Sanger sequencing were also detected by next-generation sequencing. Moreover, we discovered a novel large deletion in the FA-I patient, for whom only one truncating mutation was previously identified [4]. The exact breakpoint at nucleotide level could be distinguished as also the intronic regions of the FA genes were enriched and sequenced. We confirmed the deletion by PCR and Sanger sequencing, using a SNP in the last unaffected exon and two sets of primers amplifying the regions up- and downstream of the breakpoint (Figure 3). Besides this novel large deletion we

Table 2: Pathogenic mutation detection through filtering.

Sample[1]	Variants (total)	Variants (target genes)	NS/SS[2]	Not in dbSNP	FA genes[3]	Pathogenic clue[4]
Sam 1	2535	1579	58	11	4	1
Sam 2	2659	1700	43	6	1	1
Sam 3	2388	1537	40	5	2	2
Sam 5	2490	1570	49	5	4	2
Sam 6	2081	1362	38	5	3	2
Sam 7	2417	1541	34	0	0	0[5]
Sam 8	2267	1416	53	5	3	1
Sam 9	2284	1354	34	4	1	1
Sam 10	2570	1760	51	5	2	1
Sam 11	2277	1491	44	7	2	1

[1] Sample 4 is not included in this table, as it is composed of pooled DNA from sample 4a and 4b. Slightly different analysis parameters were used for that sample.
[2] NS: nonsynonymous, SS, splice site.
[3] Number of variations remaining after filtering for variations in FA genes only.
[4] pathogenic clues are obtained from *in silico* analysis using Align-GVGD, Polyphen-2, and SIFT [7–9].
[5] This patient is a carrier for a large deletion and a deep intronic mutation. The large deletion is detectable by a separate tool, and the intronic variation is filtered out at this stage.

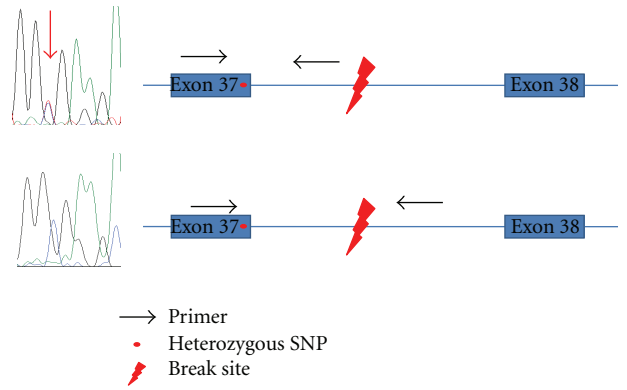

Figure 3: Confirmation of the large *FANCI* deletion by Sanger sequencing. A part of exon 37 with a SNP was amplified with primers designed either up- or downstream of the deletion breakpoint. Sequence analyses resulted in the detection of the SNP (red arrow) as heterozygous or hemizygous, respectively.

identified large genomic deletions in *FANCA* (2 samples) and *PALB2* (1 sample). The sensitivity of the method was demonstrated by mixing two DNA samples prior to library preparation and enrichment. A deletion of one allele in a background of four alleles can be detected, suggesting that the method is even applicable for the classification of mosaic FA patients. However, a thorough assessment of the method using serial dilutions with samples harboring large deletions is required to determine the detection limit of the assay. The identification of large deletions is essential for FA molecular diagnostics since about 40% of pathogenic mutations in the major FA complementation group, FA-A, are caused by large deletions in *FANCA*. As large deletions have also been demonstrated for *FANCI* (this study) and *FANCN* [6], it is plausible that these types of aberrations are present in other FA samples, which were previously unclassified by conventional molecular screening methods. Therefore, these types of mutations should be examined in the standard molecular diagnostics of FA.

The presence of *FANCD2* pseudogenes can complicate the identification of pathogenic mutations in this gene, as variations will tend to have a reduced frequency due to the occurrence of multiple highly similar copies. However, this difficulty can be resolved with bioinformatics by flagging variations that tend to have lower frequencies than expected within those regions. In cases where all other FA genes are excluded for mutations, careful inspection is required for flagged variations. Deep intronic mutations represent another type of variation, which are not analyzed with the classical molecular diagnostics approach. Indeed, the hemizygous *FANCA* c.893+920 C>A mutation in sample 7 was only identified after a heterozygous large *FANCA* deletion was detected by MLPA. This suspected the presence of a mutation on the other *FANCA* allele, which was then found after the FANCA cDNA was analyzed [5].

When we applied our novel molecular diagnostics approach on unclassified FA patients, we identified pathogenic mutations in four individuals. All the variations were confirmed with Sanger sequencing and demonstrated proper segregation with the disease. Two patients carried biallelic mutation in *BRCA2* and *FANCD2*, which have been described previously (Table 3). The remaining two patients harbored novel homozygous mutations in *FANCI* and *FANCL*; c.G2861A (p.C954Y) and c.755_756insAT (p.M252fs), respectively. The frameshift mutation in *FANCL* results in a stop codon in the last exon of the gene, which likely produces an mRNA targeted for nonsense mediated decay. The c.2861G>A substitution in *FANCI* affects a highly conserved amino acid and results in a large physiochemical difference, which is predicted to have a damaging effect on the FANCI protein by SIFT and polyphen-2. Nevertheless, functional studies are required to ultimately classify this

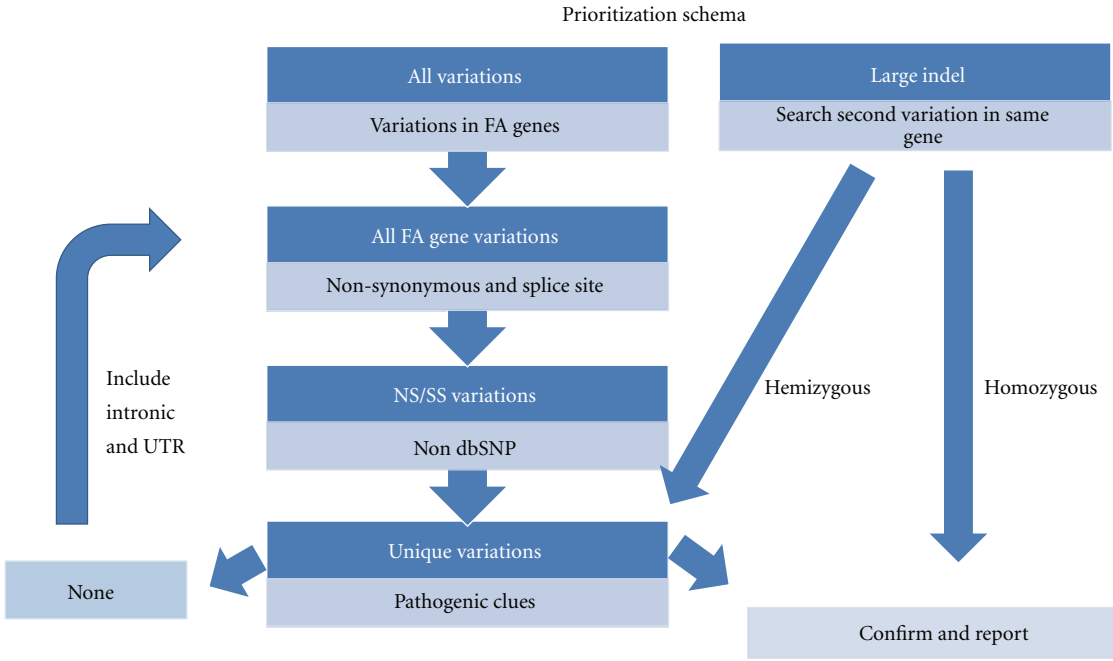

Figure 4: Prioritization scheme for the detection of disease-causing mutations by next-generation sequencing.

Table 3: Mutation detection in unclassified FA patients.

Sample	Gene	Mut 1	Effect 1	Mut 2	Effect 2	Note
Unc1	BRCA2	c.T8067A	p.C2689X	c.9672_9673insA	p.I3224fs	[10, 11]
Unc2	FANCD2	c.904C>T	p.R302W	c.2715+1G>A	splicing	[12, 13]
Unc3	FANCI	c.G3041A	p.C1014Y	c.G3041A	p.C1014Y	Novel
Unc4	FANCL	c.755_756insAT	p.M252fs	c.755_756insAT	p.M252fs	Novel
Unc5	—	NF*	—	NF*	—	—

Annotations are based on the following transcripts: BRCA2, NM_000059.3; FANCD2, NM_033084.3; FANCI, NM_001113378.1; FANCL, NM_001114636.1.
*NF = not found.

variation as pathogenic. In one FA sample we could not detect the disease causing mutations in any of the known FA genes. This patient might represent a novel FA subtype and whole-exome sequencing might be a useful approach to identify the affected gene.

Altogether, inspection of different variation types and inclusion of intronic regions warrants a comprehensive molecular FA diagnosis. Given the average number of variations of around 2500 per patient, it appears a difficult task to recognize the disease-causing mutation(s). Here we propose a prioritization approach following the recessive mode of inheritance (Figure 4). When no large deletions are identified, an initial filtering for nonsynonymous and canonical splice site variations should be performed. Subsequently, exclusion of variations in dbSNP and an in-house variant database with a frequency above 2%, reduces the number of possible pathogenic variations to less than 4. In cases where only one heterozygous pathogenic mutation is found, close examination of variations in intronic- and UTR regions in the same gene is required. When no pathogenic variations have been detected, assessment of all unique intronic and UTR variations is needed. With an ever expanding variant database of characterized FA patients, the identification of pathogenic mutations will become less complicated. Nevertheless, the necessity for functional tests, such as retroviral complementation or transfection, will remain essential to help assess the pathogenic status of unclassified missense variants.

In conclusion, multiplexed next-generation sequencing based on massively parallel sequencing is an effective molecular diagnostics approach for FA. The procedure, performed on genomic DNA, reduces the turnaround time, number of assays, and costs for a reliable detection of the disease-causing mutation. With the ever decreasing costs of enrichment and sequencing procedures, we expect that in the near future this will be the first test for patients clinically suspected of FA, thus avoiding labor-intensive chromosomal breakage assays and reducing turnaround time for FA diagnosis. To increase the efficiency of the molecular diagnosis, genes involved in other bone marrow failure syndromes (e.g., diamond-Blackfan anaemia and Shwachman-diamond syndrome) can be included, to be able to diagnose these non-FA patients that are often referred for FA diagnosis.

Authors' Contributions

N. Ameziane and D. Sie equally contributed to the work.

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Research Article

Loss of Ercc1 Results in a Time- and Dose-Dependent Reduction of Proliferating Early Hematopoietic Progenitors

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The endonuclease complex Ercc1/Xpf is involved in interstrand crosslink repair and functions downstream of the Fanconi pathway. Loss of Ercc1 causes hematopoietic defects similar to those seen in Fanconi Anemia. *Ercc1*$^{-/-}$ mice die 3-4 weeks after birth, which prevents long-term follow up of the hematopoietic compartment. We used alternative Ercc1 mouse models to examine the effect of low or absent Ercc1 activity on hematopoiesis. Tie2-Cre-driven deletion of a floxed *Ercc1* allele was efficient (>80%) in fetal liver hematopoietic cells. Hematopoietic stem and progenitor cells (HSPCs) with a deleted allele were maintained in mice up to 1 year of age when harboring a wt allele, but were progressively outcompeted when the deleted allele was combined with a knockout allele. Mice with a minimal Ercc1 activity expressed by 1 or 2 hypomorphic *Ercc1* alleles have an extended life expectancy, which allows analysis of HSPCs at 10 and 20 weeks of age. The HSPC compartment was affected in all Ercc1-deficient models. Actively proliferating multipotent progenitors were most affected as were myeloid and erythroid clonogenic progenitors. In conclusion, lack of Ercc1 results in a severe competitive disadvantage of HSPCs and is most deleterious in proliferating progenitor cells.

1. Introduction

The Ercc1/Xpf complex is an endonuclease involved in nucleotide excision repair (NER) and in repair of interstrand crosslinks (ICL) [1, 2]. Mice lacking Ercc1 (*Ercc1*$^{-/-}$) suffer from severe premature aging, which shows as small size, ruffled fur, liver polyploidy, and loss of hematopoietic progenitors from bone marrow (BM), resulting in death at 3-4 weeks of age [3–6]. Hypomorphic *Ercc1* (*Ercc1*$^{d/d}$ or Ercc1^{*292}) mice that harbor 2 C-terminally truncated alleles are also small but they survive longer (~6 months), probably as a result of their residual DNA repair capacity (~4%) [1, 2]. The hypomorphic allele has a 7 amino acid deletion at the C-terminus, which impairs dimerization with Xpf [1].

The short life span and severe aging phenotype of *Ercc1*$^{-/-}$ is shared with other models of defective NER such as the *Xpa*$^{-/-}$ *Csb*$^{m/m}$ mice that die at 3 weeks of age [7–9]. The hematopoietic defect of *Ercc1*$^{-/-}$ mice, however, is specifically linked to defective ICL repair ([5]; Verhagen-Oldenampsen et al., unpublished). The correlation of specific phenotypes with either NER or ICL repair is likely due to the activation of distinct tumor suppressor mechanisms that impact differently on specific tissues. For instance, persistent DNA damage due to defective NER results in deregulation of the growth axis and is independent of p53 and p16^{INK4a} [8]. Hematopoiesis, on the other hand, is particularly sensitive to activation of p53 (Haanstra, Verhagen-Oldenampsen in preparation).

Both fibroblasts and hematopoietic cells of *Ercc1*$^{-/-}$ mice and mice lacking Fanconi proteins are hypersensitive to the DNA crosslinker mitomycin C (MMC) [1, 5, 10]. Importantly, the endonuclease complex Ercc1/Xpf participates in the same ICL repair pathway as the Fanconi Anemia (FA) proteins [11, 12]. It associates with FancP/Sxl4 and is required for FancD2 focus formation [13, 14]. Mice lacking for instance the *Fancc* gene only develop hematopoietic

defects when challenged with MMC, or when hematopoietic cells are cultured at atmospheric oxygen prior to transplantation [10, 15]. Mice lacking Ercc1 develop hypoplasia of the BM compartment without applying an external challenge similar to FA patients [16] and Fancp/Slx4-deficient mice [14].

The Ercc1 mice are a useful model to study BM failure in FA, which is, however, limited by the short life span of *Ercc1*$^{-/-}$ mice. The BM of *Ercc1*$^{-/-}$ mice contains fewer progenitors, and the remaining myeloid and erythroid progenitors fail to proliferate *in vitro* [5]. The aim of this study was to characterize progression of BM failure in Ercc1 models with an extended life span, and to examine how low levels of Ercc1 activity impact on hematopoiesis. We used mice with a single floxed *Ercc1* allele and a Tie2-driven Cre recombinase. Tie2 is expressed in the early hematopoietic stem cell (HSC) when they dissociate from the hemogenic endothelium, and in quiescent adult HSC [17, 18]. We show that the *Ercc1* allele recombines efficiently in fetal liver. In presence of an intact *Ercc1* allele, the recombination frequency remained stable, while the frequency of cells lacking *Ercc1* rapidly decreased in BM when the second *Ercc1* allele was lacking. This indicated that Ercc1-deficient hematopoietic cells have a severe competitive disadvantage. To investigate how low levels of Ercc1 affect hematopoietic stem and progenitor cells, we compared hematopoiesis in mice harboring one or two hypomorph alleles (*Ercc1*$^{-/d}$ and *Ercc1*$^{d/d}$, encoding proteins with impaired Xpf dimerisation capacity) at 3, 10, and 20 weeks of age. At week 3, we included *Ercc1*$^{-/-}$ in this comparison. This analysis showed that proliferating stem and progenitor cells decreased, whereas the most immature cells within the LSK fraction were less affected once these cells became quiescent after 3 weeks of age. The decrease of multipotent progenitors preceded the decrease of committed progenitors indicating that the earliest proliferating progenitors are most sensitive to defective ICL repair.

2. Materials and Methods

2.1. Animals. *Ercc1*$^{+/d}$, *Ercc1*$^{+/-}$ [1], *Ercc1*$^{+/f}$ (obtained from Dr. L. Niedernhofer, University of Pittsburgh School of Medicine, Pittsburgh, PA), *Tie2-Cre* [19], and wt littermates were kept in a pure background of both C57/Bl6 and FVB/n at the Animal Resource Center (Erasmus MC). Experimental animals were generated as F1 in a mixed background of C57/Bl6 and FVB/n. *Ercc1*$^{+/-}$ and *Ercc1*$^{+/d}$ mice displayed a wild-type phenotype and were used as controls. All animal studies were approved by an independent Animal Ethical Committee. Mice were sacrificed by CO_2 inhalation between postnatal weeks 3 and 20. Neonatal mice and embryo's were sacrificed by decapitation on ice. Femurs, tibia, and sternum were isolated and BM cell suspensions were obtained by crushing the bones in HBSS supplemented with 5% (v/v) foetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Fetal livers and neonatal spleens were resuspended by pipetting in the same medium.

2.2. Colony-Forming Unit Assays. Bone marrow cell suspensions were plated in methyl cellulose medium (Methocult M3234, StemCell Technologies SARL, Grenoble, France) containing huGCSF (0.1 μg/mL), muGM-CSF (0.1 μg/mL), or Epo (4 mU/mL) plus transferrin (0.3 mM), hemin (0.2 mM), and muSCF (0.1 μg/mL). Colonies containing 30 cells or more were scored after 7-8 days of culture.

2.3. Flow Cytometry. Single-bone-marrow cell suspensions were analyzed by flow cytometry using a BD LSR II Flow Cytometer System with FCS Express Diva software (BD Biosciences, San Jose, CA). FCS files were analyzed using FlowJo (Tree Star, Inc., Ashland, OR). Cells were labelled with the following antibodies; mouse biotinylated lineage depletion kit, CD16/CD32-PE, CD117-APC, CD135-PE and streptavidin-APC-Cy7 (BD Pharmingen), Sca1-PE-Cy7, CD34-pacific blue and CD127-pacific blue (Ebioscience), and 7′AAD (Invitrogen).

2.4. Genotyping PCR and Q-PCR. Genomic DNA was isolated from tail segments or from blood (NucleoSpin Tissue XS, MACHEREY-NAGEL GmbH & Co). Genotypes were determined by PCR. Genomic Q-PCR used an Applied Biosystems 7900 instrument (Applied Biosystems, Weiterstadt, Germany) and SYBR Green PCR Master Mix (Applied Biosystems). Primers used were HPRT—forward: AGCCTAAGATGAGCGCAAGT, reverse: ATGGCCACAGGACTAGAACA; Recombined Ercc1 allele—forward: TGCAGCATGCTCTAGACTCG, reverse: CCATGAATTCCGGGATCTCTCGAC; nonrecombined Ercc1 allele—forward: TCCACTTCGCATATTAAGGTGA, reverse: AACCTGCGTGCAATCCAT; Ercc1 knock out locus—forward: TCCTCGTGCTTTACGGTATC, reverse: CAGGATCAGGAGGTACAGGA.

2.5. Histology. Livers were embedded in Tissue-Tek O.C.T (Sakura Finetek, Zoeterwoude, Netherlands). 4 μm sections were made using a cryostat (Leica) and stained with hematoxylin and eosin. Slides were imaged on a Leica DMLB light microscope equipped with Leica application suite 2.7.1 (Leica Microsystems, Switzerland).

3. Results

3.1. Ercc1-Deficient Hematopoietic Stem and Progenitor Cells Have a Competitive Disadvantage. *Ercc1*$^{-/-}$ mice have an average lifespan of 3 weeks. Because we aimed to study long-term effects of Ercc1-deficiency on hematopoietic stem cell function, we used a Cre-lox conditional mouse model expressing Cre-recombinase from the Tie2 promoter (*Tie2-Cre*). Tie2 is expressed on vascular endothelial cells and HSCs [17, 18]. Mice with a single floxed Ercc1 allele (*Ercc1*$^{+/f}$) were crossed with *Ercc1*$^{+/-}$ *Tie2-Cre* mice. We compared *Ercc1*$^{-/f}$ and *Ercc1*$^{+/f}$ mice with and without expression of Tie2-Cre. Because the recombination efficiency in Cre-lox mouse models is never 100% [20], deletion of the floxed allele was analyzed both pre- and postnatal in the most active hematopoietic organ, that is, fetal liver in the embryo, spleen

in newborn animals, and BM in adult animals. The presence of the floxed allele was analyzed by real-time genomic PCR on DNA isolated from the various tissues. The fraction of cells with a deleted floxed allele was calculated by comparing the relative signals in tissues with or without Cre. *Tie2-Cre/Ercc1*$^{+/f}$ mice showed stable deletion of the floxed allele in 50% or more of the hematopoietic cells (Figure 1). In *Tie2-Cre/Ercc1*$^{-/f}$ mice, the Ercc1 allele was deleted in 80% of fetal liver cells at prenatal days E12.5 and E15.5. In newborn *Tie2-Cre/Ercc1*$^{-/f}$ animals (postnatal day 1), ~50% of spleen cells carried a deleted floxed allele. At ten weeks of age, the recombined allele was undetectable or present in a low percentage of cells. In *Tie2-Cre/Ercc1*$^{-/f}$ animals of 1 year old, the BM contained hardly any cells with a recombined allele (Figure 1). Accordingly, blood cell parameters and colony-forming progenitors in BM were similar in *Ercc1*$^{-/f}$ mice with or without *Tie2-Cre* expression at 10 weeks and 1 year of age (data not shown). This indicates that *Ercc1*-deleted cells are outcompeted by cells in which the floxed allele was not recombined. The presence of one *Ercc1* allele is sufficient to maintain the hematopoietic cell compartment at a similar level as in nondeleted animals.

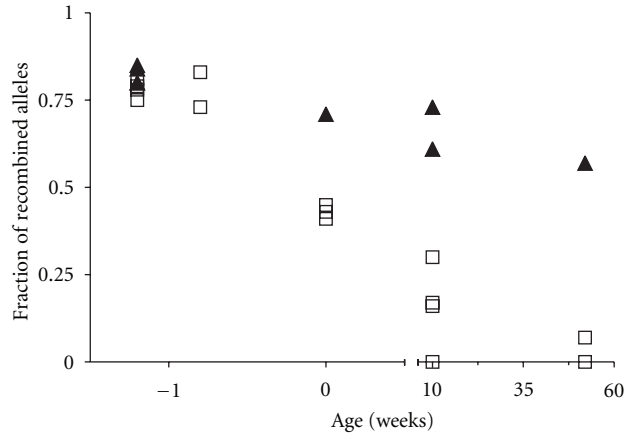

Figure 1: Recombination in Ercc1-flox Tie2-Cre model. The fraction of recombined alleles in the presence of Tie2-Cre was calculated after measuring the nondeleted floxed allele by real-time genomic PCR and comparing it to the presence of the floxed allele in absence of Cre. HPRT was measured to control for total DNA. DNA was isolated from fetal livers at embryonic days E12.5 and E15.5, from the spleen of neonatal mice, and from bone marrow of 10- and 52-week-old mice. Closed triangles: *Tie2-Cre; Ercc1*$^{+/f}$, open boxes: *Tie2-Cre; Ercc1*$^{-/f}$. Each symbol is an independent measurement.

3.2. The Composition of the Hematopoietic Stem Cell Pool Is Affected by the Level of Ercc1 Activity. To find a window of Ercc1 expression that allows for the analysis of hematopoiesis for several weeks, we compared hematopoiesis in bone marrow of *Ercc1*$^{-/-}$ mice with mice harboring one C-terminally truncated Ercc1 allele and a knock out allele (*Ercc1*$^{-/d}$), or two C-terminally truncated Ercc1 alleles (*Ercc1*$^{d/d}$). The truncated allele has been described as *293 [1] or as *delta* [21], we adopted *delta*, indicated as "*d*", that should not to be confused with a recombined floxed allele. Three-week-old mice with low or absent Ercc1 activity had a dose-dependent decrease in body size (Figure 2(a)). *Ercc1*$^{-/-}$ mice died between weeks 3 and 4. The *Ercc1*$^{d/d}$ and *Ercc1*$^{-/d}$ mice survived longer, but their low body weight persisted at 10 and 20 weeks of age (Figures 2(b) and 2(c)). A comparison of liver morphology of the various Ercc1-deficient mice at 3 weeks of age indicated that livers from both *Ercc1*$^{-/-}$, *Ercc1*$^{-/d}$, and *Ercc1*$^{d/d}$ mice contained cells with enlarged nuclei, compared to wt livers (larger than 8 μm; Figures 2(d) and 2(e)) as previously described [21].

To analyze the effect of low levels of Ercc1 on hematopoiesis, we first examined the stem and progenitor cell compartment using flow cytometry. Hematopoietic stem cells and progenitor cells (HSPCs) were defined as negative for lineage markers (Lin−) and positive for the surface markers Sca (Sca1+) and the SCF receptor cKit (cKit+), indicated as the LSK fraction. The stem cell compartment was further subdivided into long-term HSC (LT-HSC, CD34−, CD135−), short-term HSC (ST-HSC, CD34+ CD135−), and multipotent progenitors (MPP, CD34+ CD135+) [22].

Because BM cellularity corrected for body weight was comparable between the different genotypes at 3, 10, and 20 weeks of age, a comparison of the subset ratios was permitted between Ercc1-deficient mice and their wt littermates. The percentage of LSK cells in the total bone marrow of 3-week-old mice was decreased to 17% of wt for *Ercc1*$^{-/-}$, 28% of wt for *Ercc1*$^{-/d}$, and 27% of wt in *Ercc1*$^{d/d}$ mice (Figure 3(a)). At 10 weeks of age, the percentage of LSK cells present in the BM further decreased in *Ercc1*$^{-/d}$ mice to 10% of wt but stabilized to 50% of wt for *Ercc1*$^{d/d}$ mice (Figure 3(b)). At 20 weeks, the percentage of LSK was 26% of wt for *Ercc1*$^{-/d}$ and 31% of wt for *Ercc1*$^{d/d}$ (Figure 3(c)). Thus, the size of the stem cell compartment correlates with Ercc1 activity but fluctuates over time.

We next investigated how distinct subpopulations within the LSK compartment depend on Ercc1 protein activity. The distribution of LT-HSC, ST-HSC, and MPP displayed relatively minor changes at week 3 (Figure 3(d)). At 10 weeks of age, the fraction of actively dividing MPP was more than 3-fold decreased in both hypomorphic models (Figure 3(e)). The *Ercc1*$^{-/d}$ BM contained predominantly quiescent LT-HSC, while proliferating ST-HSC was the most abundant fraction in *Ercc1*$^{d/d}$ BM (Figure 3(e)). The enrichment of quiescent LT-HSC is in accordance with the further reduction of LSK in *Ercc1*$^{-/d}$ BM. In contrast, the LSK fraction in *Ercc1*$^{d/d}$ BM partly recovered at week 10, which is in accordance to the increase in ST-HSC fraction. At 20 weeks of age, the distribution of quiescent and dividing subfractions within the population of LSK cells remained similar to the distribution at 10 weeks for both *Ercc1*$^{-/d}$ and *Ercc1*$^{d/d}$ mice (Figure 3(f)).

To specify the distribution of progenitors that arise from the LSK fraction in relation to the remaining Ercc1 activity, we analyzed the following lineage committed progenitor subsets: common myeloid progenitors (CMP, Lin− cKit+ CD34+ CD16/CD32int), granulocyte-monocyte progenitors (GMP, Lin− cKit+ CD34+ CD16/CD32hi),

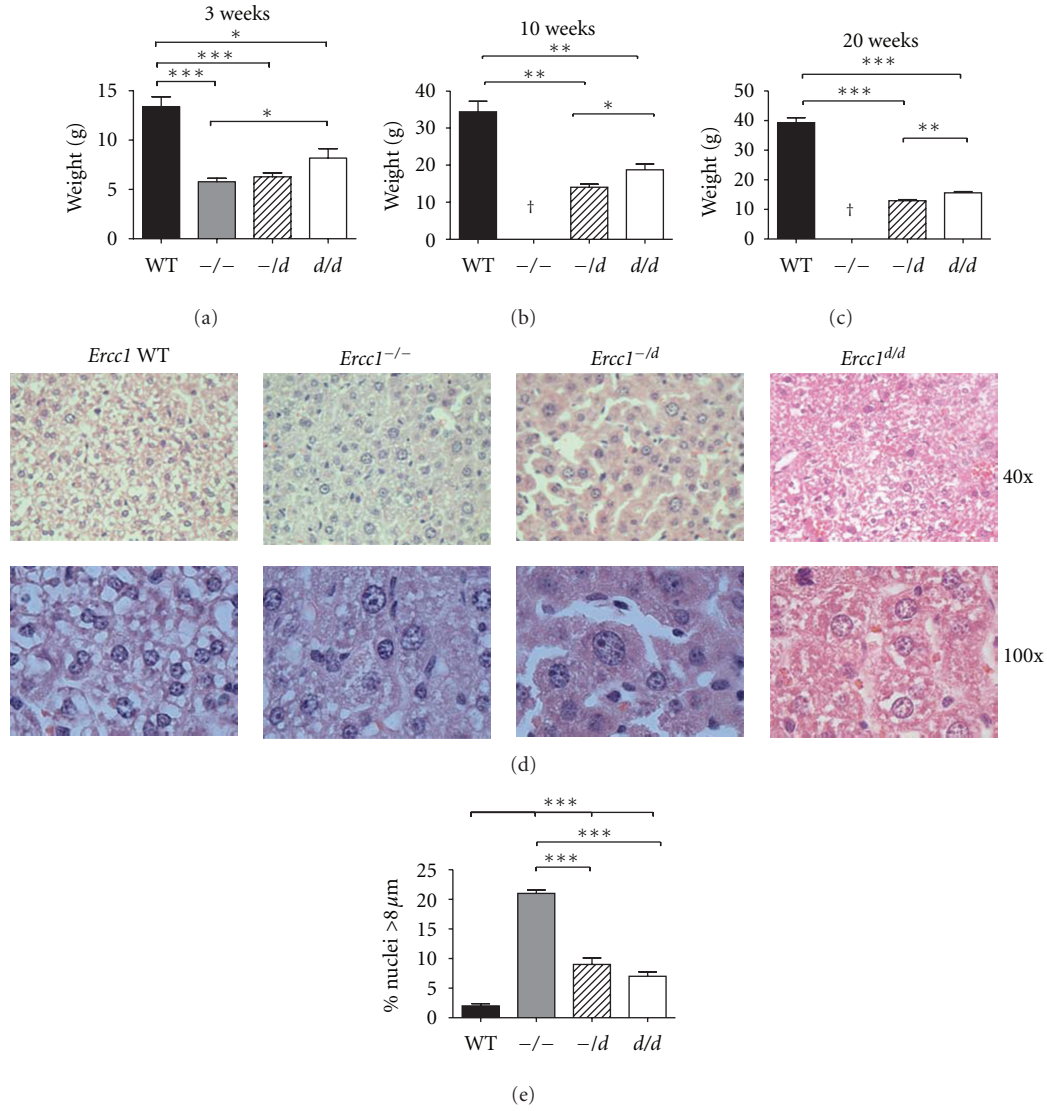

FIGURE 2: Weight and liver cell morphology of mice with distinct levels of Ercc1 activity. ((a)–(c)) Mean body weight of (a) 3-week-old $Ercc1^{-/-}$ ($n = 6$), $Ercc1^{-/d}$ ($n = 7$), $Ercc1^{d/d}$ ($n = 4$), and wt ($n = 13$) mice. (b) 10-week-old $Ercc1^{d/d}$ ($n = 3$), $Ercc1^{-/d}$ ($n = 3$), and wt ($n = 6$) mice. (c) 20-week-old $Ercc1^{-/d}$ ($n = 8$), $Ercc1^{d/d}$ ($n = 5$), and wt ($n = 12$) mice. (d) Hematoxylin- and eosin-stained sections of liver from 3-week-old wt, $Ercc1^{-/-}$, $Ercc1^{-/d}$, and $Ercc1^{d/d}$ mice. (e) Quantification of enlarged nuclei (>8 μm). Error bars indicate standard deviation. *indicates $P \leq 0.05$, **indicates $P \leq 0.01$, and ***indicates $P \leq 0.001$.

megakaryocyte-erythroid progenitors (MEP, Lin− cKit+ CD34− CD16/CD32low), and common lymphoid progenitors (CLP, Lin− CD127+ Sca1/cKit+ int). At 3 weeks of age, the CMP fraction of $Ercc1^{-/-}$ mice decreased to 46% of wt, the GMP fraction to 16% of wt, the MEP fraction to 45% of wt, and the CLP fraction to 48% of wt levels (Figure 3(g)). In $Ercc1^{-/d}$ mice, the progenitor subsets decreased to, respectively, 39%, 54%, and 88% of wt levels and no change in CLP levels (Figure 3(g)). For $Ercc1^{d/d}$ mice, these percentages were 23%, 38%, and 41% of wt levels and no difference in CLP levels (Figure 3(g)). For all myeloid subsets, except the CMP compartment, the numbers increased in $Ercc1^{-/d}$ mice as compared to $Ercc1^{-/-}$ mice.

At 10 weeks of age, BM of $Ercc1^{-/d}$ mice contained 20% of wt CMP levels, 29% of wt GMP levels, 49% of wt MEP levels, and 87% of wt CLP levels (Figure 3(h)). In $Ercc1^{d/d}$ mice, these subsets contained 32%, 27%, 39%, and 74% of wt levels, respectively (Figure 3(h)). At 20 weeks of age, $Ercc1^{-/d}$ BM contained 35% of wt CMP levels, 77% of wt GMP levels, 53% of wt MEP levels, and 67% of wt CLP levels (Figure 3(i)). In $Ercc1^{d/d}$ mice, these subsets contained 13%, 23%, 31%, and 69% of wt levels, respectively (Figure 3(i)).

In conclusion, decreased Ercc1 levels reduce all compartments of actively proliferating stem and progenitor cells except for the CLP fraction that is only moderately affected. Despite reduced numbers of progenitors in BM, we observed normal cell numbers in peripheral blood (data not shown). The presence of a hypomorphic Ercc1 allele extends the life span of the mice and marginally improves hematopoiesis in the mice. Also in $Ercc1^{d/d}$ mice, the number of HSPCs remains severely compromised.

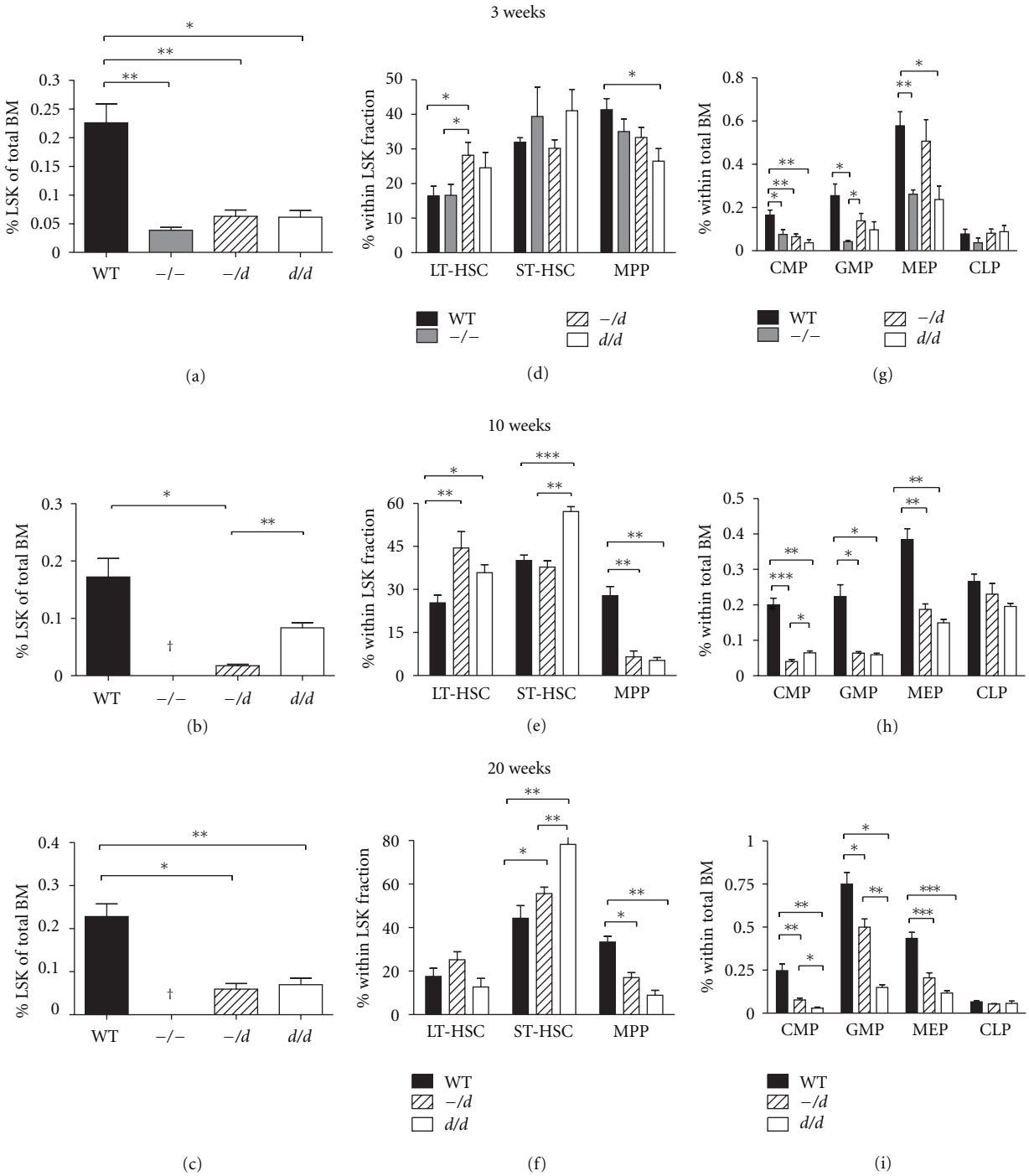

Figure 3: Ercc1 levels influence the composition of the stem and progenitor cell pool. Whole BM suspensions were stained with surface antigen specific antibodies for hematopoietic stem cells. ((a)–(c)) LSK (Lin− Sca1+ cKit+) cells as percentage of total bone marrow cells. ((d)–(f)) Distribution of stem cells within the LSK fraction (LT HSC(CD34− CD135−), ST-HSC (CD34+ CD135−) and MPP (CD34+ CD135+)). ((g)–(i)) Distribution of progenitor cells within total bone marrow (CMP (Lin− cKit+ CD34+ CD16/CD32intermediate), GMP (Lin− cKit+ CD34+ CD16/CD32high), MEP (Lin− cKit+ CD34− CD16/CD32low) and CLP (Lin− CD127+ Sca1/cKit intermediate)). Mean percentages are plotted; error bars indicate standard deviation. *indicates $P \leq 0.05$, **indicates $P \leq 0.01$ and ***indicates $P \leq 0.001$.

3.3. Ercc1 Deficiency Impairs Colony Formation by Hematopoietic Progenitors. To assess the colony-forming potential of hematopoietic progenitors, bone marrow suspensions were plated in semisolid medium supplemented with lineage-specific cytokines. At 3 weeks of age, the number of erythroid (BFU-E, Figure 4(a)), granulocytic (CFU-G, Figure 4(b)), and granulocytic-macrophage colony-forming cells (CFU-GM, Figure 4(c)) were significantly reduced in all Ercc1-deficient models relative to wt (Figures 4(a)–4(c)).

Similar results were obtained in BM of 10- and 20-week-old mice; *Ercc1*$^{-/d}$ BM formed no BFU-E colonies (Figures 4(d) and 4(g)), no CFU-G colonies (Figures 4(e) and 4(h))

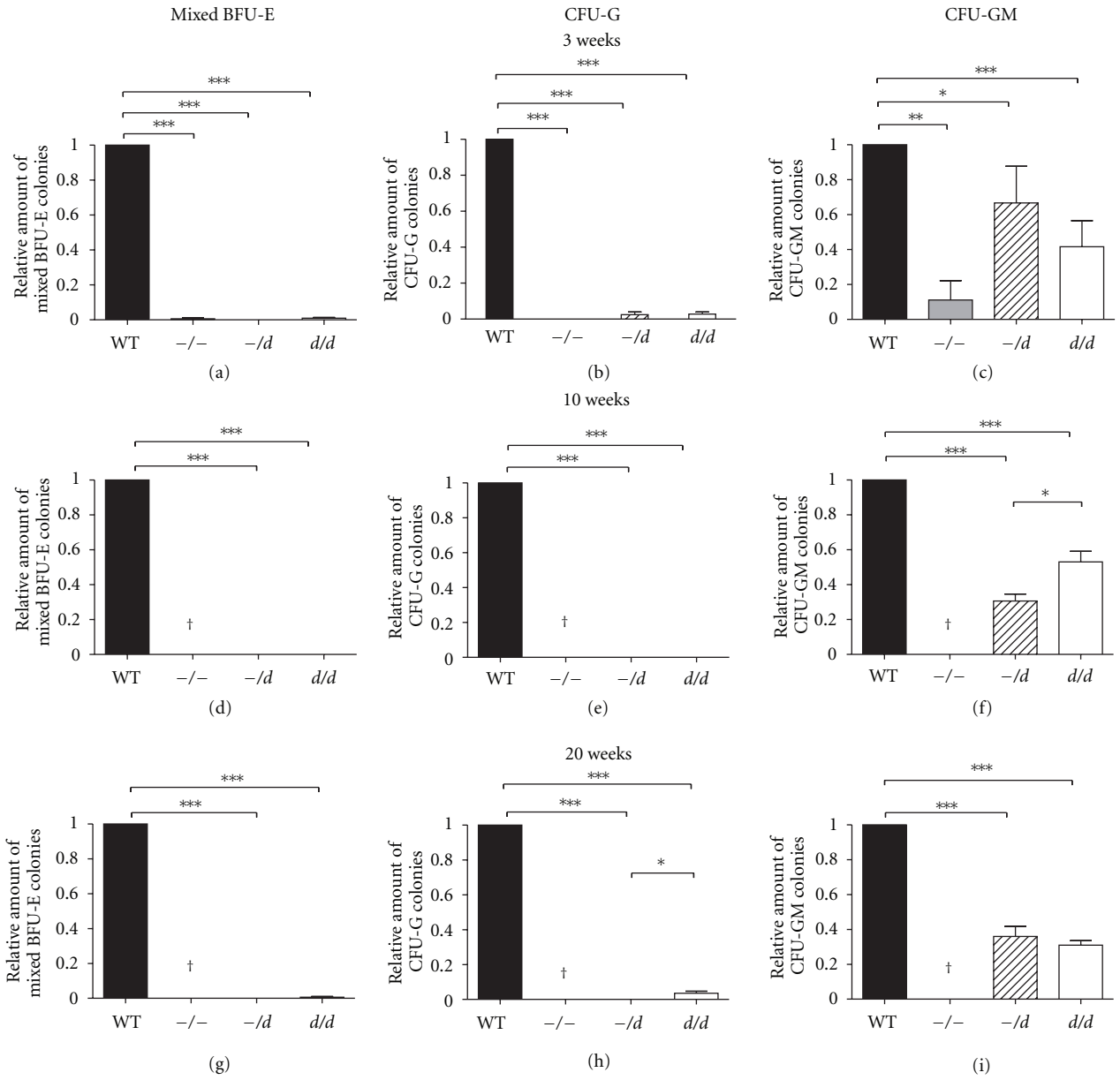

Figure 4: Colony-forming potential of bone marrow progenitors is affected in hypomorphic models or Ercc1. BFU-E, CFU-G, and CFU-GM colonies per 5×104 bone marrow cells derived from ((a)–(c)) 3-week-old $Ercc1^{-/-}$ ($n = 6$), $Ercc1^{-/d}$ ($n = 7$), $Ercc1^{d/d}$ ($n = 4$), and wt mice ($n = 7$) ((d)–(f)) 10-week-old $Ercc1^{-/d}$ ($n = 3$), $Ercc1^{d/d}$ ($n = 3$), and wt mice ($n = 3$), ((g)–(i)) 20-week-old $Ercc1^{-/d}$ ($n = 3$), $Ercc1^{d/d}$ ($n = 6$), and wt mice ($n = 8$). Error bars indicate standard deviation. *indicates $P \leq 0.05$, **indicates $P \leq 0.01$, and ***indicates $P \leq 0.001$.

and only 31% of CFU-GM colonies compared to wt (Figures 4(f) and 4(i)). In $Ercc1^{d/d}$ BM, the percentages were 0%, 0%, and 35% of wt, respectively. These results imply that the residual Ercc1 activity in $Ercc1^{-/d}$ and $Ercc1^{d/d}$ mice is not sufficient to support BFU-E or CFU-G colony formation, whereas CFU-GM colony outgrowth is only partly restored.

4. Discussion

The Ercc1/Xpf endonuclease complex acts downstream of the Fanconi pathway in ICL repair [1, 2, 12]. The hematopoietic defects in Ercc1-deficient mice are reminiscent of the hematopoietic defect of FA patients [23]. It mostly takes several years before FA patients develop anemia. In most FA mouse models, loss of HSC is only seen when the mice are challenged with Mitomycin C [24, 25]. The fact that most mouse models lacking Fanconi genes fail to display overt BM failure may reflect the time it takes to develop anemia. An important factor in the onset of BM failure and leukemia development may be the level of residual DNA repair activity. We employed Ercc1-deficient mouse models to show progressive loss of the number of hematopoietic stem and progenitor cells dependent on Ercc1 activity. Remaining progenitors were compromised in their *in vitro*

proliferation capacity, which was similarly severe in *Ercc1*$^{-/-}$, *Ercc1*$^{-/d}$, and *Ercc1*$^{d/d}$ mice.

4.1. Reduced Competitiveness of Ercc1-Deficient Hematopoietic Cells. The conditional knock out model showed that a small percentage of hematopoietic stem and progenitor cells in which the floxed Ercc1 allele did not recombine outcompeted the Ercc1-deficient cells in which Cre-driven deletion had occurred. This progressive loss of Ercc1-deficient hematopoietic cells resembles what has been found in a small fraction of FA patients. In some FA patients, a mutation was reverted because two mutated alleles were recombined and yielded an unaffected allele. Such a naturally corrected hematopoietic stem cell is able to out-compete the hematopoietic cells with two defective alleles resulting in the restoration of BM cellularity. In these patients, the fibroblasts retained two mutated alleles [26]. The conditional knock out mice that we used here underscore that defective ICL repair mainly affects continuously regenerating tissues such as the hematopoietic system. It is also in the continuously proliferating bone marrow compartment that few cells with an intact allele can out-compete cells that lack a functional FA pathway.

4.2. Reduced Hematopoietic Reserves with Normal Peripheral Blood Levels. The hematopoietic defect in Ercc1-deficient mice, and in FA, is specifically associated with DNA crosslinks that stall the replication fork. The inability to repair spontaneous DNA damage limits stress-hematopoiesis by diminishing the ability of HSCs to proliferate and self-renew. During embryo development and in young mice (<3 weeks), the HSC compartment is continuously expanded, whereas HSC become largely quiescent in adult mice [27, 28]. These quiescent HSCs are less sensitive to replication-coupled DNA damage repair defects. Progenitor cells have a higher proliferation rate compared to HSC and are, therefore, more prone to DNA damage both during development and in adult mice. Accordingly, we found that LSK numbers are 3- to 5-fold decreased compared to their wt littermates in *Ercc1*-deficient mice. At 3 weeks of age, the distribution within the LSK compartment hardly shows a tendency towards more primitive cells, most likely because all compartments contained proliferative cells. At 10 and 20 weeks of age, when the mice are adult, there is a significant shift towards the more primitive cells in the LSK compartment in the *Ercc1*$^{-/d}$ and *Ercc1*$^{d/d}$ compared to their wt littermates, indicating that maintenance of the LT-HSC fraction is less sensitive to DNA interstrand cross links than the maintenance of the proliferative MPP fraction [29].

However, the mice did not develop overt anemia, and peripheral blood contained near normal amounts of red and white blood cells. This is most likely due to compensatory mechanisms controlled by a network of cytokines and hormones: only small and transient alterations in local- and/or systemic concentrations will be needed to maintain or restore homeostasis. Notably, Epo serum concentrations were normal in Ercc1-deficient mice (data not shown), but this result was expected given that the mice were not anemic and Epo production in the kidney is activated by hypoxia.

Because cell numbers in peripheral blood are hardly affected, the hematopoietic defect in *Ercc1*$^{-/d}$ mice does not represent overt BM failure but can be regarded as a situation prone to such overt BM failure. Also in FA patients, reduced stem cell numbers precede overt BM failure and leukemia development [30, 31]. When challenged for regeneration following insult, the Ercc1-deficient stem and progenitor cells lack the robustness to do so. Analysis of BM and leukemogenesis in FA and in FA mouse models shows that hypoplasia precedes leukemic transformation [32, 33]. Hypoplastic compartments are most at risk for leukemic transformation [34]. FA patients mainly develop acute myeloid leukemia (AML) and only very rarely acute lymphoid leukemia (ALL) [35]. In the Ercc1 models, we also found that the myeloid compartment is affected by Ercc1 deficiency while the CLP compartment is hardly affected. Therefore, the hypomorphic Ercc1 mice may be a very useful model to study BM failure mechanisms and subsequent leukemogenic transformation in FA.

4.3. Comparison of the Hematopoietic Phenotype of Ercc1$^{-/-}$, *Ercc1*$^{-/d}$, *and Ercc1*$^{d/d}$ *Mice.* In myeloid and erythroid colony-forming assays, *Ercc1*-deficient progenitors show a 50% (on GM-CSF) to a 100% (on EPO/SCF or G-CSF) decrease in colony numbers. In *Ercc1*$^{-/d}$, and *Ercc1*$^{d/d}$ mice, the decrease in colony numbers was not significantly different from those in *Ercc1*$^{-/-}$ mice. This implies that low levels of functional protein cannot repair the damage inflicted by the rapid proliferation that occurs in these assays. Flow cytometry measurements indicated that the decrease in myeloid and erythroid colony-forming cell numbers was only moderate in the Ercc1-deficient models at 3 weeks of age. Thus, the progenitors are present, and they are able to generate progeny *in vivo*, but not *in vitro*. *In vitro* conditions challenge the proliferation capacity more than the *in vivo* condition and may be more mutagenic such as higher oxygen levels.

Conflict of Interests

The authors have no conflict of interests.

Authors' Contributions

J. H. E. Verhagen-Oldenampsen and J. R. Haanstra contributed equally to this paper. I. P. Touw and M. von Lindern share equal responsibility of this paper.

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Review Article

A DOG's View of Fanconi Anemia: Insights from *C. elegans*

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C. elegans provides an excellent model system for the study of the Fanconi Anemia (FA), one of the hallmarks of which is sensitivity to interstrand crosslinking agents. Central to our understanding of FA has been the investigation of DOG-1, the functional ortholog of the deadbox helicase *FANCJ*. Here we review the current understanding of the unique role of DOG-1 in maintaining stability of G-rich DNA in *C. elegans* and explore the question of why DOG-1 animals are crosslink sensitive. We propose a dynamic model in which noncovalently linked G-rich structures form and un-form in the presence of DOG-1. When DOG-1 is absent but crosslinking agents are present the G-rich structures are readily covalently crosslinked, resulting in increased crosslinks formation and thus giving increased crosslink sensitivity. In this interpretation DOG-1 is neither upstream nor downstream in the FA pathway, but works alongside it to limit the availability of crosslink substrates. This model reconciles the crosslink sensitivity observed in the absence of DOG-1 function with its unique role in maintaining G-Rich DNA and will help to formulate experiments to test this hypothesis.

1. Introduction

The helicase, FANCJ, is required for the Fanconi Anemia (FA) pathway to function properly and thus maintain genome integrity. In humans, *FANCJ* mutations have been identified in early-onset breast cancer patients [1, 2] and FA complementation group J patients [3–5]. However, the role of FANCJ in the FA pathway of DNA repair is not fully understood. Some insights have been gained from research on DOG-1 (*D*eletions *O*f *G*-rich DNA), the *Caenorhabditis elegans* functional ortholog of FANCJ [6–9]. However, even in this relatively simple model system, important questions remain. An outstanding issue is the relationship between the relatively well-known function of DOG-1/FANCJ in preventing replication blocks at unresolved secondary structures and its function in resistance to interstrand crosslinks (ICLs). Previous work from our group has shown that DOG-1 acts upstream of, or parallel to, FCD-2 in the maintenance of G-tracts [7] but is dispensable for FCD-2 focus formation in response to ICL generating agents [8]. One possibility is that DOG-1 takes on two different functions, one in G4 DNA resolution and one in FA crosslink repair. On the other hand, it is possible that its ability to unwind G-rich secondary structure may be sufficient to explain its role in both situations. Here we summarize the current understanding of DOG-1/FANCJ function and hypothesize how to reconcile the two known roles for this protein with its helicase function.

2. DOG-1 Is Required for Maintenance of G-Tracts

DOG-1 was discovered as being essential for the maintenance of G-rich DNA [6] and was subsequently shown to be the functional ortholog of FANCJ [8]. The value of *C. elegans* as a model for Fanconi Anemia and ICL repair has been thoroughly reviewed in Youds et al. [9]. An understanding of DOG-1's role in replication and repair began with the observation that it is a mutator. This was immediately recognizable in *C. elegans* because of the appearance of spontaneous morphological mutants (described in Cheung et al. [6]) and further explored by the capture and characterization of mutational changes in genes essential for survival (lethal mutations) maintained using a genetic balancer [10]. In *dog-1* mutants, the manifestation of the morphological *Vab* (*V*ariable *AB*normal) phenotype was linked to the gene *vab-1*. An examination of the molecular nature of the *vab-1* mutations revealed small deletions that were detectable by PCR. These deletions initiated at the 5′ end of poly-C or

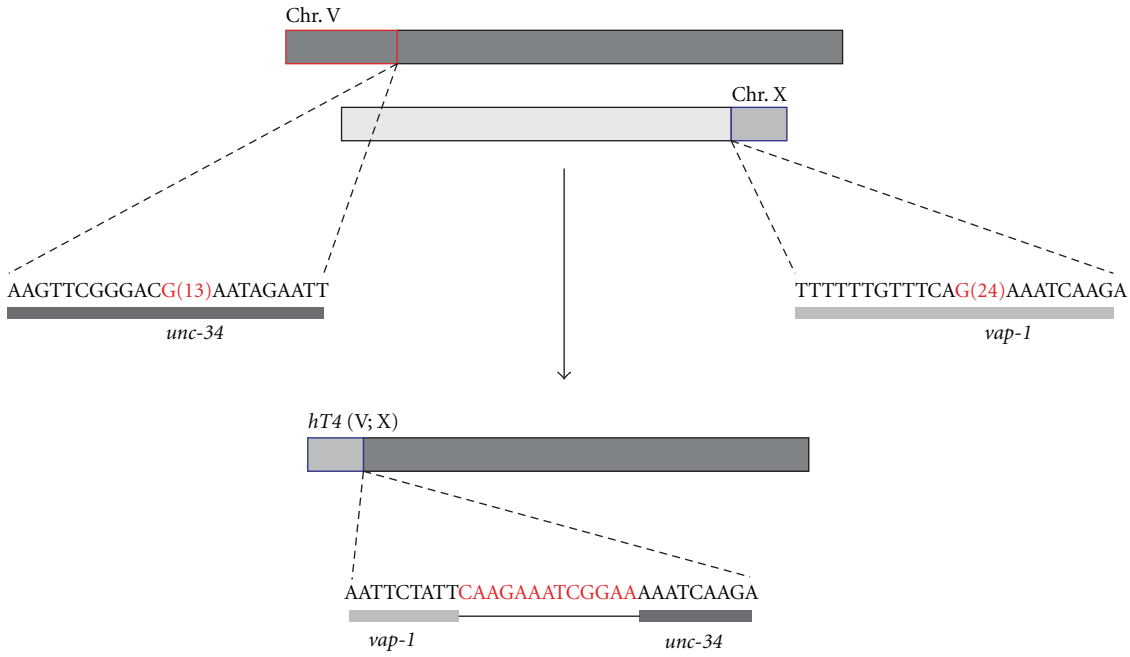

Figure 1: Schematic of the *dog-1*-derived translocation, *hT4* (V; X). Sequences near the left end of chromosome V were deleted (red box), whereas the right end of chromosome X was duplicated (blue box). PCR primers were designed and used to determine the DNA sequence across the junction of V and X in *hT4* (described in [10]).

the 3′ end of poly-G stretches of DNA and extended for variable distances. These observations led to the proposal that the deletions were occurring as a result of structural blocks to lagging-strand synthesis [6]. In this model, poly-G stretches present in the *C. elegans* genome form secondary structures. These secondary structures require the helicase function of DOG-1 to resolve them, allowing fork progression. In the absence of the helicase function, deletions are formed between the stalled fork and the upstream Okazaki fragment initiation. Another research group subsequently confirmed the prediction that Okazaki-sized deletions occurred on the lagging strand by using unbiased array comparative hybridization (aCGH) of DOG-1-minus genomes [11]. In this study, it was shown that deletions occurred exclusively at sequences that could form quadruplex structures (G4) at a frequency of 4% per site per animal generation. In the human genome, there are estimated to be >300,000 G4 forming sites [12], and these have potentially mutagenic properties implicated in development of cancer susceptibility in the absence of FANCJ function.

Further work from our laboratory revealed that in the absence of DOG-1 large chromosomal rearrangements occurred [10]. The rearrangements included larger deletions, duplications of chromosomal fragments, and translocations between chromosomes, in addition to the small deletions detectable by PCR. These large rearrangements were identified because they acquired lethal mutations, which could be isolated and characterized with the use of a balancer chromosome that provided a rescuing wild-type allele in a stable genetic construct (reviewed in [13]). The analysis showed that 1% of the chromosomes acquired lethal lesions [10], giving a forward mutation frequency greater than tenfold of the spontaneous frequency. The frequency is equivalent to that for 500 Rads of ionizing radiation [14]. Rearrangements derived from *dog-1* mutant that were examined by aCGH revealed that in most (but not all) cases the breakpoints occurred in G-rich DNA. In one example, a translocation between chromosome V and the X-chromosome was formed. In this case, the right end of the X-chromosome was duplicated and attached to the left breakpoint of a deletion at left end of chromosome V (Figure 1). The breakpoint on chromosome V is in a 24 bp G/C tract, while the breakpoint on the X is in a "short" 13 bp G-rich sequence. In vertebrates, large rearrangements have also been observed in the absence of FANCJ function. In avian DT40 cell lines, large-scale genomic deletions occurred at the rearranged immunoglobulin heavy chain locus (IgH) in the absence of *FANCJ*, but not other FA genes [15]. These researchers found that in *FANCJ* mutant cells cultured for two months, G4 sequences detected by aCGH were found at the breakpoints of one deletion. However, not all breaks occurred in G-rich DNA, suggesting that other sequences are also susceptible to breakage in the absence of FANCJ.

3. Homologous Recombination and Translesion Synthesis Compensate for the Absence of DOG-1

Repair pathways that compensate for the absence of DOG-1 in *C. elegans* have been identified. These include homologous recombination (HR) repair and translesion synthesis (TLS), but not nonhomologous end joining (NHEJ) [7]. In human cell lines, monoubiquitylation of FANCD2 is followed by HR repair. Our genetic analysis has shown that DOG-1 mutants

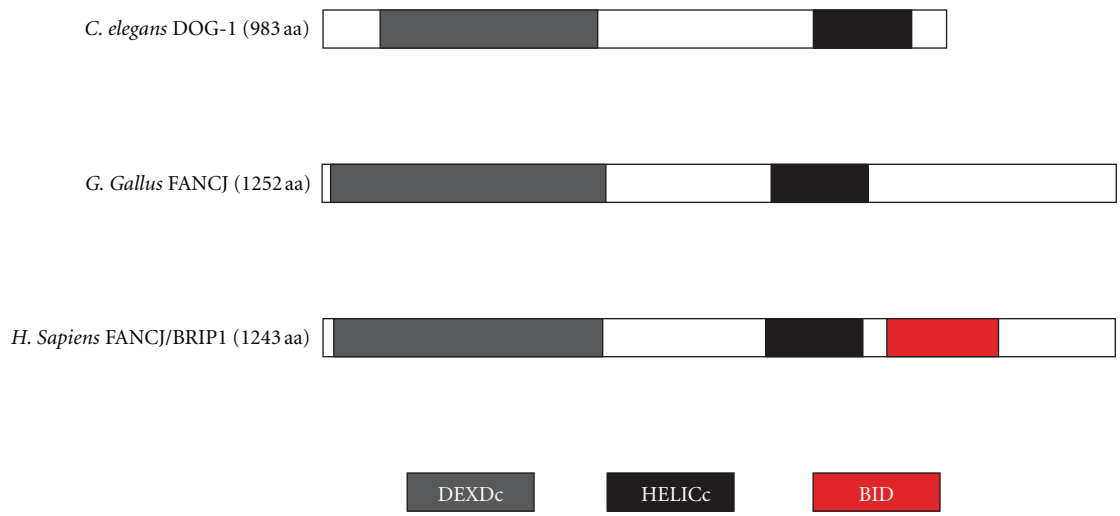

Figure 2: Protein schematic of FANCJ orthologs. *C. elegans* DOG-1, 983 aa, chicken FANCJ, 1252 aa and human, 1243 aa FANCJ proteins illustrating the position of the conserved DEAD Box (DEXDc) and helicase (HELICc) domains. The BRCA-1 interaction domain of human FANCJ is illustrated (BID). A full protein sequence alignment of DOG-1 and human FANCJ is shown in [8].

that are also mutant for FCD-2 (FANCD2) exacerbate G-tract deletions [8], as are the HR repair components, BRD-1 (BARD1), RAD-51 (RAD51), and XPF-1 (XPF). Similarly, DOG-1 mutants lacking the TLS polymerases, POL eta and POL kappa have significantly more PCR-detectable G-tract deletions than DOG-1 by itself. That the FA pathway and its downstream repair mechanisms are capable of resolving some G-tract-associated secondary structures in the absence of DOG-1 function indicates that the FA pathway is parallel to DOG-1, at least with respect to the maintenance of G-tracts.

A recent study in DT40 cells has expanded the endogenous role of FANCJ. Recently, Sarkies et al. have shown that FANCJ coordinates two independent mechanisms to maintain epigenetic stability near G4 DNA motifs [16]. These mechanisms are dependent on the function of the Y-family polymerase REV1 and the helicases WRNs and BLMs. Similar epigenetic studies have not been performed in *C. elegans*. However, G-tract instability is significantly increased in DOG-1 mutants animals deficient in the BLM ortholog HIM-6 [7]. Mutants in the *C. elegans* WRNs ortholog WRN-1 do not exacerbate G-tract deletions, indicting that if a function in G-tract resolution is conserved in *C. elegans*, it is dependent on the presence of DOG-1. The *C. elegans* REV1 ortholog REV-1 has not been studied with respect to G-tract stability.

4. DOG-1 Functions to Reduce ICL-Induced Damage

A diagnostic feature of FA defects is the cross-link sensitivity of cultured cells. The presence of ICLs can result in error-prone repair leading to chromosomal instability (CIN) and cell death. In *C. elegans*, the absence of DOG-1 also results in sensitivity to ICL-inducing agents such as UVA-activated trimethylpsoralen, nitrogen mustard, and cisplatin, but not to X-rays or UVC [8]. Treatment of DOG-1-deficient animals with ICL agents can result in checkpoint-induced cell cycle arrest and apoptosis of germ cells, as well as chromatin bridges and breaks [8]. In response to ICL treatments, animal's doubly mutant for DOG-1 and FCD-2 are as equally sensitive as each of the single mutants, potentially placing the helicase function of DOG-1 in the same pathway as FCD-2 [8]. Furthermore, DOG-1 is not required for RAD-51 or FCD-2 foci formation after replication stress or ICL induction, possibly placing DOG-1 downstream of FCD-2. This data correlates with that reported by Bridge et al. [17] who demonstrated that *FANCJ* mutant DT40 cells are also not defective for FANCD2 focus formation.

In human cell lines, monoubiquitinlation of FANCD2 is followed by HR repair. During S phase, ICLs can block replication; consequently, HR and TLS are required to stabilize the fork and restart replication (reviewed in [18]). In *C. elegans*, HR repair alleviates the loss of DOG-1. DOG-1 does not function directly in DSB repair, however, as it is not sensitive to radiation-induced DSBs [8]. Bridge et al. determined that FANCJs role in ICL repair is independent of BRCA1 function by demonstrating rescue of *FANCJ* phenotypes in DT40 cells with the expression of human FANCJ/BRIP1 lacking its BRCA1-interaction domain [17]. Since DOG-1, like the avian FANCJ, does not contain the BRCA-1 interaction domain found in human FANCJ (Figure 2), we infer that the helicase function of DOG-1 is not required for HR-mediated DSB repair following replication block or ICL induction.

The type of repair pathway recruited following replication block is important in maintaining genome stability. In *C. elegans* [8] and in human and chicken cells [19], FA proteins regulate the decision to repair double strand breaks (DSBs) resulting from replication blocks or ICLs using error-free HR repair rather than error-prone nonhomologous end joining (NHEJ). In the Adamo et al. study [20], it was shown that FA-deficient human cell lines and *C. elegans* mutants

had chromosomal abnormalities similar to those found in cell lines from cancer and FA patients. However, when the NHEJ component LIG-4 (LIG4) is lacking, the abnormalities do not occur. HR-mediated repair is proposed to be favored due to single-stranded DNA produced by FANCD2 [19]. In *C. elegans*, this result provides a potential inroad to further dissection of the role of FA in DNA repair and the maintenance of genome stability.

The relationship between TLS and HR repair in *C. elegans* has been teased apart somewhat by the characterization of two genes, *polq-1* (*POLQ*) and *hel-308* (*HELQ*) [21]. POLQ-1 has a helicase domain at the N-terminus and a polymerase domain at the C-terminus and has been implicated in recombination-independent and TLS-dependent ICL repair (reviewed in [22]). The helicase HEL-308, on the other hand, is proposed to function in HR along with the FA pathway in ICL repair. In *C. elegans*, there are two genetically distinct pathways, a BRC-1-POLQ-1 pathway and an FA (FCD-2, DOG-1)-HR-HEL-308 pathway. At least one of these pathways must be functional for animals survival as mutants in *hel-308* results in synthetic lethality when combined with *brc-1* mutants (reviewed in [9]). These results separate the helicase function of DOG-1 from the BRC-1/BRCA-1 repair pathway and further distinguish the role of DOG-1 as independent of HR repair. Initially these results may appear paradoxical. FCD-2 is not required for G-tract stability and the double mutant *dog-1*; *fcd-2* increased G-tract deletions 3-fold [8], placing DOG-1 upstream of the FA pathway. However, in the case of ICL sensitivity, the double mutant is not more sensitive. One interpretation of these data is that DOG-1 is epistatic to the FA pathway. Both findings are consistent with DOG-1 attempting unsuccessfully to remove the cross-linked structure.

How does this inform our understanding of DOG-1's helicase function and the relationship between G-rich secondary structures and ICLs? There is ample evidence that DOG-1 is unique in its role to maintain G-rich DNA that can form G4-like secondary structures [7, 8, 11]. Additionally, it has been demonstrated that purified FANCJ efficiently unwinds a variety of G4 structures dependent upon intrinsic FANCJ ATP hydrolysis and the availability of a 5′ ssDNA tail [22]. None of the other helicases that are able to unwind G4 structures can compensate for the loss of DOG-1. This is supported by the fact that in *C. elegans* DOG-1 has a unique phenotype and that in other systems only FANCJ has been shown to prevent breaks in G-rich DNA. These structures are, however, not covalently linked. There is no evidence that the DOG-1/FANCJ helicase can resolve covalently linked ICLs. So what is the connection?

We propose the following model as a resolution of this apparent paradox (Figure 3). G4 structures are known to form in a variety of circumstances as proposed by Wu et al. [22], which could include within a single strand of DNA, between DNA strands and between strands on separate chromosomes. The latter resulting in chromosomal translocations if not repaired correctly. In the absence of crosslinking agents, these secondary structures can form and unform depending upon the availability of DOG-1. In the *C. elegans* genome, there are nearly 400 poly-G regions distributed

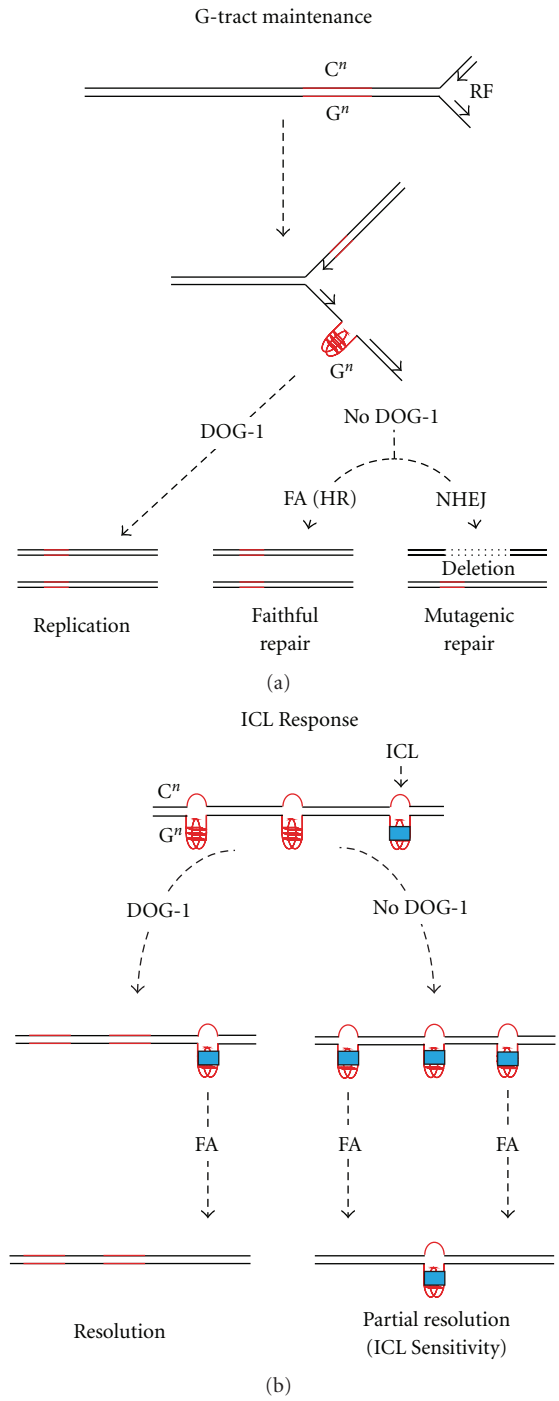

Figure 3: A model for DOG-1 function in genome stability and ICL response. The left panel illustrates DOG-1's role in G-tract maintenance. G4 formation on the lagging strand is resolved by the helicase function of DOG-1 and replication proceeds efficiently. In the absence of DOG-1 HR mediated by the FA pathway resolves a subset of stalled forks. Repair utilizing the mutagenic NHEJ repair mechanism results in deletions. The right panel describes a possible model for DOG-1 ICL sensitivity. In the presence of DOG-1, G4 structures may be resolved and not available as substrate for ICL stabilization. In the absence of DOG-1 G4 structures are available as substrate for ICL stabilization leading to an in increase in fork stalling, which is interpreted as an ICL sensitivity phenotype.

along each of the chromosomes and this pattern of distribution is conserved in a related nematode [23] providing a rich source of substrate for DOG-1. In the presence of a crosslinking agent, many of which have affinity for G's, secondary structures formed by these G-rich regions might be targets for covalent crosslinking. Here we suggest that once the secondary structures are detected by FA pathway components the first responder is DOG-1. The pathway detector may not distinguish between a noncovalent secondary structure and a crosslink. If the structure is not covalently linked, DOG-1 resolves it. If it is covalently linked, and not resolved by DOG-1, FA pathway-directed TLS and HR repair the lesion. In the absence of DOG-1, there is likely to be an increase in stabilized G-rich structures that may be beyond the ability of the FA pathway to respond to, giving the appearance of a crosslink sensitive phenotype. Further experiments will be needed to move towards a more complete understanding of the crosstalk among FA proteins.

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Review Article

Targeting the Fanconi Anemia Pathway to Identify Tailored Anticancer Therapeutics

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The Fanconi Anemia (FA) pathway consists of proteins involved in repairing DNA damage, including interstrand cross-links (ICLs). The pathway contains an upstream multiprotein core complex that mediates the monoubiquitylation of the FANCD2 and FANCI heterodimer, and a downstream pathway that converges with a larger network of proteins with roles in homologous recombination and other DNA repair pathways. Selective killing of cancer cells with an intact FA pathway but deficient in certain other DNA repair pathways is an emerging approach to tailored cancer therapy. Inhibiting the FA pathway becomes selectively lethal when certain repair genes are defective, such as the checkpoint kinase ATM. Inhibiting the FA pathway in ATM deficient cells can be achieved with small molecule inhibitors, suggesting that new cancer therapeutics could be developed by identifying FA pathway inhibitors to treat cancers that contain defects that are synthetic lethal with FA.

1. Introduction

Fanconi anemia is a rare genetic disease featuring characteristic developmental abnormalities, a progressive pancytopenia, genomic instability, and predisposition to cancer [1, 2]. The FA pathway contains a multiprotein core complex, including at least twelve proteins that are required for the monoubiquitylation of the FANCD2/FANCI protein complex and for other functions that are not well understood [3–6]. The core complex includes the Fanconi proteins FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM. At least five additional proteins are associated with the FA core complex, including FAAP100, FAAP24, FAAP20, and the histone fold dimer MHF1/MHF2 [1, 4, 7–10]. The core complex proteins function together as an E3 ubiquitin ligase assembly to monoubiquitylate the heterodimeric FANCI/FANCD2 (ID) complex. The monoubiquitylation of FANCD2 is a surrogate marker for the function of the FA pathway [11]. USP1 and its binding partner UAF1 regulate the deubiquitination of FANCD2 [12]. The breast cancer susceptibility and Fanconi proteins FANCD1/BRCA2, the partner of BRCA2 (PALB2/FANCN), a helicase associated with BRCA1 (FANCJ/BACH1), and several newly identified components including FAN1, FANCO/RAD51C, and FANCP/SLX4 [13–17] participate in the pathway to respond to and repair DNA damage (for review, see [5]).

Although FA is rare, understanding the functional role of the FA proteins in context with other DNA damage response pathways will provide broader opportunities for new cancer therapeutics. Two general strategies could accomplish this, as illustrated in Figure 1: inhibiting the FA pathway in tumor cells to sensitize them to cross-linking agents, or by exploiting synthetic lethal relationships. The latter approach depends on inhibiting the FA pathway in tumor cells that are defective for a secondary pathway required for survival in the absence of the FA pathway.

2. Chemosensitizing and Resensitizing Tumor Cells

A defining characteristic of FA cells is hypersensitivity to cross-linking agents, such as the chemotherapeutic agent cisplatin [2, 5]. Cisplatin (and other platinum-based

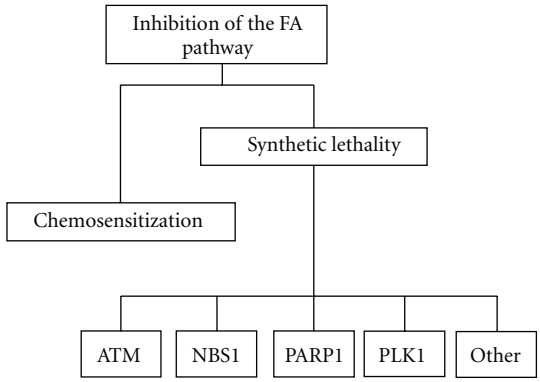

Figure 1: Inhibition of the FA pathway. Strategy for selectively targeting tumor cells by inhibition of the FA pathway by (a) chemosensitization to cross-linking agents or by (b) exploiting specific synthetic lethal interactions.

compounds) has been used as a chemotherapeutic drug for over 30 years (for review see [18]). The toxicity of platinum-based chemotherapy (nephrotoxicity, neurotoxicity, and ototoxicity) and development of cisplatin resistance are limitations of the therapy [18–20]. Once inside the cell, cisplatin enters the nucleus and forms covalent DNA interstrand cross-links via platinum-DNA adducts. These cross-links block ongoing DNA replication, and in the absence of repair, activate apoptotic pathways [18, 19]. A functional FA pathway is required for processing damage after exposure to cisplatin and other crosslinking agents, and is at least partially responsible for resistance to cisplatin. Cell-free and cell-based assays have identified inhibitors of the FA pathway, and some of these inhibitors can resensitize platinum-resistant tumors and cell lines [19, 21, 22]. Further efforts to identify small molecule compounds that specifically inhibit the FA pathway could lead to improved resensitization from treatment-induced resistance.

3. Exploiting Synthetic Lethal Interactions

In addition to sensitization, inhibiting the FA pathway may be an effective strategy to exploit synthetic lethal interactions aimed at improving targeted killing of tumor cells. Current approaches in cancer treatment are generally not selective, affecting both cancer cells and normal cells. However, inactivation of DNA repair pathways, an event that occurs frequently during tumor development [23], can make cancer cells overdependent on a reduced set of DNA repair pathways for survival. There is new evidence that targeting the remaining functional pathways by using a synthetic lethal approach can be useful for single-agent and combination therapies in such tumors. Two genes have a synthetic lethal relationship if mutants for either gene are viable but simultaneous mutations are lethal [20]. A successful example of this approach is specific targeting of BRCA-deficient tumors with PARP (poly (ADP-ribose) polymerase) inhibitors [24].

4. Defects in Homologous Recombination and Sensitivity to PARP Inhibitors

Defects in HR repair can result in an overreliance on the protein PARP1, which is responsible for repair of DNA single strand breaks by the base excision repair pathway. Unrepaired single-strand breaks are converted to double-strand breaks during replication and must be repaired by HR [25–27]. Thus, treating cells that are defective in HR with PARP inhibitors results in a targeted killing of the defective cells, while cells with intact HR are capable of repair. Defects in breast cancer susceptibility proteins BRCA1 and BRCA2 (FANCD1) result in HR defects [28]. Clinical trials investigating the effectiveness of PARP inhibitors against recurrent ovarian cancer have been promising, but rigorous stratification of tumors for HR status or "BRCA-ness" (defects in HR) is needed to identify the patients who are likely to benefit [29–31]. Future clinical trials with PARP1 inhibitors in breast cancer may require combination therapies, evaluation of resistance, and identification of non-BRCA biomarkers [32].

PARP1 Inhibition has also been shown to be selectively toxic to ATM-defective tumor cell lines *in vitro* and to increase radiosensitivity of other ATM-proficient cell lines, including nonsmall-cell lung cancer, medulloblastoma, ependymoma, and high-grade gliomas [33–35]. In addition, cell lines lacking functional Mre11 are sensitive to PARP1 inhibitors, strengthening the case for combined use of PARP1 inhibitors with inhibitors of the FA pathway [36, 37].

PTEN (phosphatase and tensin homolog) is a tumor-suppressor gene and one of the most commonly mutated genes in human tumor cells [38, 39] (see Figure 2). PTEN deficiency results in decreased expression of RAD51, which is required for homologous recombination [38, 40]. PTEN deficient tumors are thus candidates for targeted therapy by PARP1 inhibition [36, 38]. Although approximately 470,000 (48%) of 977,628 newly diagnosed cancers each year in the US may have PTEN defects, only a subset of these cancers will have PTEN mutations that result in homologous recombination defects and sensitivity to PARP inhibitors [28, 39, 41–51]. Current studies are aimed at determining the relationship between PTEN loss, RAD51 expression, and PARP1 inhibitor sensitivity [36]. Efforts to asses HR status to establish which PTEN mutations lead to an HR defect, and determining under what circumstances RAD51 expression could be used as a biomarker, will be useful to stratify and predict PARP1 inhibitor sensitivity.

Synthetic lethal interactions with the FA pathway have been explored. An siRNA-based screen of cells deficient in the Fanconi core complex protein, FANCG, showed that ATM, PARP1, NBS1, and PLK1 were among the genes with a synthetic lethal interaction [52] (see Table 1). The FA-ATM synthetic lethal relationship is particularly interesting since ATM deficiency has been reported in a subset of patients with hematological malignancies, including mantle cell lymphoma, chronic lymphocytic leukemia, and acute lymphoblastic leukemia [53, 54], making these potential targets for treatment with FA pathway inhibitors (see Table 2).

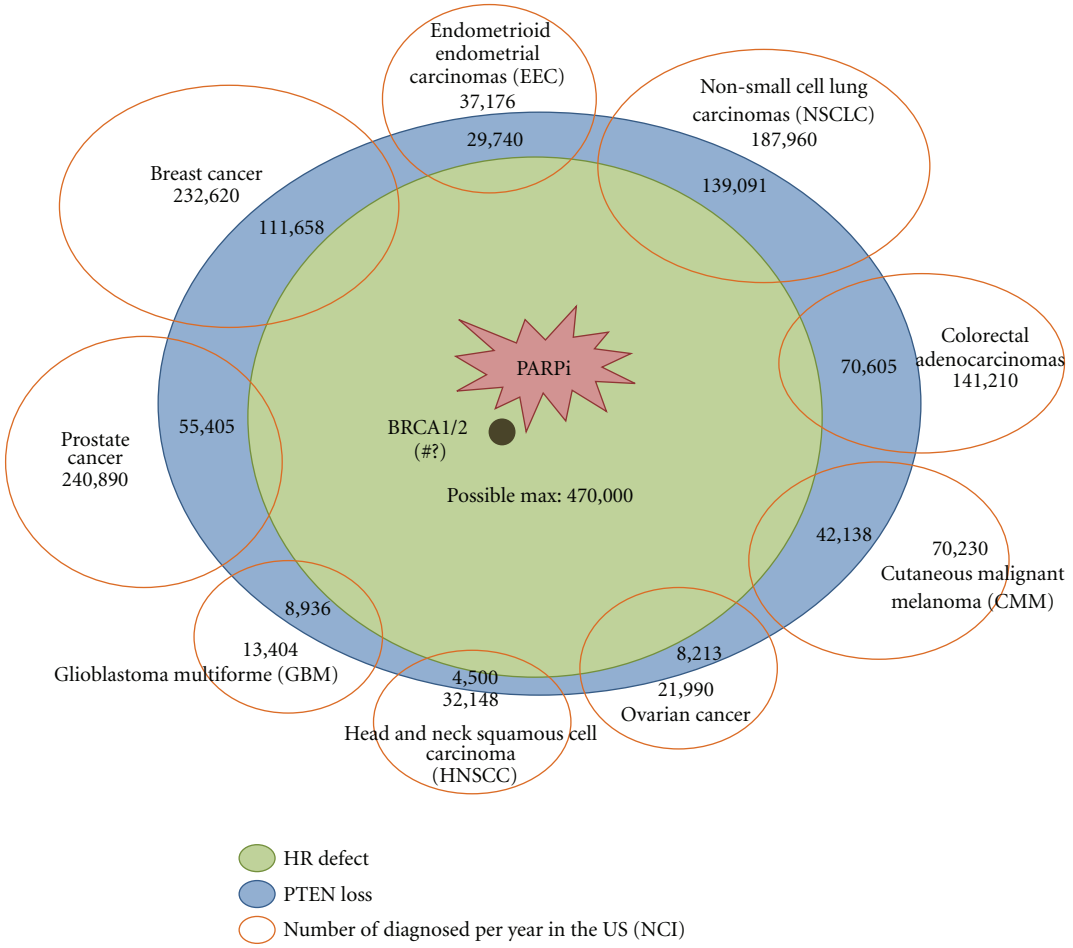

Figure 2: PTEN defects in cancers. Types of cancer diagnosed annually in the US (orange oval), with the estimates for PTEN deficiencies shown in each type (blue oval). An unknown percentage of tumors with PTEN deficiencies will have a defect in homologous recombination (HR) repair, predicting sensitivity to treatment with PARP1 inhibitors (green oval).

Table 1: Function and expression of genes synthetically lethal with FA.

Gene synthetically lethal with FA genes	Function	Expression in tumor cells
TREX2 [52]	DNA exonuclease; SAGA complex pathway	Expressed in most tumor cell lines [60]
PARP1 [52]	BER	Overexpressed in tumors, including medulloblastoma, ependymoma, HGG, melanoma, and breast cancers [35, 61–63]
PLK1 [52]	Cell-cycle progression	Over-expressed in many human tumors [64]
RAD6/HR6B [52]	Switching of DNA polymerases	Upregulated in metastatic mammary tumors [65]
CDK7 [52]	Transcription	Moderately over-expressed in tumor cell lines [66]
TP53BP1 [52]	DSB sensing; ATM activation	Underexpressed in most cases of triple negative breast cancer [67]
ATM [52]	DSB response kinase	Under-expressed in some tumors, see Figure 3
NEIL1 [52]	BER	Expression reduced in 46% of gastric cancers [68]
RAD54B [52]	HR	Known to be mutated in cancer cell lines [69, 70]
NBS1 [52]	DSB sensing; ATM activation	Over-expressed in HNSCC tumors [71]
ADH5 [6]	Formaldehyde processing	Reduced expression in melanoma cells [72]

Table 2: ATM-deficiency in cancer.

Malignancy	ATM-deficient cell lines/number tested
T-cell prolymphocytic leukemia [73]	17/32
Mantle cell lymphoma [53]	12/28
Rhabdomyosarcoma [74]	7/17
Chronic lymphoblastic leukemia [54, 73]	16/50, 38/111
BRCA1-negative breast cancer [75]	12/36
BRCA2 negative breast cancer [75]	12/40
Acute lymphoblastic leukemia [54]	4/15
Non-BRCA1/BRCA2 negative breast cancers [75]	118/1106
Other lymphomas [53]	10/97

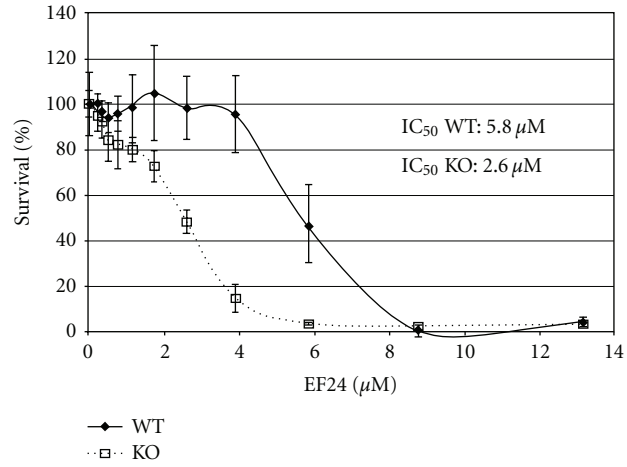

Figure 3: EF24 is selectively toxic to ATM-deficient cells [57]. 309ATM-deficient and 334ATM wild type cells were treated with the FA pathway inhibitor EF24. Cell viability was measured after 3 days by MTS assay. Each point represents the mean of 3 repeats. Error bars represent standard deviation.

5. Inhibiting the FA Pathway

Inhibition of the FA pathway could occur at any point in the multistep FA protein network, but a key predictive readout for FA function and resistance to ICLs is the monoubiquitylation of FANCD2 [11, 55]. Several inhibitors of FANCD2 monoubiquitylation have been identified including proteasome inhibitors bortezomib and MG132, curcumin, and the curcumin analogs EF24 and 4H-TTD [19, 22, 56, 57]. Curcumin, a natural product derived from turmeric, was identified as a weak inhibitor of FANCD2 monubiquitylation in a cell-based screen [19]. We developed a cell-free assay in *Xenopus* egg extracts to screen small molecules for stronger and more specific inhibitors of FANCD2 monubiquitylation. Unlike cell-based screening assays for small molecules capable of inhibiting the FA pathway, the cell-free method uncouples FANCD2 monoubiquitylation from DNA replication, thus focusing more specifically on the key biochemical steps in a soluble context enriched for nuclear proteins and capable of full genomic replication [22]. Screening in egg extracts identified 4H-TTD, a compound with structural similarity to curcumin as an inhibitor, and this inhibitory effect was verified in human cells [22, 57]. A series of curcumin analogs were also tested, including EF24, a potent monoketone analog of curcumin [58, 59]. The prediction that an FA inhibitor would selectively kill ATM-deficient cells was tested in cell-based assays for synthetic lethality in ATM-proficient and ATM-deficient cells. ATM-deficient cells treated with EF24 demonstrated an increased sensitivity compared to ATM wt cells (see Figure 3) [22, 57]. The increased lethality in ATM-deficient cells provides evidence for future synthetic lethal approaches with FA pathway inhibitors in the treatment of ATM-deficient tumors, and other tumors with deficiencies in genes that are synthetically lethal with FA (see Table 1) [6, 52].

6. Conclusion and Future Directions

Understanding how the Fanconi anemia pathway functions in concert with other DNA damage response networks is essential for understanding genomic stability and for exploiting synthetic lethality for new cancer treatments. New chemotherapeutic agents could be developed by identifying potent and specific inhibitors of the FA pathway, for example, by screening for compounds that inhibit key FA pathway steps (e.g., monoubiquitylation and deubiquitylation of FANCD2/FANCI). While a long-term defect in the function of the FA pathway would result in genomic instability, short-term inhibition could provide a treatment strategy for tumors with deficiencies in certain other DNA repair pathways. Stringent identification of additional genes with synthetic lethal relationships with the FA pathway, and identification of malignancies with deficiencies or mutations in genes that are synthetic lethal with FA will be required for these tailored therapeutic approaches.

Acknowledgments

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Research Article

Diagnosis of Fanconi Anemia: Chromosomal Breakage Analysis

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Fanconi anemia (FA) is a rare inherited syndrome with diverse clinical symptoms including developmental defects, short stature, bone marrow failure, and a high risk of malignancies. Fifteen genetic subtypes have been distinguished so far. The mode of inheritance for all subtypes is autosomal recessive, except for FA-B, which is X-linked. Cells derived from FA patients are—by definition—hypersensitive to DNA cross-linking agents, such as mitomycin C, diepoxybutane, or cisplatinum, which becomes manifest as excessive growth inhibition, cell cycle arrest, and chromosomal breakage upon cellular exposure to these drugs. Here we provide a detailed laboratory protocol for the accurate assessment of the FA diagnosis as based on mitomycin C-induced chromosomal breakage analysis in whole-blood cultures. The method also enables a quantitative estimate of the degree of mosaicism in the lymphocyte compartment of the patient.

1. Introduction

Fanconi anemia (FA) is a cancer-prone chromosomal instability disorder with diverse clinical symptoms (Table 1) [1]. Because of its rarity and variable presentation FA may be heavily underdiagnosed [2, 3]. Clinical suspicion of FA is mostly based on growth retardation and congenital defects in combination with life-threatening bone marrow failure (thrombocytopenia and later pancytopenia), which usually starts between 5 and 10 years of age. However, the clinical manifestations are highly variable, while some of the symptoms may overlap with those observed in other syndromes, making a reliable diagnosis on the basis of clinical features virtually impossible (Table 1). Even patients presenting with a number of "typical" FA symptoms may not be suffering from FA. Cells derived from true FA patients must exhibit a hypersensitivity to chromosomal breakage induced by DNA cross-linking agents such as mitomycin C (MMC), diepoxybutane (DEB), or cisplatinum.

Indications to test for FA are typical congenital abnormalities with/without thrombocytopenia and/or marrow failure. However, congenital abnormalities may be absent, while isolated thrombocytopenia may be the only presenting symptom. In all children with aplastic anemia FA should be tested as the possible underlying disease. In children and adults with cancer and an unusual response to DNA-damaging agents such as chemotherapy or radiotherapy (severe skin reactions or mucositis, longlasting aplasia), FA should also be tested for. Similarly, in adults with carcinomas (typically located in the mouth/esophagus or anogenital region) at relatively young age, FA should be considered. Cancer or leukemia may be the first symptom of FA, while congenital abnormalities and marrow failure may be absent altogether, the latter especially in cases with hematopoietic mosaicism [4–6].

The cellular phenotype typical for FA is ascertained using phytohaemagglutinin-stimulated whole-blood (T lymphocyte) cultures. Although it has been considered the gold standard for diagnosing FA, the test is not 100% specific. A few cases of Nijmegen breakage syndrome have been reported to give a false positive result [7–9], which can be excluded by screening the *NBS1* gene for mutations. In addition, patients suffering from the cohesinopathies Roberts syndrome (mutated in *ESCO2*) and Warsaw breakage syndrome (mutated in *DDX11*) may score positive in the test [10]. Additional "atypical FA" or "FA-like" patients have been reported as case reports [11, 12]. Somewhat controversially, the "FA-like" patient found to be mutated in

Table 1: General features and symptoms associated with Fanconi anemia.

Birth prevalence	0.5–2.5 per 10^5 newborns; varies with ethnic background.
Mode of inheritance	Autosomal recessive (>98%) and X-linked (~1-2%).
Carrier frequency	Traditional overall estimate: "1/300 worldwide." Needs reassessment according to subtype and ethnic background.
Congenital abnormalities*	Radial ray abnormalities (aplastic or hypoplastic radii and absent or extra thumbs) and other skeletal abnormalities; small head circumference; abnormal shape of the ears; **microphthalmia**; ectopic or horse-shoe kidney; **hypogonadism**; heart abnormalities; intestinal or anal atresia.
Other somatic abnormalities*	**Short stature/retarded growth; reduced fertility; skin pigmentation abnormalities (hyperpigmentation, café-au-lait spots)**; deafness. Endocrinopathy affecting the pancreas (diabetes mellitus), growth hormone deficiency, and hypothyroidism; early menopause.
Hematological symptoms	Bone marrow failure or aplastic anemia typically starting at 5–10 years with thrombocytopenia. Exception: D1 and N patients may die before that age from AML or other childhood solid tumors (such as medullo- or nephroblastoma).
Cancer risk	800-fold increased risk of AML, mostly occurring at age 5–15 years, typically after the onset of marrow failure. At older ages there is a similarly increased risk of solid tumors, mainly carcinomas of the head and neck or oesophagus, as well as, in females, the vulva and vagina. D1 and N patients typically develop malignancies during early childhood (<5 years).
Overlapping syndromes**	*Inherited bone marrow failure syndromes*: Dyskeratosis congenita, Diamond-Blackfan anemia, Shwachman-Diamond syndrome, severe congenital neutropenia, thrombocytopenia absent radii (TAR) syndrome, amegakaryocytic thrombocytopenia. *Other overlapping syndromes*: Baller-Gerold syndrome, *Nijmegen breakage syndrome*, Rothmund-Thomson syndrome, *Roberts syndrome*, *Warsaw Breakage syndrome*, DK-phocomelia, VACTERL hydrocephalus syndrome, Wiskott-Aldrich syndrome.

*Many symptoms show highly variable penetrance. In a sizable proportion of patients (ca. 30%), congenital abnormalities may be absent altogether. Features in bold are most consistently associated with the FA phenotype.
**For an overview of the overlapping inherited bone marrow failure syndromes, see [5, 25]. For the other overlapping syndromes, the reader is referred to the OMIM database. Three overlapping syndromes may score positive in a chromosomal breakage test (italic): Nijmegen breakage syndrome [7–9], Roberts syndrome, and Warsaw Breakage Syndrome [10].

RAD51C has been assigned to a distinct genetic FA subtype (FA-O) [13].

Approximately 80% of the patients referred for FA diagnostic testing because of bone marrow failure score negative in the chromosomal breakage test. These "true negatives" have other causes of marrow failure and most often represent cases with acquired aplastic anemia.

Lymphocyte mosaicism occurs in a sizable proportion of FA patients (estimated at 10–30%) and is caused by spontaneous genetic reversion at the disease locus in hematopoietic progenitor cells; the reverted cells may (partially) correct the bone marrow failure [14–18]. In most of these cases FA can still be diagnosed by testing peripheral blood, since a portion of the cells will still show hypersensitivity to cross-linking agents. Occasionally, the percentage of reverted cells has reached such a high level as to produce a false negative diagnosis. In such cases cross-linker sensitivity may be tested in skin fibroblasts, which are not known to be affected by mosaicism. After a positive breakage test result has been obtained, screening for mutations in the known FA genes is warranted.

Laboratory studies have revealed as many as 15 distinct "complementation groups" or genetic subtypes: FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, -N, -O, and -P [13, 19–21]. For all subtypes known to date the disease genes have been identified. Global relative prevalences are difficult to estimate, as the values may differ considerably depending on the ethnic background, due to founder effects. All FA genes are localized on autosomes, except *FANCB*, which is X-linked and subject to X inactivation in female carriers [22]. These two different modes of inheritance have important consequences for the counseling of FA families.

Recognition of FA as a chromosomal instability disorder was originally based on chromatid-type aberrations spontaneously occurring in standard cytogenetic preparations. However, this phenomenon was later found to be highly variable and considered not reliable for diagnostic purposes. After the discovery of an extreme sensitivity of FA cells to the chromosome-breaking effect of the cross-linking agents mitomycin C (MMC) [23] and diepoxybutane (DEB) [24], this feature has become routinely utilized to diagnose FA by a "chromosomal breakage test." In this test, T lymphocytes in a peripheral blood sample are cultured in the presence of a cross-linking agent, after which chromosomal aberrations are quantified in metaphase spreads. Numerous variations of the test are used in the various cytogenetic laboratories,

with significant differences in exposure times and drug concentrations. Also, the ways in which data are evaluated are diverse. We have encountered opposite conclusions from different laboratories based on the very same primary data set, due to a lack of experience in performing the test and evaluating the resulting data. Evidently, there is a great need for a clearly described reliable protocol for the accurate diagnosis of FA patients.

2. Methods and Results

Here we describe a laboratory protocol that has evolved during 30 years of experience and which we can recommend for the unambiguous diagnosis of the vast majority of FA patients, including patients with hematopoietic mosaicism. The test is based on the 72 hour whole-blood cultures as routinely applied in cytogenetics laboratories to make chromosomal preparations for karyotypic analysis. Metaphase spreads are Giemsa-stained (not banded) and analyzed for microscopically visible chromatid-type aberrations. For technical details the reader is referred to the appendices. Laboratories that are not set up to do this type of assay or have no experience with diagnosing FA on a regular basis should be advised to refer to a laboratory where the test is applied on a routine basis, rather than attempting to carry out a "similar" test that is considered a plausible alternative. The test might be omitted if a proband belongs to an ethnic population with a high carrier frequency for a specific FA gene mutation. Demonstrating this mutation in the proband would be diagnostic for FA, although the result may not provide information about possible mosaicism.

3. Discussion

It should be pointed out that, even though we have chosen to use MMC as the cross-linking agent, DEB is used in a widely accepted alternative protocol [1, 26–28]. Pros and cons for using the various cross-linking agents are further discussed in the appendices.

Cell cycle analysis via flow cytometry has been used as an alternative way to diagnose FA in skin fibroblasts [29], amniocytes [30], and peripheral blood mononuclear cells [31–34]. This test is based on the fact that cells from FA patients are hypersensitive towards DNA cross-linking agents and tend to be delayed and arrested with a 4c DNA content in the late S/early G2 phase of the cell cycle [35–38]. With the exception of overt leukemia and complete lymphocyte mosaicism, the cell cycle test reliably differentiates between FA and non-FA blood samples, including non-FA patients with aplastic anemia, Nijmegen breakage syndrome, Roberts syndrome, Baller-Gerold syndrome, VACTERL, and other thrombo- and erythropenia syndromes, as these conditions lack elevated G2-phase cell fractions [39]. For details of the cell cycle assay, readers are referred to the published protocols [39, 40].

FANCD2 western blotting is another alternative procedure to diagnose FA [40]. With this method stimulated T lymphocytes are tested for the occurrence of the ubiquitinated isoform of FANCD2, which readily reveals FA in cases where this isoform is lacking (subtypes A, B, C, D2, E, F, G, I, L, and M). This is a convenient alternative for diagnosing >90% of all FA patients. A disadvantage is that the subtypes with a defect downstream of FANCD2 ubiquitination (D1, J, M, N, O, P and possibly new subtypes) are not diagnosed as FA. In addition, true FA cases with significant lymphocyte mosaicism may also be missed by FANCD2 western blotting.

Why would a relatively laborious breakage test still be relevant now that next-generation sequencing (NGS) is available to determine mutations in FA genes? Two types of result from NGS would require assessment of the cross-linker sensitive cellular phenotype. First, unclassified sequence variations may be identified, whose pathogenic status remains uncertain until functionally tested. Second, if all known FA genes were found to be unaffected by mutations, a putative new FA gene may be found mutated. Proof of identity as a new FA gene requires the demonstration of cellular hypersensitivity to cross-linking agents and some form of functional test where introduction of a wild-type allele should correct the phenotype.

Appendices

A. Laboratory Protocol for Testing MMC-Induced Chromosomal Breakage

A.1. Materials

(1) Heparinized venous blood (≥2 mL; preferably freshly drawn, or kept at room temperature for no longer than 48 h) from the patient to be tested and from a healthy control.

(2) RPMI or Ham's F10 culture medium, including 15% fetal bovine serum, streptomycin, penicillin, and phytohemagglutinin, as utilized in standard cytogenetic whole-blood cultures.

(3) Mitomycin C (MMC, mol. wt. 334.33, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan, clinical grade), available in vials of 2 mg with 48 mg NaCl, to be stored at 4°C.

(4) Materials for the preparation of metaphase spreads.

A.2. Culturing and Cytogenetics Methods

(1) Prepare a stock solution of MMC at 1.5 mM (0.5 mg/mL) by adding 4 mL sterile water per vial; this solution is stable for 3 months at 4°C. It is mandatory "*not*" to freeze the MMC stock solution, since—upon thawing—an unknown quantity of MMC appears as crystals that do not readily redissolve.

(2) Prepare whole-blood cultures from the patient and the healthy control, as usual for a standard cytogenetic analysis [25]. You need 4 cultures for the patient and 4 for the healthy control, who should *not* be

a brother or sister of the patient. Initiate the cultures by adding 0.5 mL blood to 4.5 mL complete medium.

(3) Add, at the time of culture initiation, to each set of 4 cultures: 0, 50, 150, and 300 nM MMC, as indicated below.

(i) Dilute 1 part stock solution plus 9 parts H_2O → *solution A* (150 μM).

(ii) Dilute 1 part solution A plus 4 parts H_2O → *solution B* (30 μM).

(iii) Add to the 4 cultures from each individual:

(a) 50 μL saline → final concentration: 0 nM,

(b) 8.3 μL solution B → final concentration: 50 nM (optional),

(c) 25 μL solution B → final concentration: 150 nM,

(d) 50 μL solution B → final concentration: 300 nM.

N.B. If insufficient blood should be available, the 50 nM cultures may be omitted.

(4) Harvest at 72 h, after colcemid treatment during the last 40 min (Sigma, demecolcine final concentration 200 ng/mL). Prepare at least 4 microscope slides for every culture; make more slides if mitotic activity is low, to end up with at least several hundreds of metaphases, accounting for the possibility that a large proportion will later be judged unacceptable for microscopic analysis. Stain with Giemsa only. Do not apply any banding technique. Store remaining suspension at –20°C, for future use, if necessary.

A.3. Scoring the Aberrations. It is important to realize that quantification of chromosomal aberrations shows significant differences between laboratories. From a comparative study it appeared that the most important source of disagreement was about whether particular aberrations really existed or not, and about the definition and scoring of gaps [41]. It is therefore mandatory to score metaphases from coded slides ("blind"), that is, without knowing the identity of the preparation you are scoring. Do not score more than 25 cells per slide. This is to reduce the possibility of biased scoring, which would result from inspecting too many metaphases from the same slide. To obtain sufficient statistical power of the breakage data, attempt to find and score at least 50 scorable metaphases per culture (to be scored from at least two slides).

A.3.1. Coding and Organizing the Slides before Scoring. After staining, divide the slides into two equal sets per culture, each set containing 2, 3, or more slides (depending on metaphase yield) to allow the analysis of 25 scorable metaphases per set (see also Appendix A.2, point 4). Cover the unique identifier information on the slide with a piece of nontransparent tape. Write a random code on each set of slides and distinguish multiple slides within a set by adding A, B, C, and so forth.

Example 1. for every culture, you end up with 4 slides or more (depending on the mitotic index), coded as follows: *[random code-1]A*, *[random code-1]B*, and so forth; *[random code-2]A*, *[random code-2]B*, and so forth.

The scoring of metaphases (see below) starts with slide *[random code-1]A* until 25 metaphases have been examined. If fewer metaphases were found on the slide, proceed with slide *[random code-1]B*, and so forth, until the desired number of metaphases (in our case: 25) have been scored. Follow the same procedure for *[random code-2]A, -B, -C*, and so forth. After finishing the scoring of all preparations, the codes are uncovered and the two data sets from the various cultures are combined to provide results per 50 metaphases.

A.3.2. How to Score Chromosomal Aberrations. Systematically select the metaphases to be analysed: search, at 400x magnification, for metaphases judged suitable for evaluation of chromosomal integrity. To avoid a bias for relatively undamaged metaphases, do not at this stage select on the basis of "quality," since "nice-" looking metaphases tend to have fewer aberrations. Rather, every next metaphase encountered should—in principle—be scored, unless it must be rejected because it fails to meet the observer's criteria for adequate spreading, state of condensation of the chromosomes (not too long or too short), adequate staining and morphology (clearly recognizable chromosomes with clearly visible centromeres), and adequate ploidy. When a metaphase meets these criteria, that metaphase *must* be scored, at 1000x magnification, even if "difficult" aberrations are subsequently encountered. However, be sure to score only the *really convincing* aberrations while ignoring the unconvincing ones. Distinguish the following types of aberration:

(1) chromatid gap, an interruption in the staining of a chromatid 1-2 times the width of that chromatid (Figure 1(a));

(2) chromatid break, where the interruption is more than 2 times the width or where the broken piece of chromatid appears dislocated, as in Figure 1(b);

(3) triradial chromosome, an interchange figure presumably having resulted from the misrepair of two breaks in two distinct chromosomes (Figure 1(c));

(4) quadriradial chromosome, an interchange figure resulting from the misrepair of two broken chromatids in different chromosomes (Figure 1(d));

(5) Other chromatid interchange figures, such as illustrated in Figure 1(e).

Chromosome-type changes, such as dicentrics, acentric fragments, and ring chromosomes, may be recorded, but these aberrations, which are extremely rare with the protocol used, should not be included in the final analysis.

A.3.3. How to Record the Aberrations. The aberrations observed should be recorded with the coordinates of the metaphase, so that aberrant metaphases can be retrieved whenever considered necessary. This can be achieved manually, or with the help of an automated metaphase finder

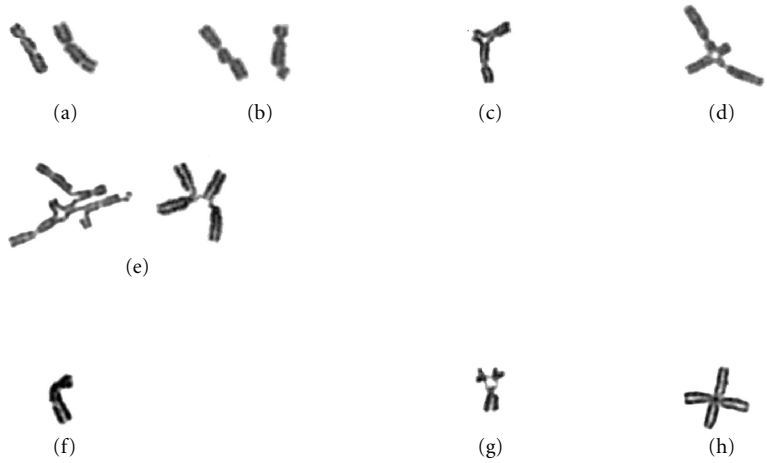

Figure 1: Examples of chromosomal aberrations typically observed in a MMC-induced chromosomal breakage assay to diagnose FA. (a) Chromatid gap (broken piece in place); (b) chromatid break (broken piece dislocated); (c) chromatid interchange figure ("triradial"); (d) chromatid interchange figure ("quadriradial"); (e) other chromatid interchange figures. In the eventual analysis, (a) and (b) are counted as one, (c) and (d) as two break events. The left figure in (e) is counted as 8 break events (5 centromeres plus 3 open breaks); the right figure is equivalent to a quadriradial as in (d) (2 break events), in which two break points remained disconnected. (f), (g), and (h), are examples of nonconvincing "aberrations" that should be ignored in the analysis. (f) A gap that is not 100% convincing and should be ignored. (g) An association of 3 acrocentric chromosomes showing "satellite association", not to be confused with a triradial, as in (c). (h) Two overlapping chromosomes, not to be confused with a true quadriradial, as in (d).

equipped with a customisable scoring sheet for the evaluation of chromosomal aberrations, such as developed by Metasystems, Altlussheim, Germany. A sheet developed for manual evaluation may be obtained from the authors, upon request.

A.4. Analyzing the Results

A.4.1. Converting Aberrations into Break Events. The ratio between gaps/breaks ("open breaks") and interchange-type aberrations ("wrongly repaired breaks") may vary considerably. Therefore, for the final evaluation, all aberrations are converted into "break events", which represent the primary type of aberration in an FA cell.

Chromatid gaps or breaks are counted as single break events, tri- and quadriradials as two break events each. Other interchange figures are converted into the minimum number of breaks required for their theoretical reconstruction; in practice, this means that the number of centromeres in an interchange figure is added up to the number of open breaks/gaps, see Figure 1. To avoid spending too much time on reconstructing complex interchange figures, cells showing more than 10 break events are not further quantified and are included in a common category "≥10 breaks/cell". Evaluate the data from a histogram, in which the percentage of cells is plotted against the number of break events/cell, as illustrated in Figure 2.

A.4.2. Evaluating the Results: "FA", "Non-FA", or "Mosaic FA". In cultures from a typical full-fledged FA patient a substantial proportion of the cells should show chromosomal breakage already at 50 nM MMC (Figure 2). At 150 nM MMC, the majority of cells should be aberrant, while at 300 nM no undamaged cells should be left and most cells should be in the category "≥10 breaks/cell". In contrast, cultures from the healthy control should hardly or not be affected, except at 300 nM, where typically 30% of the cells may show 1 to ≤5 break events/cell.

In cultures from FA patients with lymphocyte mosaicism, two cell populations are distinguished at 300 nM MMC, one behaving like typical FA cells, that is, showing ≥10 breaks/cell, and one behaving like healthy controls, that is, largely represented by the categories 0-, 1-, and 2-breaks/cell.

In the event of a positive result (FA or mosaic FA), all asymptomatic sibs of the patient should be tested as well, which is particularly important if the sibs are considered as potential stem cell donors. A positive result indicative of FA should immediately be evident from the histogram (Figure 2). If statistical analysis is considered necessary to "prove" a dubious diagnosis, the diagnosis "FA" is likely to be wrong.

If the result indicates "non-FA", an important question is whether the MMC concentration was correct. This is another reason why the highest concentration (300 nM) is included, since at this concentration the healthy control should show significantly elevated breakage. The difference between treated and untreated control cultures may be tested by comparing the percentages of aberrant metaphases, using a 2-sample Chi-square test. If the healthy control should fail to show a clear response to the MMC at 300 nM, the result "non-FA" is inconclusive and the test should be repeated.

B. Technical Notes and Comments

B.1. Breaks and Gaps. In the 1980s the distinction between chromatid gaps and breaks has been the subject of much

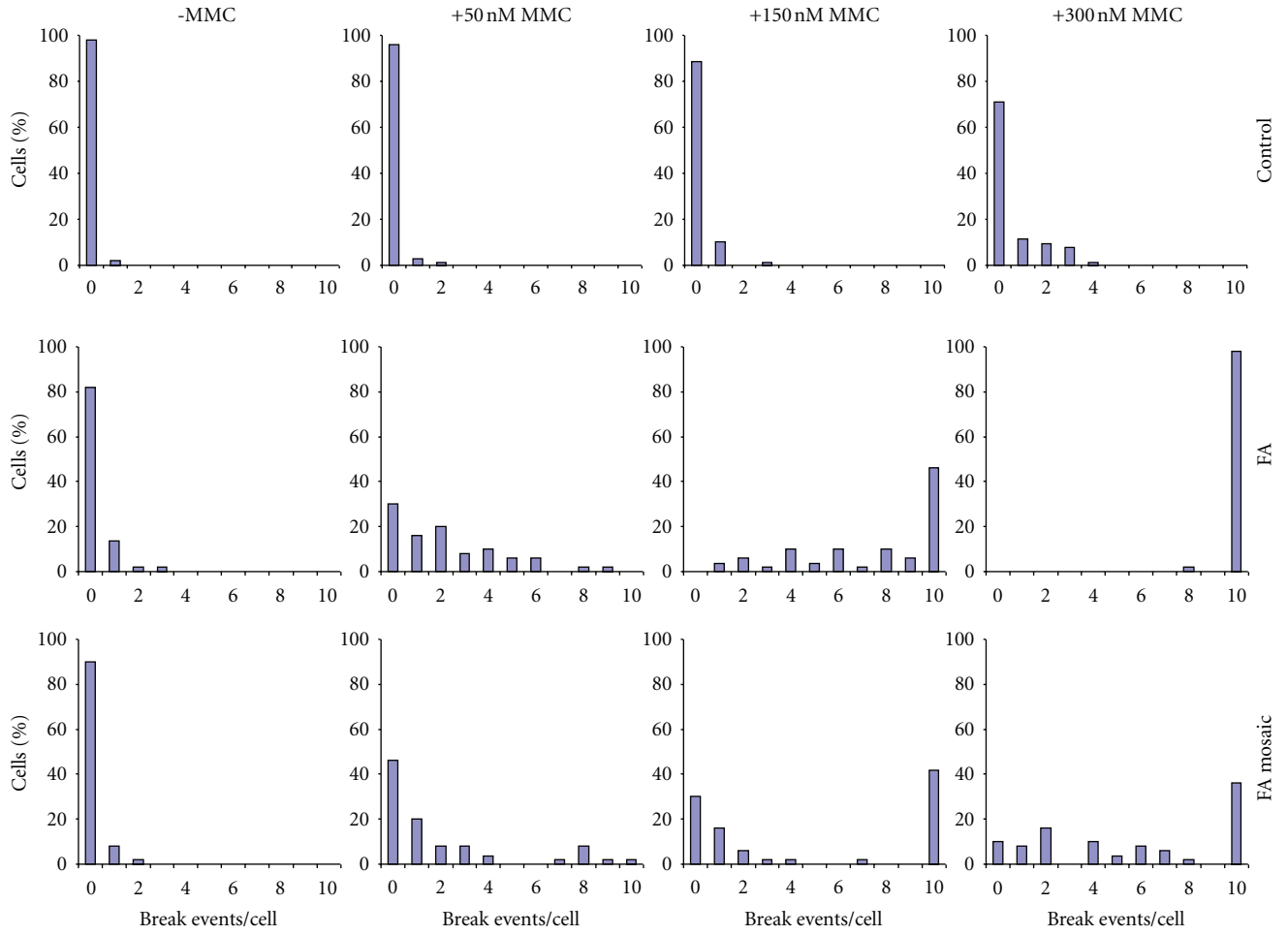

Figure 2: Evaluation of MMC-induced chromosomal breakage in stimulated T lymphocyte cultures. Upper row: healthy control; middle row: FA patient; lower row: mosaic FA patient. The healthy control shows breakage only at 300 nM, where the FA patient shows massive breakage (no normal cells present). Mosaicism is evident from the two highest concentrations of MMC, where there are still normal cells present next to cells showing an FA-like breakage rate (>10 breaks/cell). A crude estimate of the proportion of reverted T cells in this mosaic patient would be ~40%.

discussion, the issue being whether a gap represented a true double-stranded break in the DNA of a chromatid. A problem during the evaluation of aberrations is to decide which gap-like feature should be scored as a true aberration. A consensus was reached by accepting an aberration as a gap if the discontinuity in the staining of a chromatid is at least as wide as the width of the chromatid. If wider than twice the width of the chromatid, the aberration may be scored as a break [42]. If the "broken" piece appears dislocated the aberration is always scored as a break. If the interruption is considered doubtful, it should be ignored (Figure 1); this holds for all other aberrations that appear not entirely convincing.

The main reason to distinguish between chromatid gaps and breaks is that their biological impact may be different; conclusions based on significant differences in the frequency of gaps only, should be viewed with caution.

B.2. Saving Time on the Breakage Test

B.2.1. Finding Metaphases. In cases of low mitotic activity considerable time may be gained by utilizing a metaphase finding apparatus, which can perform unattended metaphase searches on multiple slides. Such apparatus may also be equipped with software for chromosomal aberration scoring, see for example, http://www.metasystems-international.com/

B.2.2. Scoring Aberrations. To save time, the scoring process may be divided into phases. Score first the cultures exposed to 0 and 300 nM MMC, which may already give you an unambiguous answer.

(1) The diagnosis "FA" is warranted if all cells from the proband contain multiple aberrations, whereas the majority of the control cells is normal.

(2) A result *excluding* FA should be based on a modest but significantly elevated breakage level at 300 nM MMC, both in the proband and in the healthy control.

If there are too few evaluable metaphases, or if there is an indication of mosaicism, score the samples exposed to 150 nM, and—with again too few metaphases present—the 50 nM samples as well. If, however, the 300 or 150 nM cultures have provided a conclusive answer, the 50 nM cultures may be skipped.

Some laboratories score only chromatid interchanges (often referred to as "radials") as aberrations, while ignoring chromatid breaks and gaps. Even though this is a considerable time saver, there are several disadvantages. First, with full-fledged (nonmosaic) FA patients "normal" cells (i.e., cells without interchange aberrations) are still observed at the higher MMC concentrations, leading to a false impression of mosaicism. Second, at the highest MMC concentration the aberration rate in the control does not reach statistical significance, which eliminates the internal check for drug activity. Third, chromatid interchanges are generated from chromatid breaks by an unknown joining mechanism, the precise nature of which is unclear, while variations in this process will affect the ratio between breaks and interchanges. As this ratio may vary from patient to patient, some FA patients might go unrecognised when scoring chromatid interchanges only.

B.2.3. High-Level Mosaicism. With the protocol described, most patients with mosaicism will be correctly diagnosed as FA, because even a minor proportion of FA-like lymphocytes will show up in the $\geq$10 breaks/cell category. When no FA cells can be detected in a patient with a "compelling" clinical phenotype, fibroblasts can be used to establish the diagnosis. We have encountered several FA patients whose T lymphocytes' response was indistinguishable from that in the healthy control, but whose skin fibroblasts' response clearly revealed the cellular FA phenotype (see, e.g., [6, 15]).

Breakage Test Using Fibroblasts. Add MMC (50 nM) or saline to either of two 80 cm^2 tissue culture flasks containing 1-2 $\times$ 10^6 freshly trypsinized fibroblasts (preferably fewer than 8 *in vitro* passages) from the following individuals: (1) the patient to be tested, (2) a healthy control, and (3) a known FA patient (positive control). After 48 h at 37°C, harvest the cultures by trypsinization, following colcemid treatment for 45 min, and prepare chromosome slides. Code the slides and score for aberrations (50 cells per culture). Typical results are as follows.

> *"Control (non-FA) fibroblasts", untreated*: 3% aberrant cells (0.03 breaks/cell); *MMC-treated*: 13% aberrant cells (0.2 breaks/cell).
>
> *"FA fibroblasts", untreated*: 7% aberrant cells (0.07 breaks/cell); *MMC-treated*: 95% aberrant cells (7 breaks/cell).

B.2.4. Alternative Cross-Linking Agents. Several other DNA cross-linking agents, besides MMC, have been used to demonstrate the hypersensitive phenotype of FA cells, for example, diepoxybutane (DEB), and cis-diamminedichloroplatinum(II) (cisplatin). DEB is on the Special Health Hazard Substance List because it is a (volatile) carcinogen that should be handled with great caution. DEB is hygroscopic and—upon contact with water—slowly loses activity, with a half-life of approximately 4 days, because it hydrolyzes into 1,2,3,4-tetrahydroxybutane, a compound with no cross-linking activity. DEB is commercially available from Sigma/Aldrich. Since different batches may vary in activity, comparative testing is required, since relatively small differences may lead to incorrect conclusions regarding mosaicism in a patient (compare the standard concentration of 0.1 μg/mL with 0.15 μg/mL in [14]). MMC, which—as a clinically approved chemotherapeutic agent produced by Kyowa Hakko Kogyo (not by Sigma)—is under rigorous quality control and is stable when stored in the vials provided by the manufacturer. A similar argument would favour the use of cisplatin, which is also clinically approved, over DEB as the diagnostic reagent for FA. On the other hand, provided the reagents are properly handled, DEB, MMC, and cisplatin are similarly effective in establishing the FA diagnosis in a chromosomal breakage assay. According to a single comparative study, MMC appeared slightly more suitable for the assessment of lymphocyte mosaicism [14]. It should be pointed out that, unlike DEB and cisplatin, MMC requires metabolic activation in order to become active as a cross-linking agent. If metabolic activation were a variable parameter, this may be considered a disadvantage for MMC and an argument in favour of choosing cisplatin as the diagnostic cross-linker.

B.2.5. Conversion of Interchanges into Break Events. Although the idea of two breaks underlying each interchange between two chromosomes (often referred to as "radial") has been considered commonplace in the genetic toxicology literature, this hypothesis is challenged by the observations of Godthelp et al. [43], who found the frequency of interchanges to increase linearly with drug dosage (rather than exponentially), implying single-hit rather than two-hit kinetics. If the single-hit principle were to be accepted, this would change the conversion factor for the quantification of interchange aberrations into break events from 2 to 1; however, adopting a conversion factor of 1 would not affect the general principle of the FA diagnosis, as described here.

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Review Article

Chromosomal Aberrations Associated with Clonal Evolution and Leukemic Transformation in Fanconi Anemia: Clinical and Biological Implications

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Fanconi anaemia (FA) is an inherited disease with congenital and developmental abnormalities, bone marrow failure, and extreme risk of leukemic transformation. Bone marrow surveillance is an important part of the clinical management of FA and often reveals cytogenetic aberrations. Here, we review bone marrow findings in FA and discuss the clinical and biological implications of chromosomal aberrations associated with leukemic transformation.

1. Introduction

Fanconi anemia (FA) is an inherited disease with bone marrow failure, variable congenital and developmental abnormalities, and extreme cancer predisposition. The most common malignancies in FA are myeloid leukemia and squamous cell carcinoma. On a cellular level, FA is characterized by chromosomal instability and cross-linker sensitivity, which is the diagnostic hallmark of FA. For diagnostic testing, this is determined by demonstration of hypersensitivity to mitomycin C (MMC) or diepoxybutane (DEB) of patient derived peripheral blood cells or fibroblasts [1–3]. FA cells also display hypersensitivity to proapoptotic stimuli of certain cytokines, such as TNF-α and IFN-γ, which has been implicated in haematological manifestations of FA [4–6]. Cell cycle analysis of FA cells shows a characteristic arrest in the G2 phase, which is exacerbated by exposure to MMC [7–9]. This clinical and cellular phenotype results from a defect in a DNA damage response (DDR) pathway (FA/BRCA pathway), in which FA and associated proteins interact. So far, 15 FA genes *(FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN/PALPB2, FANCO/RAD51C,* and *FANCP/SLX4)* have been identified that can be mutated in FA [2, 10–12], of which *FANCA*, *FANCG*, and *FANCC* are the most commonly mutated genes in studied FA populations [2]. Importantly, the discovery that mutations in *BRCA2* causes FA in the subgroup FA-D1, which comprises less than 5% of all FA patients, linked the FA DNA damage response pathway to hereditary breast and ovarian cancer (HBOC) [13, 14]. Hematopoiesis in the bone marrow (BM) is the most commonly affected organ system in FA, and most FA patients will develop clinically relevant hematological complications in their first or second decades of life [15]. BM complications of FA can manifest with hypoplasia, often initially being limited to thrombocytopenia in peripheral blood counts, or general aplasia. When the diagnosis of FA is made,

which might happen with considerable delay, bone marrow appearances can already be more advanced and consistent with myelodysplasia. In FA, this often presents as refractory cytopenia with multilineage dysplasia, with or without excess of blasts on morphologic evaluation. Common morphologic abnormalities on bone marrow examination include irregular nuclear contours, budding nuclei, and karyorrhexis [16]. In some patients, the diagnosis of FA is only made on presentation with overt myeloid leukemia. How common undiagnosed FA presents with AML is not known, but FA should be considered especially in young patients with AML, even in the absence of sometimes only subtle congenital malformations such as short stature and microcephaly, in particular when excess toxicity or prolonged aplasia after chemotherapy of extreme toxicity is encountered [17–19]. Less than ten cases of lymphoblastic leukemias have been reported in FA, which have been mostly of T-lineage, and appear to be limited to patients with mutations in *FANCD1/BRCA2* and *FANCD2* [19–21].

Given the high incidence of hematological complications of FA, BM surveillance for morphological and cytogenetic changes makes an important contribution to the clinical management of FA patients. With improved and more sensitive methods for the detection of chromosomal aberrations over the last decade, and better understanding of clinical implications of BM cytogenetic findings in general for preleukemic changes and the diagnosis and management of hematological malignancies, FA bone marrow surveillance provides important information for clinical decision making. In addition, clonal evolution and associated chromosomal aberrations in FA are important for understanding malignant transformation in general and therefore wider implications. Here, we review bone marrow chromosomal aberrations in FA and discuss the clinical and biological implications.

1.1. Bone Marrow Surveillance: Clinical Aspects for Patient Management. In view of the relative infrequency and clinical variety of FA, no evidence-based data exist on how and how frequent bone marrow should be surveyed in FA patients. In view of variable practice, a recent survey carried out in the UK (S. Meyer, unpublished data), confirmed broadly the recommended practice, in that FA patients with normal blood counts should have at least a yearly assessment of bone marrow morphology and cytogenetics [3, 22]. If there is evidence of bone marrow failure, most centers would consider increasing the frequency and monitor more closely for appearance and evolution of chromosomal aberrations. For the initial management planning at diagnosis, it is worth considering that absent radius and a severe phenotype is statistically associated with earlier bone marrow failure, and that of the most commonly mutated genes, *FANCG* and *FANCC* have a statistically higher incidence of early hematological complications than *FANCA* [23]. However, BM failure is a common presentation of FA caused by mutations in all complementation groups. Surveillance for FA-associated BM manifestations should include morphology and assessment of cellularity as well as cytogenetic evaluation [3]. The cytogenetic evaluation should include conventional karyotyping. Importantly, however, cytogenetic analysis should specifically include investigations for FA-characteristic chromosomal aberrations as outlined below. Only with the application of more sophisticated cytogenetic methodologies the incidence and significance of FA-specific aberrations can be determined. These would include routine application of or/and comparative genomic hybridization (CGH) or more sensitive whole genome analysis such as array CGH, in addition to fluorescent in situ hybridization (FISH), targeting FA-specific chromosomal gains and losses on a single cell level.

1.2. Spectrum of FA-Specific BM Chromosomal Aberrations. Clonal bone marrow aberrations in individuals affected by FA were first reported as far back as the 1970s and early 1980s, when several studies recognized cytogenetic abnormalities on bone marrow examination of FA patients, many of them noting a high frequency of monosomy 7, detected by conventional karyotyping [25, 26]. The clinical observation that the detectability of chromosomal aberrations in bone marrow aspirates of FA patients can vary over time, with clones becoming transiently undetectable, has led to an underestimation of clinical relevance of chromosomal aberrations in FA [27]. In addition, the absence of nonrandom chromosomal rearrangements that are frequently found in AML in particular in childhood, has delayed the recognition and the clinical and prognostic significance of specific aberrations frequently seen in FA [24]. A better understanding of the clinical relevance and biological implications of chromosomal aberrations in FA was achieved over the last decade by analysis of larger case series and the application of modern molecular cytogenetic technologies in addition to conventional karyotyping [16, 28–31]. This has led to the identification and delineation of specific patterns of chromosomal aberrations in FA. In contrast to aberrations seen in sporadic AML in childhood, these are characteristically unbalanced, with gains and losses of chromosomal material during clonal evolution. Frequent for FA are gains of the chromosomal regions 1q and 3q, as illustrated in Figure 1, and partial or complete loss of chromosome 7 [16, 29–33]. Of these, 3q gains are in particular characteristic of FA. By studying larger numbers of FA patients sequentially, not only the high specificity for FA became evident, but also the clinical implication of 3q gains, of which occurrence indicate transformation to MDS and AML [30, 31]. In four independent studies, two from Europe and two from North America, the association of 3q gains with progression to or presence of FA-related myelodysplasia was confirmed [16, 29–31]. Importantly, gains involving 3q are only rarely seen in BM from non-Fanconi patients [34–36], while balanced chromosomal aberrations, such as inversions or translocations involving the 3q are well documented in myeloid malignancies from non-FA patients, in particular in adults [35, 36]. Therefore, cytogenetic detection of 3q gains in apparently sporadic cases of MDS or AML would indicate testing for FA. The impact on gene expression resulting from FA-specific gains in the area of common amplification, 3q26-3q29, has only recently been studied and point to an important role of the

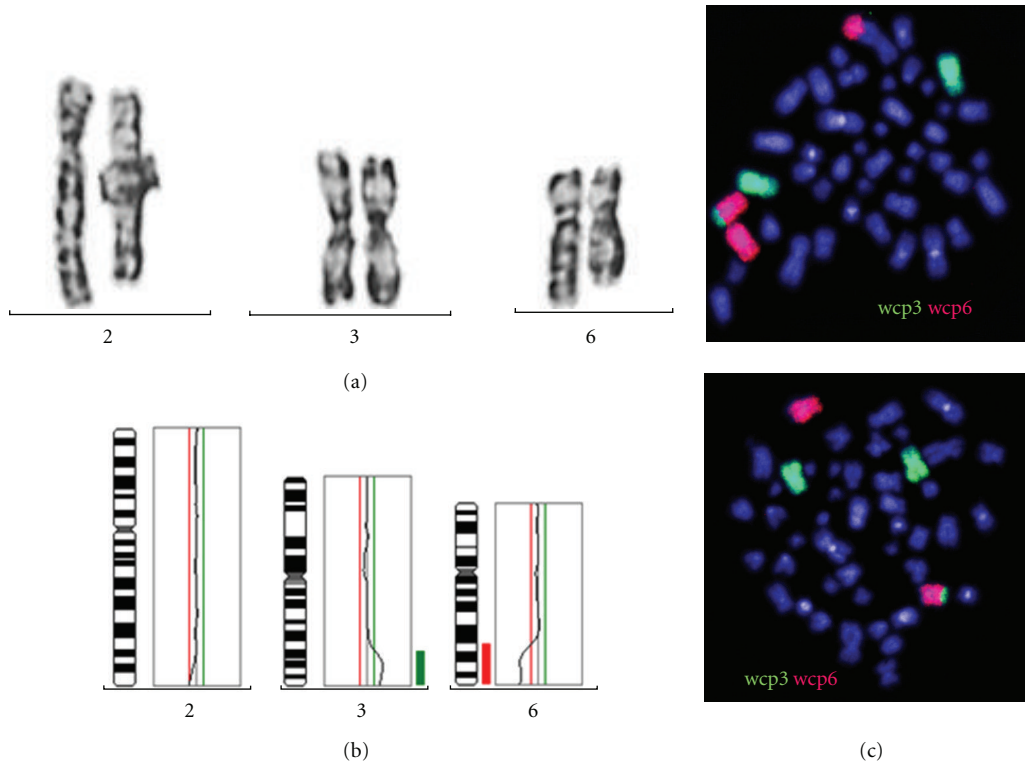

Figure 1: FA-associated 3q aberrations. (a) conventional cytogenetics: outcuts of chromosome 2, 3, and 6 showing additional material at 2q, normal chromosomes 3, and an apparent deletion of 6q. (b) the conventional CGH shows a slight deviation at 2q, a gain of 3q25 to 3qter (enh), and a loss of material from 6q23 to 6qter (dim). (c) the FISH with whole chromosome paints wcp3 und wcp6 demonstrates two cell lines: one with material of chromosome 6 translocated to 2q and with material of 3 translocated to 6q; another cell line which carries only the translocation of material of chromosome 3 to 6q. Thus, the apparent deletion detected by conventional cytogenetics proved to be not a sole deletion of 6q but in addition a unbalanced translocation of 3q onto 6q. In addition, the patient had a monosomy 7 (data not shown). The karyotype according to ISCN 2009 in bone marrow cells was 45,XY,-7[2]/45,der(6)(6pter → 6q22::3q25 → 3qter),-7[27]/45,der(2)(2pter → 2q37::6q22 → 6qter),der(6)(6pter → 6q22::3q25 → 3qter),-7[8].

transcriptional regulator *EVI1* (ecotropic viral integration site 1) for leukaemic transformation in FA [37, 38]. Another frequently observed aberration in FA is gain of chromosomal material at 1q. This aberration can also be present in morphologically relatively normal BM and is a finding also in non FA-hematological diseases. Its presence is often the sole finding in the early stages of clonal evolution and can persist for years, but also occurs frequently with 3q gains and other aberrations. Chromosomal aberration involving chromosome 7 include -7/-7q, which, as in the non-FA population, is significantly correlated with more advanced dysplasia and commonly part of a clone with a more complex karyotype that frequently also shows gain of 3q material [39]. Sequential analysis of clonal progression in FA has revealed that 3q-gains often precede changes involving partial or whole loss of chromosome 7 [30, 37]. Another more recently recognized frequent finding in FA-associated clonal evolution is 11q-in advancing FA-associated MDS. This lesion occurs in FA frequently with a more complex karyotype that also shows 3q gain and/or -7 [31]. The recent detection of involvement of the *RUNX1* locus at 21q in FA-associated genomic abnormalities, which in all cases were associated with advanced MDS [31], has also some important biological implications for the understanding of clonal evolution with FA, which is discussed below. Gains and losses of chromosomal material can also involve other chromosomes, but not with the same FA-specific patterns as for 3q. Balanced translocations have been described in FA, and occur usually as part of more complex clonal rearrangements. Single cases also had involvement of the 3q region as well a single report of an 11q23 translocation [21, 38]. Importantly, common balanced nonrandom chromosomal rearrangements that are seen in AML, such as t(8; 21) or inv(16) translocations, have never been reported in FA [24].

1.3. Clonal FA-Associated Bone Marrow Aberrations: Clinical Implications. Hematological complications are the most common manifestation of FA. Over the last three decades according to large studies carried out in North America and Europe, the cumulative incidence of any hematological abnormality in FA approaches 90%, and the cumulative incidence of leukemia has been approximately 30% by 40 years of age [20, 23]. Therapeutically, options to treat bone marrow failure in FA are limited to interventions with growth factors and androgens in order to improve peripheral blood counts [3], but this does not alter the high risk of leukemic

transformation. The most important management decision for hematological complications of FA is when and how to proceed to hematopoietic stem cell transplantation (HSCT). The outcome of HSCT in FA has improved dramatically [40, 41], and the incidence and survival patterns quoted above, as well the clinical course of FA are changing accordingly [23]. Many centers would elect HSCT for FA in the presence of significant hematological abnormalities and availability of a suitable donor. Cytogenetic information would certainly inform decision making, and the presence of chromosomal aberrations, in particular those associated with high risk of malignant transformation would potentially justify a more aggressive approach that could include partly mismatched donors [41, 42]. Leukemia in FA is very difficult to treat. From sparse published data of relatively few reported cases, overt leukemia in FA is associated with poor prognosis and short survival. Conditioning regimes for FA have empirically been tailored for the intrinsic chemosensitivity of FA patients and are increasingly based on fludarabine with low-dose cyclophosphamide. There is little evidence that the presence of BM cytogenetic aberrations should influence conditioning regimes, as long as there is no evidence of overt leukemia. However, numbers of reported cases are very small [40, 41, 43], and several cases of leukemic relapse after HSCT have been reported, of which intriguingly one was from donor cells [44].

1.4. FA-Characteristic Chromosomal Aberrations: Implications for Malignant Transformation. The emergence of characteristic patterns of chromosomal aberrations in FA has relevance for the management of FA patients. Detection of chromosomal aberrations that confer a high risk of transformation to MDS and AML warrants a more aggressive approach in order to prevent leukemia development. However, the study of chromosomal aberrations in this disorder has some more generally relevant implications, giving insight of secondary events in clonal evolution arising associated with an inherited defect in the DNA damage response. The FA-characteristic clonal evolution with dominance of chromosomal gains and losses is likely to be a specific result of the disruption of the FA/BRCA pathway and at least partially caused by FA-related unresolved DNA damage during S phase. This unresolved DNA damage is thought to lead to the FA-specific G2 arrest and could lead to the accumulation double-strand breaks and switch to more error-prone repair by nonhomologous end joining. Indeed, nonhomologous end joining is largely efficient in FA cells in contrast to homologous repair, which is grossly impaired in FA [45–47]. However, this possible and extremely simplified explanation could only partly explain the occurrence of aberrations, but not characteristic patterns of chromosomal aberrations involving typically 3q and 1q. The striking overrepresentation of 1q and 3q could imply that these chromosomal regions are particularly susceptible to FA/BRCA disruption-associated damage. An alternative or additional explanation would be that resulting genetic changes confer a growth advantage, possibly in particular in the presence of a defect in the FA/BRCA pathway. Cytogenetic analysis of sporadic AMLs that occur in a comparable age group of older children and young adults shows some marked differences when compared with FA-associated leukaemic transformation, exemplified by the rarity of any 3q aberrations in less than 5% of childhood the AML in the NPO studies and the MRC trials [48, 49], and exceedingly rare findings of gains of chromosomal material in this region, implying an alternative pathogenesis of FA-associated leukaemic transformation. The FA-characteristic 3q gains nearly always harbor one of the most aggressive leukemogenic oncogenes, *EVI1*, which was first detected to be amplified and overexpressed as an initial event in FA-derived AML transformation in patient material and cell lines from a patient with biallelic FANCD1/BRCA2 mutations [16, 31, 38] and subsequently shown to result in overexpression of *EVI1* [37]. This suggest that FA-associated leukemia shares its biology with one of the most aggressive forms of sporadic AML [35, 36]. Intriguingly, *EVI1* overexpression in childhood AML, which has been detected in approximately 10% of cases, is normally not a result of chromosomal rearrangements of the 3q region, but appears to be associated other chromosomal rearrangements and is not of the same prognostic relevance as *EVI1* overexpression resulting from chromosomal rearrangements [50]. The other specific gene that appears to be targeted by FA-associated chromosomal rearrangements is *RUNX1*, which points to the question as to how chromosomal rearrangements in FA promote leukemic transformation. One important observation comes from studies with FANCC -/- mice. Leukaemic clones in bone marrow of these mice that were outgrown under the selective pressure of TNF-α showed abrogated cytokine sensitivity occurring together with chromosomal aberrations [51]. In addition, analysis of patient derived BM cells of FA patients with chromosomal aberrations led to the detection of an attenuated cellular FA phenotype. Cells with this phenotype maintained lack of FANCD2 ubiquitination associated with FA core complex gene mutations, and MMC hypersensitivity, but did not display the FA-specific G2 arrest on cell cycle analysis [28]. It will be important to explore to what extent and by which mechanism individual or combined the genetic changes associated with leukemic transformation in FA modulating the cellular FA phenotype. Taken together, these observations point to a modulatory effect mediated by chromosomal aberrations on the cellular FA phenotype, which is likely to be of general relevance for oncogene-mediated malignant progression and the DNA damage response [52].

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Review Article

Disrupted Signaling through the Fanconi Anemia Pathway Leads to Dysfunctional Hematopoietic Stem Cell Biology: Underlying Mechanisms and Potential Therapeutic Strategies

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Fanconi anemia (FA) is the most common inherited bone marrow failure syndrome. FA patients suffer to varying degrees from a heterogeneous range of developmental defects and, in addition, have an increased likelihood of developing cancer. Almost all FA patients develop a severe, progressive bone marrow failure syndrome, which impacts upon the production of all hematopoietic lineages and, hence, is thought to be driven by a defect at the level of the hematopoietic stem cell (HSC). This hypothesis would also correlate with the very high incidence of MDS and AML that is observed in FA patients. In this paper, we discuss the evidence that supports the role of dysfunctional HSC biology in driving the etiology of the disease. Furthermore, we consider the different model systems currently available to study the biology of cells defective in the FA signaling pathway and how they are informative in terms of identifying the physiologic mediators of HSC depletion and dissecting their putative mechanism of action. Finally, we ask whether the insights gained using such disease models can be translated into potential novel therapeutic strategies for the treatment of the hematologic disorders in FA patients.

1. Introduction

Fanconi anemia (FA) is a rare, autosomal recessive and X-linked hereditary disorder, which is characterized by progressive bone marrow failure (BMF), congenital developmental defects, and an early onset of cancers such as leukemia and some solid tumors [1]. In general, the hematologic manifestations of FA remain the primary cause of morbidity and mortality, with patients suffering from a markedly increased risk of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). In addition, FA patients are also predisposed towards various forms of solid tumor such as squamous cell carcinoma of the head and neck, esophagus, and gynecologic area [2, 3].

FA is a genetically heterogeneous disorder caused by inactivating mutations in genes that are thought to function in an epistatic signaling pathway. Loss of function of any of the FA family members results in inefficient repair of DNA damage and deregulation of signaling pathways controlling cell proliferation and apoptosis. To date, 15 genes associated with FA in patients have been identified and cloned: *FANCA*, *FANCB*, *FANCC*, *FANCD1*/*BRCA2*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*/*BACH1*/*BRIP1*, *FANL*/*PH F9*/*POG*, *FANCM*, *FANCN*/*PALB2*, *FANCO*/*Rad51C* [4], and *FANCP*/*SLX4*/*BTBD12* (Table 1) [5–7]. The FA proteins appear to function in a common biochemical ubiquitin-phosphorylation network, the FA signaling pathway, that is involved in controlling multiple functions related to DNA repair and the cellular response to stress [8]. Upon DNA damage, FA proteins are recruited to the site of damage and assemble to form the FA core complex. This nuclear multiprotein complex consisting of FANCA, FANCB, FANCC,

Table 1: Members of the FA signaling pathway found in different species.

Homo sapiens	*Mus musculus*	*Danio rerio*	*Drosophila melanogaster*	*Caenorhabditis elegans*	*Archaea*	*Saccharomyces cerevisiae*
FANCA	*Fanca*	*fanca*				
FANCB	*Fancb*	*fancb*				
FANCC	*Fancc*	*fancc*				
FANCD1/BRCA2	*Fancd1*	*fancd1*	*brca2*	*brc-2*		
FANCD2	*Fancd2*	*fancd2*	*fancd2*	*fcd-2*		
FANCE	*Fance*	*fance*				
FANCF	*Fancf*	*fancf*				
FANCG	*Fancg*	*fancg*				
FANCI	*Fanci*	*fanci*		*fnci-1*		
FANCJ/BACH1/BRIP1	*Fancj*	*fancj*		*dog-1*		
FANCL/PHF9/POG	*Fancl*	*fancl*	*fancl*			
FANCM	*Fancm*	*fancm*	*fancm*	*fncm-1*	*hef*	*MPH1*
FANCN/PALB2	*Palb2*	*palb2*				
FANCO/Rad51C	*Rad51*		*rad51C*			
FANCP/SLX4/BTBD12	*Slx4*		*mus312*	*him-18*		
References	[10]	[11]	[12–14]	[12, 13, 15]	[16]	[16]

FANCE, FANCF, FANCG, FANCL, and FANCM functions as an E3 ubiquitin ligase and mediates the activation of the ID complex, which is a heterodimer composed of FANCD2 and FANCI. Once monoubiquitinated, it interacts with classical tumor suppressors downstream of the FA pathway including FANCD1/BRCA2, FANCN/PALB2, FANCJ/BRIP1, and FAN CO/Rad51C and thereby contributes to DNA repair via homologous recombination (HR) [9].

Defects in any of the gene products associated with the FA pathway result in similar cellular abnormalities. Firstly, cells isolated from FA patients show elevated levels of chromosomal aberrations and are hypersensitive to DNA interstrand crosslinking agents such as mitomycin C (MMC), cisplatin, diepoxybutane (DEB), and melphalan [17–21]. These DNA alkylating agents covalently link two bases on opposite strands of the DNA and thereby cause replication arrest and DNA double-strand breaks, which ultimately leads to cell death. The increased susceptibility of FA cells to these compounds indicates a defect in the DNA repair machinery that is usually involved in the resolution of these crosslinks. The evaluation of such abnormal structures in response to the clastogenic effect of crosslinking agents provides a reliable cellular marker for the diagnosis of FA and allows the identification of patients presenting with aplastic anemia or leukemia that would not be recognized in the absence of the characteristic physical signs associated with FA. The so-called chromosome breakage test exposes cultured FA cells to alkylating agents such as DEB and MMC, in order to provoke chromosomal abnormalities. While MMC causes radial chromosomes [18], DEB mainly functions as a bifunctional crosslinking agent inducing chromosomal breakage or rearrangements [17]. More recently, the FA pathway has been shown to be involved in the cellular response to DNA damaging agents that do not cause crosslinks. One example is the O^6-alkylating agent temozolomide, which is commonly used in the treatment of glioblastomas. It has been shown that inactivation of the FA pathway, in particular FANCG and FANCD1/BRCA2, renders cells more susceptible to apoptosis following treatment with temozolomide, suggesting that a functional FA pathway is required for the sensing and/or resolution of the DNA adducts formed by this agent [19, 20].

An additional cellular phenotype, which can be observed in response to the treatment of FA cells with DNA damaging agents like MMC and melphalan, is an exaggerated arrest of cells in the G2/M phase of the cell cycle. Cultured cells exhibit a prolonged G2 phase transit and frequently arrest in G2. This phenotype can be analyzed by flow cytometry and is useful as an additional diagnostic tool for FA [22–24]. Recently, this assay has been further modified to allow the rapid and accurate determination of complementation groups in FA patients using retroviral-mediated gene transfer of FA cDNAs to correct the melphalan-induced G2/M arrest [25, 26].

Finally, cells with a defective FA signaling pathway demonstrate hypersensitivity to the inhibitory action of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), and macrophage inflammatory protein-1-α (MIP-1-α) [27, 28]. Both TNF-α and IFN-γ are produced at abnormally high levels in the serum and bone marrow (BM) of FA patients and are hypothesized to play a role in hematopoietic failure [29, 30]. Although the mechanistic bases for the hypersensitivity of FA cells to IFN-γ and MIP-1-α remain to be elucidated, it has been shown that murine BM cells defective in the FA signaling pathway produce excessive reactive oxygen species (ROS) in response to treatment with TNF-α [31]. These elevated levels of ROS may in turn comprise a source of DNA interstrand crosslinks and therefore provide a direct link between proinflammatory cytokines and the defective DNA damage response seen in FA cells.

Although FA cells are well documented to be hypersensitive to both DNA crosslinking agents and to proinflammatory cytokines, it has not been formally demonstrated whether these two phenomena are related to each other. Perhaps more importantly, the exact identity of the key players driving BMF in FA patients remains unclear. In this paper we summarize evidence that the hematopoietic problems associated with FA are rooted in a stem cell defect and aim to highlight research that reveals insight into the mechanisms through which this stem cell depletion is mediated. We also discuss how this mechanistic data can be used as a starting point to identify new targets for therapeutic intervention.

2. Hematologic Abnormalities in FA

Hematologic abnormalities, which are found in virtually all FA patients, include cytopenias such as thrombocytopenia (abnormally low platelet counts in the peripheral blood), neutropenia (low neutrophil counts), and progressive pancytopenia (abnormality in two or three blood cell lineages) [3]. At birth, FA patients usually do not show any signs of these defects and have normal blood cell counts, but, as the patient grows older, the hematologic complications start to develop, mainly within the first decade of life. Macrocytosis (enlargement of red blood cells) is usually the first to be detected, followed by thrombocytopenia and aplastic anemia (insufficient production of red blood cells, leukocytes, and platelets in the BM), finally resulting in the characteristic progressive BMF phenotype [3, 32, 33]. Unless treated, BMF represents the primary cause of morbidity in FA patients.

In addition to the observed low levels of mature hematopoietic cells across all lineages, FA patients have also been found to possess severely compromised hematopoietic progenitor compartments. Growth of BM hematopoietic progenitors from FA patients has been shown to be impaired as they are compromised in their ability to produce colony-forming unit granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) colonies in semisolid culture media *in vitro* [34, 35]. While hematopoietic progenitors of normal individuals, or even patients with aplastic anemia, respond to recombinant stem cell factor (SCF) by improved colony output, FA progenitors largely fail to respond to stimulation with SCF [34]. In a separate study, knockdown of FANCC expression in human BM cells using antisense oligonucleotides resulted in significantly reduced clonal growth of erythroid and myeloid progenitor cells *in vitro* [36]. Taken together, these data clearly implicate a defect at the level of FA progenitor cells.

As well as suffering from the progressive depletion of normal BM cells, FA patients are also predisposed towards malignant transformation. Approximately half of all FA patients present with MDS and/or AML before they are 40 years old [33]. Both of these disorders result from the dominance of abnormal hematopoietic clones, likely caused by genetic instability within the hematopoietic stem and/or progenitor compartments.

3. The Evidence for Hematopoietic Stem Cell (HSC) Defects in Patients

Since all hematopoietic lineages are compromised in FA patients, it would seem reasonable to assume that a defective FA signaling pathway may negatively impact upon the biology of HSCs, which comprise the top of the hematopoietic system hierarchy. However, while it is relatively straightforward to assess the depletion of mature hematopoietic cells and myeloid progenitors in FA patients, it is more difficult to directly examine HSC function. Nonetheless, there are several lines of evidence in FA patients that suggest that the HSC pool is compromised.

Firstly, at the immunophenotypic level, FA patients demonstrate significantly decreased frequencies of BM CD34+ cells, which are comprised of the HSC and progenitor compartment. As this is also true for patients that do not yet show any evidence of BMF, this finding supports the hypothesis that FA patients have a defect at the level of the stem cell [37]. Importantly, this correlates with the finding that there are reduced stem/progenitor numbers in umbilical cord blood taken from newborn FA patients [38]. Thus, HSC/progenitor depletion appears to precede the decreased production of mature lineage committed hematopoietic cells and may have already begun during early development.

Secondly, the BMF syndrome can be corrected by allogeneic HSC transplantation using minimal conditioning [39, 40]. Thus, in a mixed chimera setting, normal donor HSCs are able to permanently reconstitute the failing hematopoietic system to a corrective level, regardless of the environment of patient FA hematopoietic and niche cells that the donor cells are infused into. As well as eliminating the possibility that the FA HSC niche has a dominant role to play in progressive BMF, this also suggests that FA HSCs are defective relative to their normal counterparts.

Finally, the phenomenon of reverse mosaicism reveals that the FA defect can be corrected with a single functionally normal HSC. Reverse mosaicism occurs as a consequence of the correction of one of the patient's nonfunctional FA alleles within a somatic cell clone. In FA patients, this correction has been documented to arise from either the genetic recombination of two compound heterozygote mutations in a FA gene, resulting in the production of a single functional allele; or from spontaneous point/frame shift mutations that restore function to the inactivated gene [41–43]. Importantly, the corrected clone must demonstrate a survival advantage over its noncorrected counterparts in order to be able to expand to sufficient numbers to facilitate detection and confer a therapeutic effect. This survival advantage must be present in FA patients, as in several independent instances, a single corrected hematopoietic clone has been shown to support the hematopoietic function and survival of the patient, despite the fact that cells within other somatic tissues did not contain the correcting mutations [41, 42]. Although these spontaneously reverting clones have been documented to persist for a number of years in several patients, it is still possible that the correcting mutation occurred in a long-lived progenitor

cell as opposed to an HSC. However, one intriguing study has described the occurrence of reverse mosaicism from a single clone in monozygotic FA twins. Although the twins presented with nonhematologic symptoms of FA and their skin fibroblasts were sensitive to DNA crosslinking agents, their hematopoietic cells appeared to function normally [43]. Upon molecular analysis, it was revealed that the twins both possessed a revertant clone with exactly the same correcting mutation. Presumably, a single somatic hematopoietic cell must have been subject to this correcting point mutation *in utero,* and then its descendants must have been distributed between both twins during gestation. Since this single clone was able to reconstitute the hematopoietic system of both twins for more than two decades, the original mutation must have occurred within an HSC. This observation clearly demonstrates the selective advantage of corrected FA HSCs over noncorrected HSCs and therefore reveals a survival defect at the level of HSCs in FA patients.

4. Model Systems to Study FA HSCs

Clinical observations from FA patients provide some evidence that allows us to implicate a defect at the level of HSCs in driving the BMF disease phenotype. Nonetheless, experimental model systems must be employed to directly interrogate the function of HSCs defective in the FA signaling pathway in a reproducible manner. The "gold standard" for assessing the capacity of HSCs to be able to differentiate to form all mature lineages of the hematopoietic system, while also being capable of self-renewal, is to perform BM transplantation and measure long-term multilineage engraftment within the recipient. Ideally, this assay would be performed with a limiting dilution of HSCs and involve at least one serial transplantation into a secondary recipient. While this rigorous assessment clearly cannot be performed in patients, a number of surrogate assays have been developed in order to dissect human HSC biology.

As briefly discussed above, it is possible to measure the frequency of a range of human hematopoietic progenitor compartments by their ability to form colonies in semisolid media supplemented with various hematopoietic growth factors. However, in the case of FA, while a defective HSC pool may cause a decreased frequency of progenitor cells, this assay does not allow the direct evaluation of whether this is the case, as it only enumerates the downstream progeny of HSCs. One technique that has been developed to measure the frequency of more primitive cells based on their ability to maintain the output of hematopoietic progenitor cells during extended periods of *in vitro* culture is the long-term BM culture assay, also known as Dexter culture [44–46]. This approach has been applied to the study of patient-derived FA HSCs with varying results. While Butturini and Gale were able to demonstrate that FA BM cells were able to generate long-term cultures that were able to initiate secondary long-term cultures with robust output of differentiated myeloid cells, Martinez-Jaramillo et al. found that long-term cultures seeded with FA patient marrow were drastically curtailed in terms of output of myeloid and erythroid progenitor cells [47, 48]. These differences likely reflect either disparities in the culture conditions employed, which FA HSCs may be particularly sensitive to, or to interpatient variation, for example due to differences in the degree of BMF at the time of BM biopsy. Nonetheless, these two studies clearly serve as a proof of principle for the use of this approach in assessing FA HSC function and may be useful in the evaluation of potential novel therapeutic strategies using primary patient material.

While the long-term BM culture approach can be successfully employed as a surrogate assay for HSC function, it does suffer from some critical drawbacks. Hence, to date, it has proven extremely difficult to engineer an *in vitro* system that encompasses the extrinsic cues that maintain HSCs within their *in vivo* niche. One possible solution to this problem is the murine xenotransplantation system. This approach takes advantage of the fact that HSCs derived from human umbilical cord blood, BM, and mobilized peripheral blood can be successfully engrafted into immune-deficient mice [50–53]. To date, there have been no reports of successful direct transplantation of FA patient HSCs using the immune-deficient mouse models that are currently available. However, Cohen-Haguenauer and colleagues were able to demonstrate long-term engraftment of retroviral gene corrected CD34+ cells from an FA patient in complementation group A, using the nonobese diabetic/severe-combine immunodeficiency (NOD/scid) immune-deficient mouse model as a recipient [54]. Moreover, a second group took advantage of the "humanized" NOD/scid IL-2R$\gamma^{-/-}$/SGM3 (NSG/SGM3) mouse model, which combines a profoundly immune-compromised background with transgenic expression of the recombinant human growth factors SCF; granulocyte macrophage colony-stimulating factor (GM-CSF); interleukin-3 (IL-3), in order to facilitate robust engraftment of FA patient AML cells [55]. Taken together, these studies suggest that it may be possible to develop a human FA HSC xenotransplant system given an appropriate murine recipient model and an adequate supply of patient CD34+ cells. Unfortunately, given the fact that FA patients tend to already be suffering from marrow hypoplasia upon diagnosis, it is unlikely that this approach will be widely accessible to the research community.

A potential novel source of FA HSCs for use in the laboratory is through the directed differentiation of patient-specific induced pluripotent stem (iPS) cells. A recent study by Tulpule et al. showed that *in vitro* differentiation of FA-deficient pluripotent cell lines, in this case human embryonic stem (ES) cells knocked down for either FANCA or FANCD2, can be used to show a developmental defect in hematopoietic specification, thereby highlighting pluripotent cell lines as a valuable tool to study FA [56]. However, there are two major barriers to the application of iPS cell technology in the study of FA HSCs. Firstly, there are currently no robust protocols available for the directed differentiation of *bona fide* HSCs from either human ES or human iPS cells [57]. The second barrier is it appears that the absence of a functional FA signaling pathway restricts the attainment of pluripotency when somatic tissues are reprogrammed using the approach devised by Takahashi and colleagues, namely,

via the retroviral delivery of exogenous OCT4, SOX2, KLF4, and c-MYC [58, 59]. However, recent work from Müller and colleagues would seem to suggest that somatic cells from FA patients can be successfully reprogrammed to a state of pluripotency, albeit at lower efficiencies than normal counterparts, by incorporating slight modifications to the reprogramming procedure, such as reducing oxygen tension [60].

Recently, an alternative cellular reprogramming approach was established for the generation of transplantable HSC/progenitors from normal adult somatic tissues. It was demonstrated that it is possible to convert human fibroblasts directly to multipotent hematopoietic progenitors via OCT4-dependent cellular programming without transiting through a pluripotent state [61]. Notably, using this approach, the authors could demonstrate multilineage engraftment potential using the murine xenotransplant model. It would be intriguing to establish whether this approach could be used to directly bypass the reprogramming deficiency that FA patient fibroblasts suffer from when they are subject to the iPS cell-derivation methodology.

5. Alternate Model Systems for FA

Studying the etiology of human disease ideally involves the use of human model systems. However, given the previously discussed constraints that are associated with studying HSC biology in FA, namely, the lack of an abundant source of patient HSCs to act as a starting material and the absence of a transplantation system to assess HSC function, it has been necessary to develop animal model systems for this disease. Fortunately, the FA signaling pathway has been well conserved throughout evolution; thus, there are several potential model systems available (Table 1).

Five of the 15 human genes can be found in plants, including components of the core complex (FANCM and FANCL), the FANCD2 component of the ID complex, as well as the downstream effectors FANCD1 and FANCJ [62]. Orthologs for FANCM can also be found in *Saccharomyces cerevisiae* (MPH1) and Archaea (Hef), while six of the FA proteins have clear orthologs in *Caenorhabditis elegans*: BRC-2 (FANCD1/BRCA2), FCD-2 (FANCD2), FNCI-1 (FANCI), DOG-1 (FANCJ), FNCM-1 (FANCM), and HIM-18 (FANCP/SLX4/BTBD12) [12, 13, 16]. In the genome of *Drosophila melanogaster*, six orthologs of the FA complementation groups are encoded: Brca2, Fancd2, Fancl, Fancm, Rad51C and Mus312 (FANCP/SLX4/BTBD12) [12–14]. Meanwhile, in vertebrates, the chicken DT40 B cell line has been extensively used to study the effects of loss of function in the FA signaling pathway, including inactivation of FANCG, FANCD2, FANCC, FANCI, and FANCP [63–68]. Notably, the complete FA gene family can only be found in vertebrates; thus, the DT40 cell line gives a good system to study molecular interactions of the whole FA pathway [69]. Although these model systems are suitable for pathway analysis, a hematopoietic transplantation system is required for the study of HSC biology.

6. Transplantation Models for HSC Research in FA

There are a number of vertebrate model systems that have been used to interrogate HSC biology. In the zebrafish, transplantation of whole kidney marrow cells into lethally irradiated recipient fish was shown to be radioprotective, specifically rescuing the ablation of the hematopoietic system that is observed in nontransplanted fish [70]. This demonstrated that transplantable zebrafish HSCs were to be found in the adult kidney. The recently identified existence of histocompatibility genes in the zebrafish has allowed the further improvement of this transplant system [71]. Although zebrafish contain the full complement of FA family members found in humans, loss of function models have only been described for a few complementation groups [11]. The knockdown of the zebrafish ortholog of FANCD2 using an antisense morpholino approach leads to similar developmental defects as those observed in some FA patients, including decreased body size, microcephaly, and microphthalmia [72]. This suggests that the FA pathway plays a similar role in zebrafish and humans. While the morpholino approach is particularly useful for the study of a gene product during development in the zebrafish, it is not appropriate for the ongoing evaluation of gene function in the adult organism. To date, zebrafish mutant lines for FANCL and FANCD1 have been described [73]. Although these fish have an interesting defect in sex determination, they have no documented defects in hematopoiesis. Nonetheless, it is possible that a more detailed assessment of the HSC function of these mutant fish using the transplantation assay described above may yield a phenotype.

The mouse is by far the most frequently used model system employed to study mammalian hematology. The transplant system for mice is well established, and the immunophenotypic identification of murine HSCs and their committed progeny has dramatically improved over the last decade such that HSCs can be prospectively purified to a frequency of around 1 in 4 cells using flow cytometry [74, 75]. A number of murine models for FA have been developed. Knockout models exist for components of the FA core complex (FANCA, FANCC, FANCG, FANCF, and FANCM), FANCD2, and the downstream effectors FANCD1 and FANCP [5, 76–78]. As in FA patients, a number of common cellular phenotypes are present across the different mouse models. These include defective regulation of the cell cycle and apoptosis; spontaneous genomic instability including chromosome breakage and radial chromosomes; increased sensitivity towards DNA interstrand crosslinking agents such as DEB, MMC, and cisplatin [79]. Our discussion will focus on the hematologic defects in these mice, but a detailed overview of the nonhematopoietic phenotypes can be found in [80].

Of the models for FA core complex loss of function, the phenotypes of FA core complex *Fanca*$^{-/-}$, *Fancc*$^{-/-}$, and *Fancg*$^{-/-}$ mice are almost identical [78]. Their splenocytes and BM progenitors show MMC hypersensitivity. In addition, the BM progenitors are also hypersensitive towards IFN-γ and TNF-α *in vitro* and *in vivo* [28, 49, 81–85]. The

peripheral blood is normal in *Fancc*$^{-/-}$ and *Fancg*$^{-/-}$ mice; however, *Fanca*$^{-/-}$ mice show mild thrombocytopenia in young (8–10 weeks), but not in older mice [86]. Regarding the BM, *Fancg*$^{-/-}$ mice demonstrate a defect in the proliferation of mesenchymal stem/progenitor cells and a compromised ability to promote HSC engraftment *in vitro* or *in vivo* [87]. Although *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ mice do not demonstrate reduced numbers of HSCs as determined by flow cytometry analysis, they do have impaired proliferation of progenitors *in vitro* and show a decreased long-term repopulating ability of HSCs in competitive transplantation assays *in vivo* (Figure 1), which may be related to the development of BMF in FA patients [88–90]. Relating to this, *ex vivo* expanded *Fancc*$^{-/-}$ HSCs demonstrate a dramatically reduced repopulation ability indicating impaired HSC maintenance during stress [91, 92]. Notably, the HSC phenotype of *Fanca*$^{-/-}$ and *Fancc*$^{-/-}$ mice can be corrected via retroviral-mediated delivery of the corresponding FA cDNA, formally demonstrating that the defect in the FA signaling pathway is responsible for this phenotype [93–95].

Of the two other murine models which are knocked out for components of the core complex, neither the *Fancf*$^{-/-}$ nor the *Fancm*$^{-/-}$ mice have been shown to have any hematologic defects to date, although extensive analysis of the HSC compartment using transplantation assays may not have been performed [76, 77].

Two independent murine *Fancd2*$^{-/-}$ models have been developed [96, 97]. *Fancd2*$^{-/-}$ mice have a reduced HSC content, leading to significantly reduced frequencies of late-developing cobblestone area forming cells *in vitro* and defective short- and long-term repopulating ability *in vivo* [96]. Of potential importance is the fact that both *Fancd2*$^{-/-}$ models demonstrate a reduction in the frequency of quiescent HSCs, which may relate to their reduced engraftment capacity. Interestingly, treatment with the antioxidant resveratrol is able to partially correct the hematopoietic defects in *Fancd2*$^{-/-}$ mice [97].

The mice that harbor a hypomorphic mutation in the FA downstream effector *Fancd1* (*Fancd1*/*Brca2*$^{\Delta 27/\Delta 27}$) have a more severe hematopoietic phenotype than the FA core complex knockout mice [80]. The peripheral blood is normal, but the function of the more immature hematopoietic compartment is significantly compromised, including decreased proliferative capacity of the progenitor compartment and a profoundly reduced competitive repopulation capacity of HSCs [98]. Notably, wild-type or gene-corrected *Fancd1*$^{\Delta 27/\Delta 27}$ HSCs transplanted into *Fancd1*$^{\Delta 27/\Delta 27}$ mice demonstrated a selection advantage, which would appear to recapitulate the phenomenon of reverse mosaicism that has been observed in some FA patients [98, 99].

Despite the fact that a number of the FA murine models described above do demonstrate a defect in HSC function during BM transplantation, none of the mice suffer from the progressive BMF that is almost universally prevalent in FA patients. As described below, it is possible to provoke a BMF-like phenotype in some of these murine models by the application of stress situations other than BM transplantation, such as *in vivo* exposure to crosslinking agents or oxidative stress [86, 100]. However, the only single gene deleted FA mouse model that appears to demonstrate aspects of spontaneous BMF is the recently described *Btbd12*$^{-/-}$ (*Fancp*$^{-/-}$) mouse [5]. *Btbd12* is the mouse ortholog of the evolutionarily conserved SLX4 protein, which is a key regulator of nucleases and critical for DNA damage response. These mice are born at sub-Mendelian ratios; have reduced fertility; are growth retarded and suffer from developmental defects including microphthalmia. In addition, cells from *Btbd12*$^{-/-}$ animals spontaneously accumulate chromosomal damage and are particularly sensitive to DNA crosslinking agents. However, of particular import is the observation that *Btbd12*$^{-/-}$ mice are prone to peripheral blood cytopenias and have decreased myeloid and pre-B-cell progenitor content in the BM. The HSC phenotype of these mice has not been reported to date, but it would be particularly informative to assess how this relates to the other murine FA models and whether there is a progressive, spontaneous loss of the HSC pool as these mice age. The fact that *Btbd12*$^{-/-}$ mice more closely mimic the phenotype of FA patients begs the question as to why this is not the case for other murine models of FA. One possibility is that the FA proteins targeted in the other FA mice do not have an equivalent role in humans and mice. While this is certainly possible, the cellular phenotype relating to their DNA damage response; cell cycle defects; proapoptotic phenotype; hypersensitivity to proinflammatory cytokines appears to be conserved across the species barrier. A second possibility is that most of the FA murine models developed to date are in fact hypomorphic. Again, this would not seem to correlate with the abnormal cellular phenotypes that are obtained in these models. A third possibility is that the mice are not exposed to an environmental stimulus that drives BMF. While this possibility will be discussed at some length below, it would not explain the fact that BMF is observed in the *Btbd12*$^{-/-}$ model, which is presumably exposed to a similar environment as all the other FA mice models. Another alternate explanation is that BTBD12 has additional functions outside the FA pathway. As a putative scaffolding protein for DNA nucleases, it could certainly be involved in other forms of DNA repair. Conversely, it is apparent that the phenotypes of the FA murine models become more severe the further downstream within the pathway the targeted gene is proposed to act. While this may again relate to the other knockout models actually being hypomorphic in terms of FA pathway signaling, it is also possible that the downstream proteins, which are directly involved in DNA repair, can retain some function in the complete absence of upstream signaling. In this context, it would be interesting to examine BTBD12 activity in the other existing murine FA models.

While the *Btbd12*$^{-/-}$/*Fancp*$^{-/-}$ mouse is the only single FA gene loss of function model to recapitulate some aspects of the BMF seen in patients, the compound loss of function of *Fancc*$^{-/-}$ and *Fancg*$^{-/-}$ also results in spontaneous hematologic defects including BMF, AML, MDS, and complex random chromosomal abnormalities [101]. This would again seem at odds with the idea that the FA signaling pathway is epistatic and suggests that some of the FA proteins have divergent functions. However, an alternative interpretation would be that the single *Fancc*$^{-/-}$ and *Fancg*$^{-/-}$ mutations

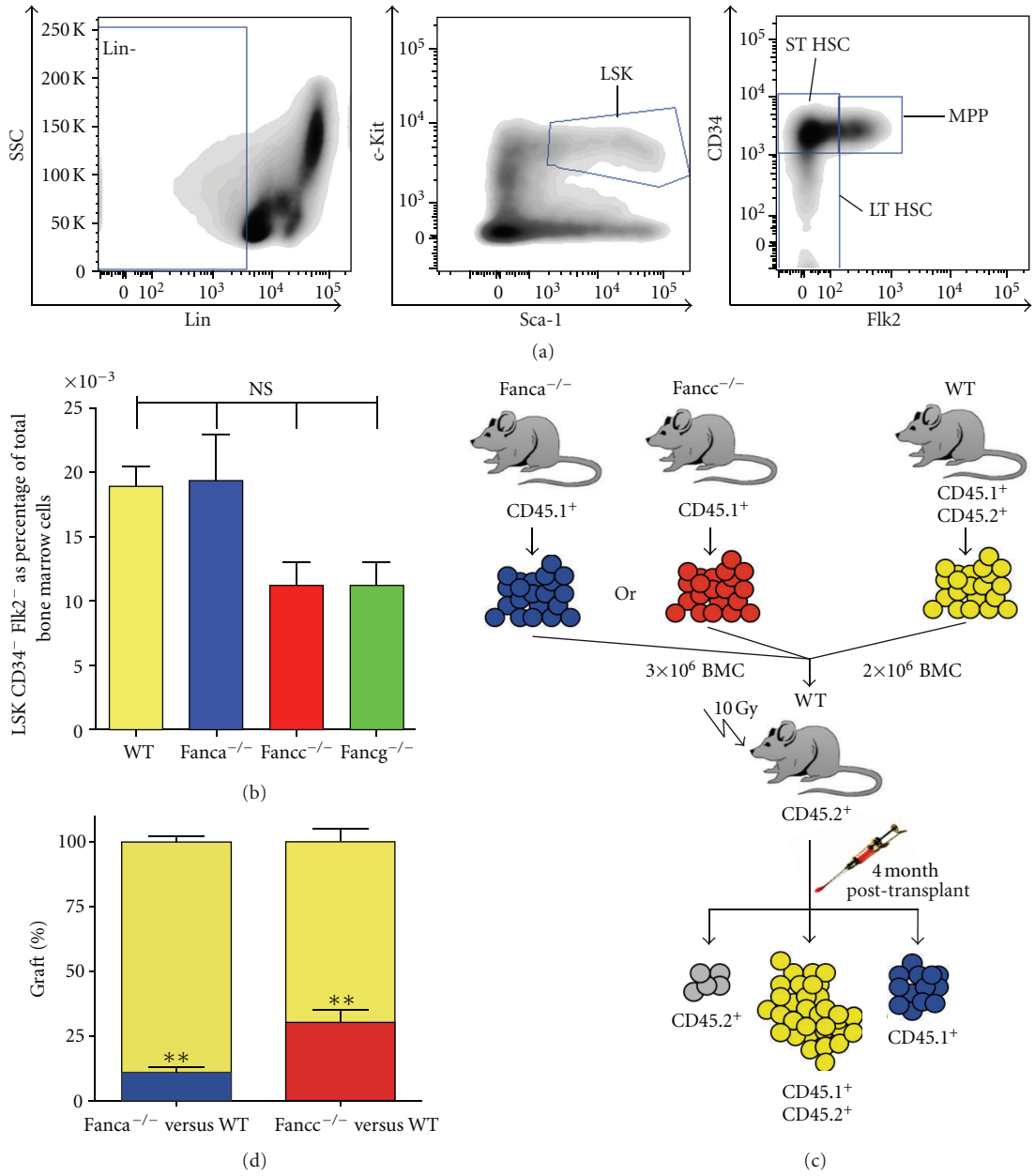

Figure 1: Murine models of FA do not have reduced numbers of immunophenotypically defined HSCs but do have lower frequencies of functionally defined HSCs. (a) Representative FACS plots showing the gating scheme which is employed to enumerate long-term HSCs (LT-HSC) in the BM which are defined by the following combination of immunophenotypic markers: Lineage-, c-Kit+, Sca-1+, Flk2−, and CD34−. For illustrative purposes, the compartments enriched for short-term HSC (ST-HSC) and multipotent progenitors (MPP) are also shown. (b) Based on the FACS methodology shown in (a), the frequency of LT-HSCs does not differ significantly in wild-type (WT) mice compared to FA mice. The mean frequency of immunophenotypically defined LT-HSC found in the BM of WT ($n = 21$), *Fanca*$^{-/-}$ ($n = 12$), *Fancc*$^{-/-}$ ($n = 6$), and *Fancg*$^{-/-}$ ($n = 6$) mice is shown, ± SEM. NS = $P > 0.05$ by comparison using ANOVA. (c) Schematic representation of the competitive repopulation assay employed to assess the relative frequency of HSCs in WT versus either *Fanca*$^{-/-}$ or *Fancc*$^{-/-}$ mice. BM from FA (3×10^6 total BM cells) and WT (2×10^6 total BM cells) mice were coinjected into lethally irradiated (10 Gy total body irradiation) recipient mice at a 3 : 2 ratio, respectively. At four months posttransplant, peripheral blood was harvested from recipient mice and the percentage contribution of FA cells to the peripheral blood was determined by FACS analysis, taking advantage of the differential expression of CD45 subtypes on the surface of FA (CD45.1+ and CD45.2−) and WT (CD45.1+ and CD45.2+) leukocytes. If FA BM contained the same number of functionally defined HSCs as WT BM, then the FA HSCs would be predicted to contribute to 60% of the peripheral blood chimerism at 4 months posttransplant. (d) FA HSCs have a severe engraftment defect compared to WT HSCs. The mean relative frequency that FA or WT cells contributed to peripheral blood leukocyte engraftment at 4 months post-transplantation is shown ± SEM. ** = $P < 0.001$ for comparison of WT versus FA chimerism using ANOVA. $n = 21$ for WT versus *Fanca*$^{-/-}$, and for WT versus *Fancc*$^{-/-}$. *Fancc*$^{-/-}$, *Fancg*$^{-/-}$ and *Fanca*$^{-/-}$ mice have all been previously described [49].

are in fact hypomorphic for HSC function. Clearly this is a very interesting model system that may be used to gain some insight into the role of abnormal HSC biology in BMF.

While the murine models of FA seem to be the best system currently available for interrogating the HSC defect in this disease, it is possible that they will never fully recapitulate the hematologic disorders observed in patients. Therefore, it may be beneficial for those in the FA research community to develop a large animal model of FA. While such an undertaking would require a significant financial investment, large animal transplant models such as the existing canine and primate systems have already given us unique insights into human HSC biology and have been invaluable in helping to develop novel therapeutic modalities for other diseases [102].

7. Cellular DNA Crosslinking Agents and HSC Depletion in FA

The observation that none of the currently available models of FA fully phenocopies the progressive BMF observed in patients may relate to the lack of an environmental and/or endogenous factor that drives HSC loss. Since FA cells are invariably hypersensitive to DNA crosslinking agents, resulting in cell cycle arrest and apoptosis, it is not unreasonable to focus upon the identification of potential crosslinking agents in FA patients in the search for a physiologic mediator of HSC depletion (reviewed in [103]).

Cellular ROSs are one of the chemical entities that are frequently proposed to act as a source of DNA damage in FA cells. Fuelling this speculation is the observation that primary FA cells from patients and murine models of the disease are more susceptible to karyotypic abnormalities induced by oxygen; have a proapoptotic response following exposure to elevated oxygen and/or ROS; are propagated much more effectively under low oxygen tensions [54, 104–108]. Confirmatory evidence is provided by a study in which compound inactivating mutations of FANCC and the ROS detoxifying enzyme Cu/Zn superoxide dismutase (SOD) in mice led to BM hypocellularity [86]. Relating to this, a recent study by Li and colleagues demonstrated a direct functional interaction between FANCD2 and FOXO3a in human lymphoblast cell lines following challenge with H_2O_2 [109]. This interaction was specific for treatment with ROS-inducing agents and was absent in cell lines where the FA pathway was nonfunctional. Using a retroviral overexpression strategy, it was also possible to demonstrate that the FANCD2-FOXO3a interaction was involved in protecting cells from oxidative stress via enhancing the induction of antioxidant genes that are direct targets of FOXO3a. Intriguingly, several groups have also shown that the balance of normal HSC homeostasis, including their genetic integrity, is dependent upon ROS levels, suggesting that the ROS-sensitive phenotype of FA cells may represent an exaggerated form of normal HSC behavior [110–115].

Reactive aldehydes comprise another potential source of cellular DNA crosslinking agent that may deplete FA HSCs *in vivo*. FA deficient cell lines are hypersensitive to both acetylaldehyde and formaldehyde *in vitro*, while exposure of FA proficient cells to acetylaldehyde *in vitro* results in activation of the FA signaling pathway [116]. Moreover, it has recently been shown that in mice which have compound inactivating mutations of FANCD2 and the acetylaldehyde detoxifying enzyme ALDH2, postnatal exposure to ethanol, the metabolic precursor for acetylaldehyde, results in BMF [117]. These $Fancd2^{-/-}Aldh2^{-/-}$ mice are also predisposed to spontaneously develop acute leukemia, hence further phenocopying the disease progression in patients.

8. Replicative Stress and DNA Damage in HSCs

Since DNA synthesis can be considered a form of DNA damage, replicative stress may also be a candidate for HSC depletion in FA. Indeed, in FA competent cell lines, upon induction of replicative stress, FANCD2, FANCM, and the Blooms complex are localized to discrete fragile sites on sister chromatids during mitosis [118, 119]. These fragile sites comprise common chromosomal break points and are also the location at which stress-induced ultrafine DNA bridges form. In human and murine FA-deficient cells, including FA HSC/progenitors, there is an increased number of ultrafine DNA bridges compared to their wild-type counterparts [120]. This correlates with an increased frequency of cytokinesis failure, as assessed by the number of binucleated HSC/progenitors, and an increased rate of apoptosis. Thus, it is hypothesized that the FA pathway is involved in the resolution of these spontaneously occurring ultrafine bridges and that the absence of a functional FA pathway leads to cytokinesis failure followed by programmed cell death, or to genetic instability. Such a mechanism would be an attractive explanation for the progressive BMF seen in FA patients, as HSCs would potentially be depleted as they were induced into cycle.

9. Proinflammatory Cytokines as Potential Mediators of HSC Depletion

In addition to an inability to resolve some forms of DNA damage, FA cells are also hypersensitive to the inhibitory action of certain proinflammatory cytokines. Proinflammatory cytokines are potential physiologic mediators of BMF in FA, since HSCs are routinely exposed to a range of proinflammatory cytokines during either infection or as part of the etiology of diseases with an inflammatory component, such as rheumatoid arthritis.

The investigation into the role of proinflammatory cytokines in the hematologic defects found in FA patients was driven by the observation that such signaling molecules are expressed at elevated levels in some FA patients due to aberrant activation of intracellular stress-response signaling [29, 30, 121–124]. Although this abnormal expression pattern could conceivably be a cause or an effect of progressive BMF, subsequent work established that FA-deficient hematopoietic progenitor cells were subject to an inhibition of proliferation and increased apoptosis following treatment with TNF-α, IFN-γ, and MIP-1-α [27, 29, 125–127]. Although there is no mechanistic data to explain the sensitivity of FA cells to MIP-1-α, some signaling pathway analysis has been performed to

dissect how the inhibitory effect of IFN-γ and TNF-α on FA deficient cells is mediated.

In FANCC deficient cells, IFN-γ exposure results in decreased phosphorylation of STAT1, 3, and 5 and, in the case of STAT1, is the result of FANCC binding to STAT1 and being required for its docking with the IFN-γ receptor [128, 129]. Although JAK/STAT signaling is involved in cell survival, the suppression of STAT1 signaling alone in FANCC-deficient cells may not explain IFN-γ inhibitory effects since hematopoietic progenitors from *Stat*1$^{-/-}$ mice are resistant to IFN-γ treatment [129]. In addition, double-stranded RNA-dependent protein kinase (PKR) is constitutively activated in FANCC$^{-/-}$ cells and demonstrates an increased binding affinity for double-stranded RNA [127]. While overexpression of PKR leads to an increased apoptotic response of FANCC$^{-/-}$ cells to IFN-γ, inhibition of PKR signaling partially rescues the hypersensitivity phenotype, thus establishing a role for PKR downstream of IFN-γ. In a separate study, overexpression of a dominant negative form of the PKR activator RAX resulted in increased resistance of *Fancc*$^{-/-}$ murine embryonic fibroblasts to IFN-γ [130]. Although the FANCC protein does not directly bind to PKR, it may promote resistance to IFN-γ-mediated apoptosis via its interaction with Hsp70 [127]. Intriguingly, Pang and colleagues were able to generate mutant versions of the FANCC protein that were able to correct the crosslink repair defect of FANCC-deficient cells and rescued FANCD2 monoubiquitination yet were unable to facilitate a normal activation of STAT1 and resulted in continued hypersensitivity of the cells to IFN-γ and TNF-α [131]. This uncoupling of the characteristic DNA repair defect of FA cells and their hypersensitivity to treatment with proinflammatory cytokines suggests that some members of the FA pathway may hold multifunctional roles outside of the canonical function in the sensing and repair of DNA interstrand crosslinks. Whatever its mechanism of action, it would seem that IFN-γ can elicit its effect at the level of FA HSCs as well as in progenitors, since pretreatment of either *Fancc*$^{-/-}$, *Fanca*$^{-/-}$, or *Fancg*$^{-/-}$ mice with IFN-γ facilitates the depletion of endogenous HSCs to such an extent that wild-type HSCs can be successfully engrafted into these mice without additional myeloablative conditioning [132, 133].

In the case of TNF-α-mediated suppression of FA hematopoietic cells, there is some evidence to implicate a link between DNA damage and TNF-α signaling. Treatment of *Fancc*$^{-/-}$ mice with either LPS or TNF-α results in an exaggerated production of intracellular ROS within HSC/progenitors that may be linked to the requirement for FANCD2 monoubiquitination in the FOXO3a-mediated suppression of oxidative stress [31, 109, 134, 135]. Elevated ROS expression in response to TNF-α treatment correlates with increased DNA damage and HSC/progenitor senescence, which can be rescued by the addition of the ROS scavenger molecule N-acetylcysteine (NAC) or by inhibition of the TNF-α signaling axis. Interestingly, *ex vivo* treatment of *Fancc*$^{-/-}$ HSC/progenitors with TNF-α results in a ROS-dependent increase in genetic instability; development of TNF-α resistant clones; an increased predisposition towards AML upon transplantation of treated cells into recipient mice [31]. Relating to this, extended *ex vivo* culture of *Fancc*$^{-/-}$ cells leads to an exaggerated reduction in HSC content via induction of apoptosis in addition to an increased frequency of cytogenetic abnormalities and risk of malignant transformation which appears to be related to an acquired resistance to TNF-α [92]. Inappropriate activation of the apoptosis signal-regulating kinase-1/p38 MAP kinase signaling pathway has been identified as mediating the proapoptotic response of *Fancc*$^{-/-}$ cells to TNF-α, although it is not clear whether this is downstream of the induction of DNA damage [134, 136]. TNF-α would also appear to exert its effect upon FA HSCs, since inhibition of TNF-α signaling in *Fancc*$^{-/-}$ HSCs via ectopic expression of the homeobox transcription factor HOXB4 partially rescues their engraftment defect [137]. Of particular note, TNF-α would also appear to act as a negative regulator of wild-type HSC function in mice, again suggesting that FA HSCs act as a hypersensitive version of normal HSCs [138]. Taken together, it would appear that TNF-α is able to mediate DNA damage, HSC depletion, and hematologic transformation in the setting of FA-deficient HSC/progenitors.

It is important to note that the majority of the experiments linking proinflammatory cytokines to a role in FA HSC depletion have been performed using only the *Fancc*$^{-/-}$ murine model. However, the previously discussed studies, which demonstrate an inhibitory role of either TNF-α or IFN-γ in FA patient-derived cells or in murine models of alternate complementation groups, would seem to suggest that this hypersensitivity may be a generic feature of FA cells [29, 133].

10. Current Therapeutic Modalities for FA

Hematopoietic stem cell transplantation (HSCT) derived from the BM, mobilized peripheral blood, or umbilical cord blood of a human-leukocyte-antigen-(HLA-) matched donor currently remains the only curative treatment option for the hematologic abnormalities in FA [139]. In fact, FA was the first disease that was successfully treated by transplantation using cord blood from an unaffected HLA-identical sibling as a starting material [140]. Significant barriers to successful transplantation of FA patients include the challenge of creating a satisfactory preparative regimen in light of the patient's acute sensitivity to chemotherapeutics and radiotherapy [39, 141, 142]; the availability of an HLA-matched donor who does not suffer from the disease. Most patients are dependent on alternative donor grafts from an HLA-unmatched donor as only a very small number of patients have an unaffected, matched sibling donor (less than 25%). However, advances in the conditions used in preparative regimens allow the achievement of almost similar transplant outcomes for both cases, with survival rates of 52–88% for mismatched family members or matched unrelated donors and 69–93% for HLA-identical sibling donor HSCTs [39, 143, 144]. While FA patients are waiting for a suitable HSC donor, supportive care can be provided via red blood cell and platelet transfusions; oral administration of androgens such as oxymetholone, methyltestosterone, the androgen analogue danazol; or the direct injection of growth

factors such as granulocyte-colony forming factor (G-CSF) [145, 146]. However, androgen application can also lead to adverse sideeffects like masculinization of female patients, acne, hyperactivity and diverse problems associated with the liver such as deranged liver enzymes, hepatic adenomas and the potential risk of hepatic adenocarcinoma [147]. While hematopoietic growth factors such as G-CSF and GM-CSF are capable of enhancing peripheral blood neutrophil counts, and, in some cases platelets [148, 149], they should be avoided in patients with clonal cytogenetic abnormalities because of the risk of inducing leukemia. In any case, they are only effective for the short-term treatment and HSCT is ultimately the definite therapy.

11. Gene Therapy of FA HSCs

Since the lack of availability of HLA-matched disease-free donor HSCs is a major limitation in HSCT, one attractive novel therapeutic modality is the genetic correction of autologous patient HSCs via the reintroduction of the defective FA cDNA using a delivery system such as a retroviral vector. Recent major advances have been made in the field of gene therapy, which has allowed the correction of a range of different inherited genetic disorders with a hematologic basis via the retroviral-mediated delivery of correcting cDNAs into patient HSCs [150, 151]. Since the input cells are patient derived, there should be no issues with immunologic rejection of the graft unless the vector system or transgene payload is immunogenic. The phenomenon of reverse mosaicism, that we have previously discussed, would appear to indicate that FA would be an ideal candidate disease for treatment via gene therapy, since correction of an individual HSC can result in sustained reversal of BMF [43]. Indeed, this finding has been recapitulated in murine models of FA [99]. Unfortunately, FA also presents some unique problems, which means that it may be an incredibly difficult disease to treat with gene therapy using existing technologies. These include the extremely low yield of CD34+ cells that can be collected for gene modification relative to those routinely achieved in non-FA patients; the extreme sensitivity of FA cells to *ex vivo* culture [37, 92]. To date, the clinical gene therapy trials for FA have all failed to achieve robust engraftment of corrected patient HSCs, although advances have been made in the ability of clinicians to transduce FA CD34+ cells with retroviral vectors [37, 152, 153]. Fortunately, some of the model systems that have been developed for FA have been able to assist in the formulation of new strategies that may help overcome the barriers to effective gene therapy of FA.

The clinical gene therapy trial performed by Kelly and colleagues highlighted the low collection yield of CD34+ cells that are typically obtained from FA patients [37]. Initially, HSC collection was attempted by apheresis of peripheral blood following mobilization with G-CSF. Unfortunately, the cohort of FA patients appeared to be refractory to the HSC-mobilizing effects of G-CSF. However, the ability of murine models of FA to recapitulate this mobilization defect allowed the evaluation of two alternate mobilization protocols. While Milsom et al. were able to show that coadministration of G-CSF with the Rac GTPase small molecule inhibitor NSC23766 is able to rescue the mobilization defect of $Fanca^{-/-}$ HSCs, Pulliam and colleagues were able to demonstrate that the addition of the SDF1-α/CXCR4 antagonist AMD3100 to a G-CSF mobilization regimen was able to achieve similar results in both $Fanca^{-/-}$ and $Fancc^{-/-}$ mice [154, 155].

An alternate route to overcome the barrier of limited patient HSCs being available for genetic manipulation is to enhance the ability of the gene-corrected cells to reconstitute the patient. Murine models have been used to demonstrate that the homing of transplanted HSCs to their niche in the BM following intravenous injection into the recipient is not 100% efficient [156]. The postulation that direct injection of HSCs into the intramedullary cavity might enhance engraftment rates was first demonstrated as a proof of concept using the murine xenotransplantation assay and may be of interest for the treatment of FA patients [157].

In order to reduce the loss of FA HSCs during *in vitro* culture, it would be beneficial to develop a transduction protocol that minimized the *ex vivo* manipulation period. To these ends, lentiviral and foamy retroviral vectors hold the distinct advantage over gammaretroviral vectors in that they are able to efficiently transduce nondividing HSCs and therefore do not require *ex vivo* manipulation protocols that involve a lengthy prestimulation step in order to drive the largely noncycling HSC population to proliferate. Again, using murine models of FA, two groups have independently shown that the use of either lentiviral or foamy viruses to deliver a correcting FA cDNA using a drastically shortened transduction protocol, significantly improves the engraftment capacity of gene corrected cells [158, 159].

One further possibility to completely overcome the problems associated with *ex vivo* transduction of FA HSCs is to perform the stem cell gene transfer *in situ*. The efficacy of this novel approach was demonstrated by intrafemoral injection of a lentiviral vector expressing GFP into adult immune-competent mice. FACS analysis four months after injection showed significant transduction efficiency with up to 12% of the cells, observed in myeloid and lymphoid subpopulations, being positive for GFP. After secondary transplant, 8.1–15% GFP-positive CFU were detectable and integration site analysis confirmed multiple transduced clones contributing to hematopoiesis in these animals [160]. Importantly, Habi and colleagues were able to extend these findings to the FA model and show that intrafemoral injection of a lentivirus encoding the *FANCC* gene resulted in correction of $Fancc^{-/-}$ HSCs *in vivo* [161]. Altogether, this data indicates that *in situ* transduction of adult stem cells using lentiviral vectors is a promising improvement to *ex vivo* gene therapy. Indeed, Frecha et al. have recently improved on this approach, using normal donor and FA stem and progenitor cells to demonstrate that specially modified lentiviral vectors can selectively target human CD34+ cells *in vitro* and *in vivo*. Using a novel vector system, which expresses an envelope pseudotyped with a fusion of SCF and a mutant cat endogenous retroviral glycoprotein, they were able to selectively transduce target human CD34+ cells from nontarget cells in unfractionated total cord blood and BM from FA patients.

In addition, the glycoprotein prevents degradation by the human complement system and therefore makes the vector suitable for *in vivo* use [162]. Ultimately, this vector system may provide improved alternatives for gene therapy of FA *in vivo*, as it allowed the selective transduction of human CD34+ cells *in vivo* using the murine xenotransplantation model.

One potential adverse side effect of the use of integrating vector systems to deliver correcting genes into HSCs is the phenomenon of insertional mutagenesis. Insertional mutagenesis arises as a result of the integrated vector causing a change in expression of cellular genes that are proximal to the integration site (reviewed by Muller et al.) [163]. As documented in several clinical gene therapy trials using retroviral vectors, this can sometimes result in deregulated expression of protooncogenes, which can in turn lead to malignant transformation [163]. In the setting of FA, insertional mutagenesis might be particularly important as promalignant mutations caused by the vector integration may synergize with mutations that spontaneously arise as a result of the genetic instability that is inherent to FA cells. However, recent advances in the development of new vector systems that may have less mutagenic potential could prove a way to overcome this problem. These systems include self-inactivating retroviral vectors with weaker promoter enhancer elements [163]; recombinant transposons such as the sleeping beauty retrotransposon [164]; vectors which facilitate the codelivery of zinc finger nucleases in order to facilitate the directed repair of the endogenous FA allele via HR [165, 166]. The latter approach may prove particularly challenging in the setting of FA, where the cells are deficient for some aspects of HR-mediated DNA repair.

12. Alternative Novel Therapeutic Modalities Targeting FA HSCs

In addition to gene therapy, data obtained from experimental models of FA have been used to devise other alternative novel therapeutic modalities for FA (summarized in Figure 2). Metabolism is predicted to generate reactive by-products, such as ROS and aldehydes, which are believed to be one potential physiological source of DNA damage that precipitates the FA phenotype. While healthy cells protect against these threats through the combined action of enzymatic detoxification and DNA repair, FA cells clearly show a defect in this respect.

As previously discussed, there is strong evidence that FA cells are intolerant of oxidative stress as oxygen-induced chromosomal aberrations were observed in cultured FA cells [104] and hematopoietic cells from FA knockout mice exhibit extreme oxidant sensitivity [107]. Therefore, antioxidants promise to improve cell viability by conferring resistance to apoptosis. FANCC-deficient cells pretreated with either selenomethionine or NAC are resistant to H_2O_2 treatment and demonstrate enhanced survival [107]. The antioxidant drug resveratrol has been shown to partially correct the hematopoietic defects of FANCD2-deficient mice such as reduced spleen colony-forming capacity and abnormal cell cycle status. Colony forming unit-spleen (CFU-S) assays with whole BM revealed a significantly improved frequency of primitive spleen colony forming cells in resveratrol-treated *Fancd2*$^{-/-}$ mice. HSC/progenitors cells derived from *Fancd2*$^{-/-}$ mice initially showed a significantly lower frequency of cells in G0 when compared to wild-type counterparts; however, resveratrol treatment was capable of increasing the total amount of cells in G0 by 27%. Most importantly, a trial of treating FA patients with a two-week infusion of recombinant human SOD was shown to be effective in decreasing ROS levels. Half of the patients enrolled in this study (two out of four) were less sensitive to DNA crosslinking agents and one had improved BM progenitor numbers [167].

Recently, it was also found that the FA pathway genes seem to be required in conferring resistance to reactive aldehydes, such as acetaldehyde and formaldehyde [117, 168]. They are thought to be a potential source of DNA damage as they can directly modify DNA bases *in vitro*, which may then lead to crosslinks with DNA or proteins. These findings raise new therapeutic options for the treatment of FA patients. For instance, it may be possible to therapeutically enhance acetaldehyde/formaldehyde catabolic activity using small molecule agonists of the ALDH2 and alcohol dehydrogenase 5, respectively, to provide an intrinsic mechanism for enzymatic detoxification. Based on this data, it would also seem prudent to recommend that FA patients severely restrict their intake of alcohol in order to prevent the accumulation of acetylaldehydes.

A number of FA patients display enhanced serum levels of TNF-α and IFN-γ, which were previously shown to cause apoptosis of hematopoietic progenitor cells and may therefore be one of the major driving factors in the pathogenesis of BMF [27, 28, 30, 83, 122]. Pharmacologic agents that inhibit the TNF-α-signaling pathway, such as etanercept and infliximab, have been suggested as a possible therapeutic intervention directed against the pathogenesis of proinflammatory cytokines and have already proven to be effective in clinical use for the treatment of inflammatory diseases, such as rheumatoid arthritis and Crohn's disease [169]. They hold significant promise for prolonging the hematologic output in FA patients as preclinical models have already demonstrated that inhibition of TNF-α using etanercept was effective in increasing the size and number of CFU-E and BFU-E in BM cultures of FA patients [29]. In addition, neutralization of TNF-α was shown to be effective in preventing excessive ROS production in FANCC-deficient mice and thereby significantly reduced the DNA damage phenotype as well as senescence [135].

13. Summary

FA is a fatal inherited disorder, which almost universally results in severe defects of the hematopoietic system, likely as a direct consequence of defective HSC biology. Advances in our ability to model the HSC defect in FA patients have not only enhanced our understanding of the underlying

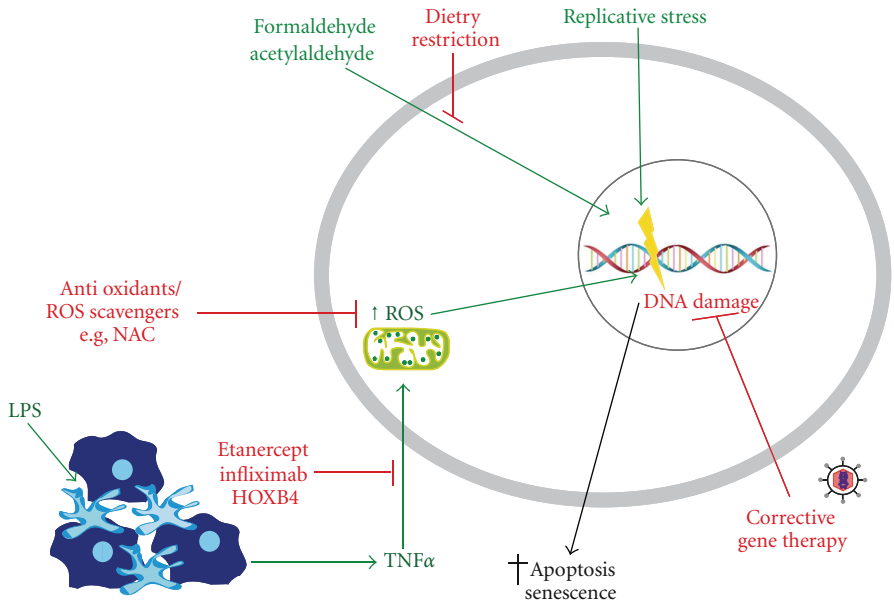

Figure 2: Potential novel therapeutic approaches for the prevention of FA HSC depletion. The potential physiologic mediators of FA HSC depletion, including replicative stress, TNF-α, ROS, lipopolysaccharide (LPS), and reactive aldehydes, are depicted as green arrows. Exposure of FA HSCs to these agents/conditions would be predicted to result in DNA damage and ultimately loss of the cell via apoptosis or senescence. Novel therapeutic modalities that directly target these HSC-depleting stimuli are shown in red. Controlling dietary consumption of certain food types (e.g., alcohol) may reduce the production of high levels of reactive aldehyde species in FA patients. Intracellular ROS could be decreased via treatment with antioxidants or ROS scavengers. Proinflammatory cytokine signaling could be targeted by interfering with ligand-receptor binding (e.g., using etanercept or infliximab to block interaction of TNF-α with its receptors) or by inhibiting downstream signaling cascades, as demonstrated for HOXB4 overexpression in the context of TNF-α signaling. Finally, gene therapy approaches offer the possibility of restoring expression of a functional FA gene into patient HSCs.

etiology of this disease but have also highlighted novel targets for therapeutic intervention. One challenge for the immediate future is to determine whether the defects that have so far been identified in FA HSCs can be extrapolated to explain the abnormal biology of other tissues that are commonly impacted upon by a defect in the FA signaling pathway. This is of particular importance since there are very limited treatment options for the serious nonhematologic complications observed in FA patients such as the increased predisposition towards solid tumors.

Although FA is a relatively rare disease, it is predicted that FA HSCs can, in some instances, act as an extremely sensitive experimental model system to interrogate the biology of normal HSCs. Therefore, understanding the behavior of FA HSCs not only benefits the community of FA patients, but may potentially impact upon our knowledge of more common disorders such as leukemias, myeloproliferative disorders, and other BMF syndromes such as acquired aplastic anemia.

Conflict of Interests

The authors have no conflict of interests to disclose.

Authors' Contribution

MDM performed the experiments described in the paper. AG, AL, DW and MDM wrote the paper.

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Review Article

Towards a Molecular Understanding of the Fanconi Anemia Core Complex

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Fanconi Anemia (FA) is a genetic disorder characterized by the inability of patient cells to repair DNA damage caused by interstrand crosslinking agents. There are currently 14 verified FA genes, where mutation of any single gene prevents repair of DNA interstrand crosslinks (ICLs). The accumulation of ICL damage results in genome instability and patients having a high predisposition to cancers. The key event of the FA pathway is dependent on an eight-protein core complex (CC), required for the monoubiquitination of each member of the FANCD2-FANCI complex. Interestingly, the majority of patient mutations reside in the CC. The molecular mechanisms underlying the requirement for such a large complex to carry out a monoubiquitination event remain a mystery. This paper documents the extensive efforts of researchers so far to understand the molecular roles of the CC proteins with regard to its main function in the FA pathway, the monoubiquitination of FANCD2 and FANCI.

1. Introduction

Fanconi Anemia (FA) patients present a variety of symptoms including skeletal and developmental defects, bone marrow failure, and a high predisposition to cancer [1]. The predisposition to cancer is attributed to the FA pathway being involved in DNA damage repair, particularly interstrand crosslinks (ICLs). FA patients are highly susceptible to crosslinking agents such as mitomycin C (MMC) and cisplatin. Such treatment results in chromosome abnormalities, and sensitivity to these agents is used as a diagnostic tool for FA [2]. Currently there are 14 verified FA genes [3–22], with a possible additional gene FANCO/Rad51C [23, 24], that make up the FA pathway. Mutations in any of the 15 genes results in the loss of ICL repair. The key event of the FA pathway is the monoubiquitination of FANCD2 and FANCI [4–7], which triggers the downstream factors, FANCP/SLX4, FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2 [9, 10, 17–22] to repair DNA damage (Figure 1). Only one protein to date has been shown to have E3 ubiquitin ligase activity, FANCL [8]. FANCL is a member of the Fanconi Anemia Core Complex (CC), consisting of 7 other FA proteins: FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, and FANCM (Figure 1) [3, 25–28]. Additionally, there are the Fanconi Anemia Associated Proteins (FAAPs), which are not yet found mutated in patients but form part of the CC: FAAP100, FAAP24, and most recently FAAP20 [29–33]. MHF1 and MHF2 (also known as FAAP16 and FAAP10) have also been implicated in the FA pathway through their association with FANCM [34, 35]. Approximately 90% of patient mutations reside in the CC, most of which are found in FANCA (60%) (Table 1) [36]. Importantly a single mutation in any of the 8 genes that make up the CC prevents the key monoubiquitination event from occurring. Extensive research efforts have been made to understand the role of the individual CC proteins and the requirement of all CC proteins for the monoubiquitination event. This paper outlines our current understanding of the molecular interactions within the core complex and highlights key remaining questions for a full molecular understanding of the CC.

2. E3 Ligase Function of the CC

Patient mutations in any member of a CC protein result in the loss of the critical monoubiquitination of the

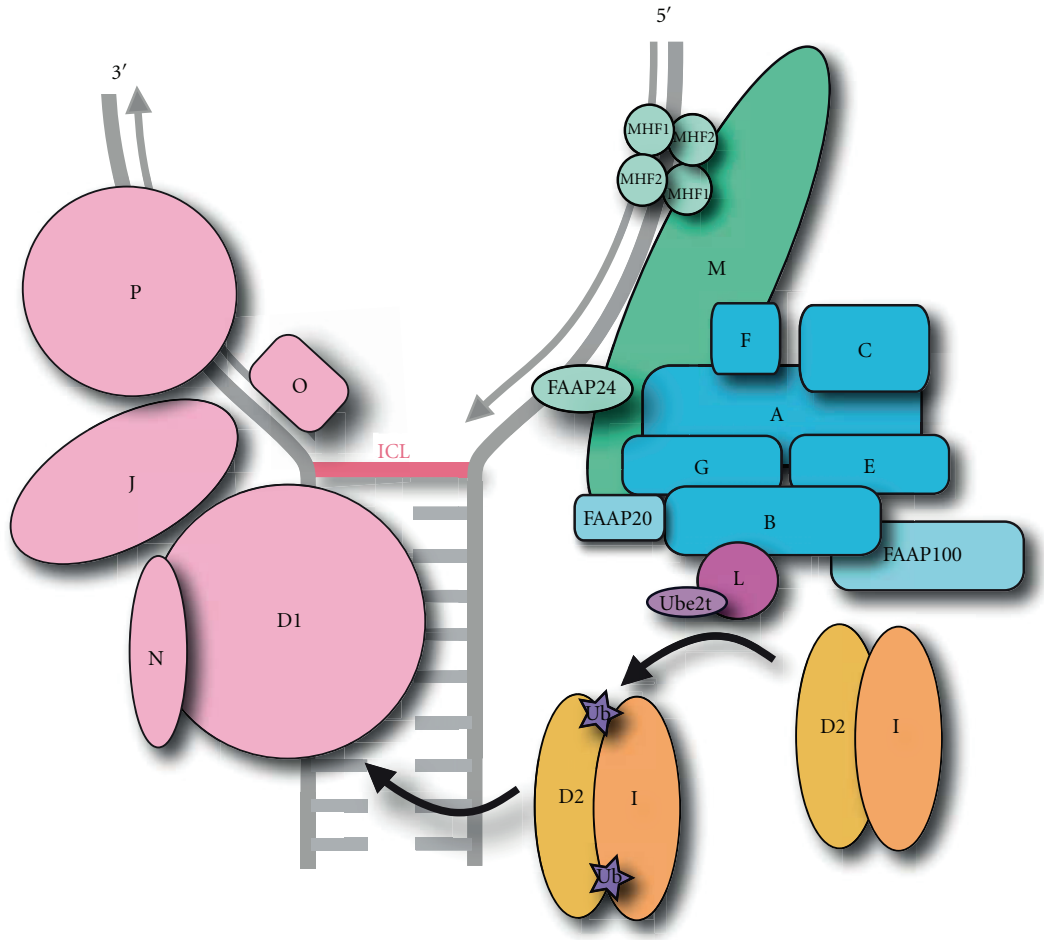


Figure 1: The Fanconi Anemia Pathway. A model of the Fanconi Anemia Pathway at a stalled DNA (grey) replication fork, caused by an interstrand crosslink (ICL). FANCM and its associated genes are coloured green, which assemble on DNA at the stalled replication fork. The other CC proteins are represented by blue with FANCL as the E3 ligase of the CC represented by mauve. The substrates for ubiquitination FANCD2 and FANCI are coloured gold and peach, respectively, with their associated ubiquitins represented by purple stars. The DNA repair machinery is coloured pink.

FANCI/FANCD2 complex. All CC proteins appear to be required for this event *in vivo;* therefore, historically the CC has been regarded as a multisubunit E3 ligase. Multisubunit E3 ligases such as the Cullin-RING ligases (CRLs) and the Anaphase Promoting Complex (APC) are well understood at the molecular level, with their modularity essential for function.

The CRLs consist of a Cullin scaffold protein, which associates with either Rbx1 or Rbx2 RING proteins, the subunit responsible for binding the E2 carrying the activated ubiquitin moiety [37]. The Cullin and RING therefore form the catalytic unit of the CRL. In order for a substrate to become ubiquitinated, it must be recognised by the CRL. This is achieved by the substrate receptor proteins, which associate through an adaptor protein onto the Cullin scaffold, forming the complete CRL (Figure 2(a)) [37]. A plethora of different substrate recognition proteins for a single Cullin achieves flexibility within the CRLs to target a repertoire of substrates.

The APC also targets a variety of substrates to control cell cycle progression from metaphase to anaphase. Similarly to the CRLs the APC comprises of a Cullin repeat protein Apc2, which binds the RING protein Apc11, and also Apc10 involved in substrate association. Together, these 3 subunits form the catalytic unit. However, in contrast to CRLs the APC contains an additional 10 proteins (Figure 2(b)). Apc9 and 13 and Cdc26 are structural stabilizers, whereas Apc1, 4, and 5 form a scaffold platform for the catalytic unit [38]. The scaffold platform along with the tetratricopeptide repeat (TPR) proteins Cdc23, Cdc27, Cdc16, and structural stabilizer Cdc26 forms the TPR subcomplex, orientating the catalytic unit for its association with coactivators, Cdc20 and Cdh1 [38]. The co-activators are required along with Apc10 for substrate recognition [38–40]. In common with

Table 1: Fanconi Anemia genes and their products.

Gene	MW (kDa)	No. of amino acids	Patient mutations
A	163	1455	60%
B	98	859	2%
C	63	558	13%
D1	384	3418	2%
D2	164	1451	3%
E	59	536	3%
F	42	374	3%
G	68	622	9%
I	150	1328	1%
J	141	1249	2%
L	42	375	0.2%
M	232	2048	0.2%
N	131	1186	0.6%
O	42	376	0.5%
P	200	1834	0.5%
FAAP24	24	215	—
FAAP100	100	881	—
MHF1	16	138	—
MHF2	10	81	—

Amino acids numbers were taken from the NCBI webserver, and patient mutational information was obtained from the Rockerfeller FA Mutations Database and was calculated as a percentage of all individuals recorded in the database.

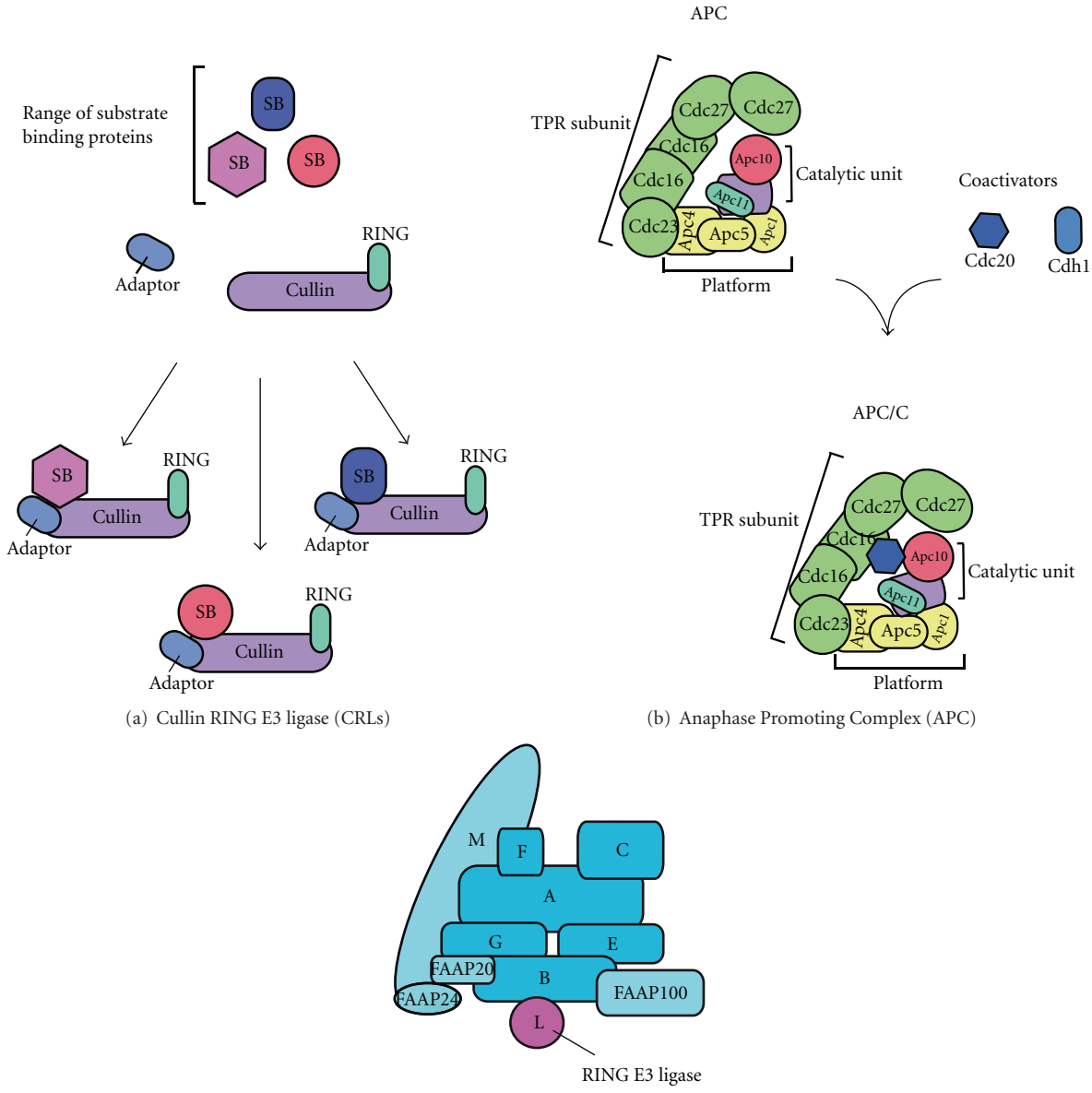
the CRLs, this ensemble allows flexibility and diversity in substrate recognition.

By contrast, the CC has one subunit with E3 ligase activity, FANCL [8] (Figure 2(c)), shown to be the only subunit of the CC required for FANCD2 monoubiquitination *in vitro* [41]. FANCL is a RING E3 ligase [42], which binds the E2 of the FA pathway, Ube2t [41, 43] in a canonical fashion through its RING domain [43, 44]. Earlier *in vitro* and *in vivo* work indicated a FANCE-FANCD2 interaction [45] and a series of yeast and mammalian 2-hybrid studies further support this interaction [46–48]. The interaction of FANCD2 with a CC component prompted the idea that FANCE may bring the substrates FANCD2 and FANCI into close proximity of FANCL for their subsequent monoubiquitination. As with other multisubunit E3 ligases, this would leave FANCL as the "catalytic" subunit, indirectly ubiquitinating substrates through its interaction with E2. However, not only is FANCL sufficient *in vitro* for the monoubiquitination event [41], but also has been shown to interact directly with both FANCD2 and FANCI *in vitro* [42, 44], and with FANCD2 in cells [49]. Although mutations in other CC proteins result in a loss of the monoubiquitination event, these more recent findings suggest FANCL possesses all the requirements to be able to carry out the monoubiquitination, unlike the multisubunit E3 ligases.

3. Protein-Protein Interactions Required for CC Stability

Although the monoubiquitination event *in vitro* requires only FANCL, it is clear from patient mutations that all members of the CC are required *in vivo*. The reasons for this are not clear, although numerous groups have shown the CC proteins interact with one another and are required to form a stable CC. Part of the challenge of gaining a molecular understanding of the core complex lies in the lack of obvious domain structures from primary sequences in any of the proteins except FANCL and FANCM. This section will describe the efforts to understand the molecular biology of the core complex to date.

3.1. FANCG-FANCA CC Interactions. FANCG and FANCA have been shown to interact directly and indirectly through yeast 2-hybrid, co-immunoprecipitations (co-IPs), cell-based studies, and *in vitro* translational (IVT) work [25, 26, 46, 50–55] (Figure 3). Co-IPS and IVT studies suggested the N-terminal 300 residues of FANCA bind FANCG [50, 51]. This region has been further narrowed down to the first 40 amino acids by a yeast 2-hybrid assay and the first 37 amino acids by a co-IP study [25, 46], with a requirement for basic amino acids within this region for the interaction [25]. IVT studies indicate residues 18–29 of FANCA are sufficient for a FANCG interaction, specifically Arginine 18, Arginine 19, and Leucine 25 [50]. Studies aimed at identifying the regions of FANCG involved in a FANCA interaction are more conflicting. Hussain et al. [55] reported via yeast 2-hybrid analysis that the predicted TPR motifs 5 and 6 of FANCG, which reside in the C-terminal 170 residues, were required for the interaction. Consistent with this finding, IVT assays also revealed two FANCA binding regions in the C-terminal 222 residues of FANCG: one encompassing the same predicted TPR motifs 5 and 6, and the second



(a) Cullin RING E3 ligase (CRLs)

(b) Anaphase Promoting Complex (APC)

(c) Fanconi Anemia Core Complex

Figure 2: Models of multisubunit E3 ligases. (a) A model of the proteins that make a Cullin-RING E3 ligase (CRL). The Cullin protein (lilac) acts as scaffold and binds the RING domain (cyan) required for E2 binding and the substrate binding proteins (pink, red, blue) via an adaptor. The variety of substrate binding proteins allows the CRLs flexibility in binding a range of substrates. (b) A model of the Anaphase Promoting Complex (APC). The catalytic core consists of a Cullin repeat protein Apc2 (lilac), which acts a scaffold for the RING protein, Apc11 (cyan), and the substrate binding protein Apc10 (red). For substrate recognition the APC also binds coactivators (dark blue). The APC also consists of a TPR subcomplex (green) and a platform (yellow) which orient the catalytic unit and aid binding to the co-activators. The range of subunits allows a variety of substrates to be recognised. (c) A model of the Fanconi Anemia Core Complex. The catalytic activity resides in one protein FANCL (mauve). The rest of the CC proteins are coloured blue, with light blue representing proteins associated with the CC.

residing in the last 37 residues of FANCG [50]. However, in contrast, a yeast 2-hybrid study found the C-terminal 142 amino acids of FANCG to be dispensable for interaction with FANCA [46]. To add to the complexity, other studies report a requirement for additional regions throughout FANCG for a FANCA interaction [52, 54, 56] with Wilson et al.'s [56] *in vivo* study indicating several regions, TPR motifs 1, 2, 5, and 6 throughout FANCG are required.

FANCG null lymphoblasts have a defect in FANCA nuclear accumulation, which can be rescued by the addition of FANCG, suggesting that FANCG plays a role in the subcellular localization of FANCA [26]. Indeed, the FANCG interaction with FANCA appears to promote FANCA nuclear accumulation [26]. However, a thorough analysis of multiple FANCA patient mutations suggests that FANCG binds FANCA even when the nuclear localisation of FANCA

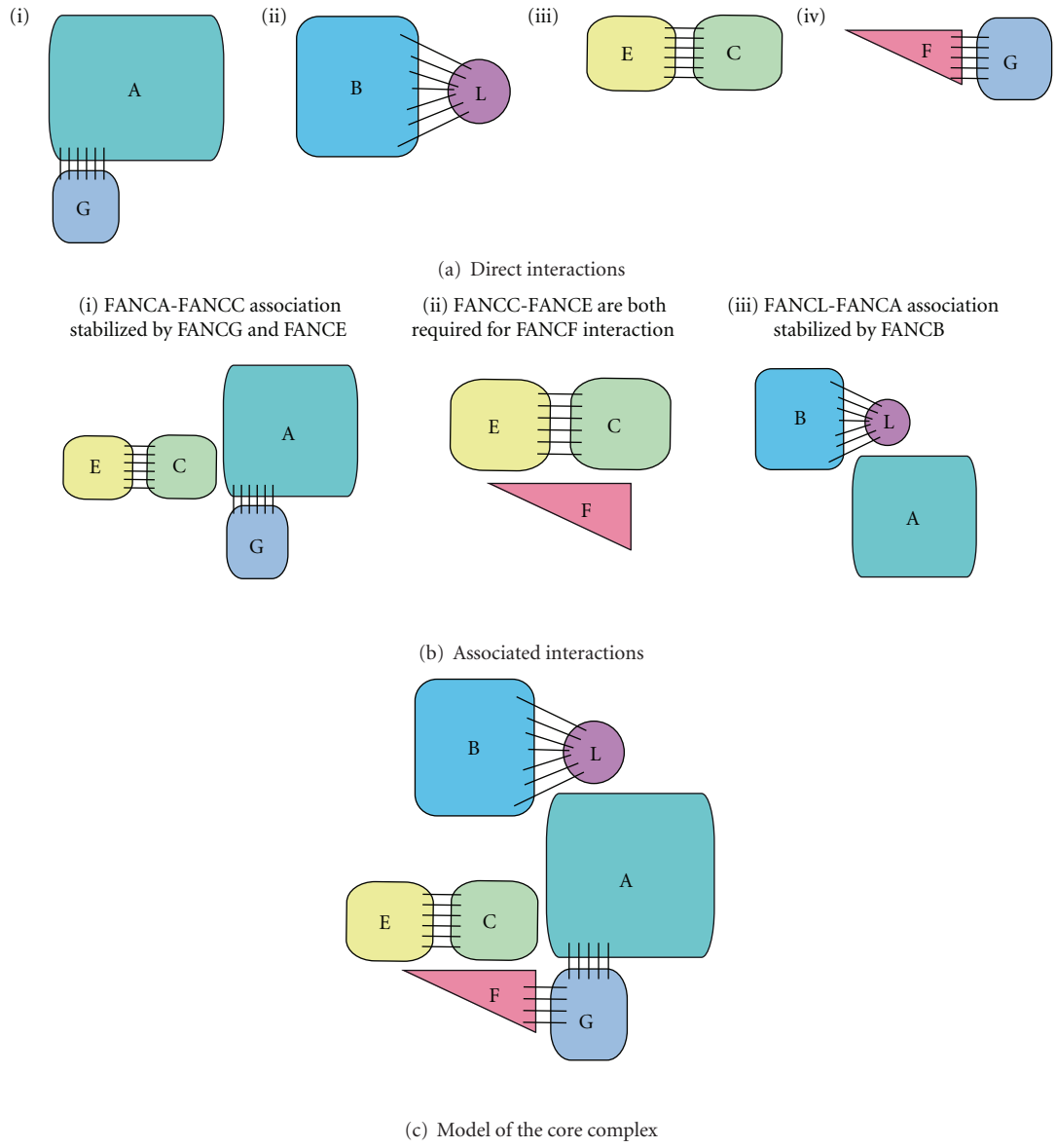


Figure 3: A model of CC interactions. (a) Models of the CC proteins that directly interact with one another, represented by black lines. Yeast and mammalian 2-hybrid and *in vitro* and translational studies have shown these interactions. (b) Associated CC interactions as shown by mammalian and yeast 3-hybrid experiments. (c) A model of how all the CC proteins interact to from the full CC, the requirement for the monoubiquitination event.

is lost [57]. The FANCA extreme N-terminus contains a nuclear localisation signal (NLS) [25, 58]. FANCA patient mutations are varied and account for 60% of all FA cases (Table 1) and predominantly result in loss of FANCA nuclear accumulation [57]. It appears likely that a combination of the NLS on FANCA and FANCG-binding stabilise, and supports the nuclear subcellular localisation of the core complex.

These studies all indicate a likely physical interaction between FANCG, and FANCA, but the molecular details have yet to be fully resolved.

3.2. FANCF CC Interactions. FANCF has been implicated in the physical stability of the majority of other CC proteins, FANCC, FANCE, FANCG and FANCA by several groups [27, 28, 59, 60]. X-ray crystallographic analysis of a C-terminal portion of FANCF (residues 156–357) revealed an architecture of helical repeats [60], similar to those found in scaffolding proteins. Structure-based mutations were then generated for use in mammalian co-IP assays. Using both point mutations L209R and F251R and a hydrophobic patch mutation Y287A/L289A/F339A/V341A/L344A, Kowal and coworkers [60] verified and provided the molecular details of the FANCF associations with FANCA and FANCC reported from earlier co-IP studies [27]. In addition, a further association with FANCE was identified [60]. In contrast to the structure-based analysis, Léveillé et al. [59]

use coimmunoprecipitation from cultured lymphoblasts and report the requirement of the last 31 amino acids (343–374) of FANCF for a FANCG and FANCA interaction and additionally report that the first 15 N-terminal amino acids are required for a FANCE and FANCC interaction.

Yeast and mammalian 3-hybrids revealed that a FANCC-FANCE interaction was required for a direct interaction with FANCF [59, 61]. In accordance with their co-IP studies, Léveillé et al. [59] show residues Leu5/Leu8/Leu15 are required for a FANCC-FANCE interaction in their mammalian 3-hybrid assay and report two additional regions, Arg10/Phe11/Arg47/Phe48 and Ser18/Ser19/Thr20/Thr21, also required for this interaction [59]. Whilst these findings are not necessarily incompatible, the molecular details of the interactions between FANCF and FANCG, FANCA and FANCE are still unresolved.

The FANCA-FANCF interaction is mediated by FANCG, from yeast 3-hybrid analyses [46]; conversely co-IP studies indicate a FANCA-FANCG interaction is stabilized by FANCF [27]. Both these observations are supported by yeast 2-hybrid experiments that show a direct interaction of FANCG with FANCF [28, 46, 55, 59]. Gordon and Buchwald [46] show that this interaction resides in the last 131 C-terminal residues of FANCF, and Léveillé et al. [59] narrow this down to the last 40 amino acids in their yeast 2-hybrid assay. Several groups have attempted to map the region of FANCG responsible for a FANCF interaction by yeast 2-hybrid analysis, all of which conclude that several sites are required throughout the full amino acid sequence of FANCG [46, 52, 55].

Importantly, structure-guided mutagenesis of FANCF increased MMC sensitivity, thereby directly showing FANCF interactions are critical [60]. Although there are conflicting results regarding FANCF associations with other CC members, numerous studies all support the role for FANCF in coordinating and stabilizing other CC proteins, as seen for FANCG.

3.3. FANCE-FANCC CC Interactions. A FANCC-FANCE interaction and their association with other members of the CC have been documented by yeast and mammalian 2- and 3-hybrid assays, IVT studies and co-IPs [28, 45–47, 59, 62]. The central part of FANCE, residues 149–371, is required for the FANCC interaction as seen by yeast and mammalian 2-hybrid experiments [46, 47]. However, the corresponding regions of FANCC required for the interaction were not determined. More recent studies employing mammalian and yeast 3-hybrid assays indicate the importance of a FANCC-FANCE interaction to facilitate a direct interaction with FANCF [59, 61]. This interaction of FANCE with FANCF explains early co-IP findings of FANCEs associations with FANCA, FANCG, and FANCF [45, 62], as FANCF has been shown to directly interact with FANCG, and FANCG directly interacts with FANCA, indicating a possible indirect association of these proteins.

3.4. FANCL-FANCB CC Interactions. Research has indicated FANCL is required to form a stable CC using co-IP experiments and size exclusion chromatography [63, 64]. Alpi et al. [64] show that in a wild-type chicken DT40 lymphoblastoid cell line, a complex of 1.5 MDa pulled out using a Tandem-affinity tagged FANCC exists. In corresponding FANCL-null cells, this complex is both less abundant and a lower molecular weight. However, the 1.5 MDa complex is established again upon expressing FANCL [64]. Consistent with these data, co-IPs in FANCL-null cells show a disruption of the interactions of FA CC proteins [63]. In the same study, a mammalian 2-hybrid assay indicates that FANCL forms a direct interaction with the CC via FANCB [63] (Figure 3). Medhurst et al. [63] also suggest that FANCL-FANCA interactions are mediated through FANCB and that FANCG is required to stabilize FANCA in this interaction. Additionally Alpi et al. [64] demonstrate that the stabilizing role of FANCL for the CC is independent from its E3 ligase activity, as point mutations that disrupt the RING domain and inhibit the monoubiquitination activity can still form a stable CC when introduced into the FANCL-null cell line. It is clear that FANCL is an important member of the CC for stability; however, molecular details, including the stoichiometry and domain requirements of how FANCL interacts with the CC are still lacking.

The intricacy of, these CC protein-protein interactions is further complicated by the findings that a FANCA-FANCG interaction is required in stabilizing a FANCC-FANCA interaction and the need for FANCE to support the FANCA-FANCC interaction [25, 62].

The extensive research described here reflects a complex network of interactions between the CC proteins (summarized in Figure 3), all of which seem to be a requirement for a fully stable CC.

4. Subcomplexes, Stoichiometry, and Assembly of the CC

As discussed above FANCA has an NLS and monoubiquitinated FANCD2 locates at nuclear foci on chromatin as seen by fluorescent microscopy and co-IP studies [7, 65, 66]. However, FANCD2 has also been located in the cytoplasm [67–70] as have several of the CC components [8, 27, 51, 71]. Such findings give rise to the possibility that subcomplexes of the CC exist and localize in different cellular regions.

Meetei et al. [72] reported different ratios of CC proteins observed in their co-IPs studies suggesting the idea of subcomplexes, although this could also reflect different stoichiometry of the CC proteins. An analysis of CC proteins isolated from the cytoplasm at different stages of the cell cycle revealed different molecular weight protein complexes by size exclusion chromatography [73]. A complex that consists of a single copy of each FA protein of the CC would give an approximate 737 kDa complex, which would increase to 861 kDa if FAAP24 and FAAP100 were included. Thomashevski et al. [73] report a 600 kDa cytoplasmic complex that increases to a 750 kDa complex during mitosis, supporting the idea of subcomplexes. One study documents such a subcomplex: FANCL-FANCB-FAAP100, which was tandem affinity purified from HeLa cell extracts [29].

Ling et al. [29] also suggest this subcomplex has a stoichiometric ratio of 1 : 1 : 1 in both cytoplasmic and nuclear extracts, with a more prominent association with FANCA in the nucleus. They speculate FANCA along with FANCM may localize the FANCL-FANCB-FAAP100 subcomplex to the nucleus [29]. Medhurst et al. [63] also support the idea of subcomplexes as they reveal from immunoprecipitation experiments of FANCG that FANCA and FANCL are coprecipitated independently of FANCE, FANCC, and FANCF. Studies identifying the localization of individual CC proteins by fluorescence microscopy in cells indicate FANCF and FANCE, which give a joint molecular weight of 101 kDa, are predominantly located in the nucleus, independently of any other CC proteins [27, 45, 47, 62]. FANCEs absence from any cytoplasmic complex described by Thomashevski et al. [73] further supports FANCEs localization in the nucleus. As a full complement of CC proteins is required for the monoubiquitination event, the observations of cytoplasmic subcomplexes and nuclear localization of certain CC proteins prompt the idea of the assembly of the full CC in the nucleus.

The study by Thomashevski et al. [73] supports the idea of the full CC residing in the nucleus, as they report a large nuclear complex of 2 MDa and a 1 MDa chromatin associated complex, both containing CC proteins. As stated above, a CC consisting of one copy of each protein would give an approximate molecular weight of 861 kDa (including FAAP24 and FAAP100). Their reports suggest the nuclear and chromatin complexes may contain multiple copies of CC proteins and indicate the associations of the CC proteins and their stoichiometry differ between the two nuclear and chromatin complexes. Additionally components of these large nuclear complexes are likely to include other nuclear proteins, such as the BLM proteins and MHF1 and MHF2. Meetei et al. [72] report a 1.5–2 MDa complex when immunoprecipitating the BLM complex and found this complex to contain CC proteins. FANCM has since been appointed the CC protein interacting with the BLM proteins, as shown by co-IPs, *in vitro* translational work and fluorescence microscopy [74]. Likewise, Yan et al. [35] report a 1 MDa complex containing CC proteins and the FANCM-associated histone-fold proteins 1 and 2 (MHF1 and MHF2). Elucidating the existence of subcomplexes and a large nuclear CC certainly complicates the understanding of the CC. However, these studies highlight the importance of understanding both the assembly and stoichiometry of the CC and its subcomplexes. Whether there are additional roles for the subcomplexes is not yet understood.

5. FANCM: A Member of the CC?

FANCM is considered a member of the CC, as it co-immunoprecipitates with other CC proteins and the loss of FANCM results in a loss of DNA damaged induced monoubiquitination and nuclear localization of other CC proteins [3, 30, 75, 76]. Indeed FANCM is thought to promote DNA damage-induced monoubiquitination of FANCD2 by recruitment of the CC via FANCF through its MM1 region [74]. Deans and West [74] also show deletions of regions throughout FANCF reduce an interaction with FANCM and deletion of FANCF residues 1–158 completely disrupt this interaction. The C-terminal end of FANCM associates with FAAP24 and both are thought to stabilize one another, [30, 76]. Ciccia et al. [30] also suggest the stability of FANCM-FAAP24 complex may be dependent on FANCB. However, Kim et al. [77] suggest FANCM recruits the CC proteins to chromatin and is not required for a stable CC. The histone-fold proteins MHF1 and MHF2 form another complex with FANCM [78] and are suggested to aid with the remodelling of DNA, as seen by co-IPs, size exclusion chromatography and DNA binding assays [34, 35]. Although the loss of MHF1 and MHF2 results in a loss of FANCD2 DNA damage inducible monoubiquitination, in agreement with Kim et al. [77], Yan et al. [35] report more than 70% of this complex is independent from the CC. A recent structural analysis reveals that MHF1 and MHF2 form a heterotetrameric complex and that disrupting the heterotetrameric interfaces results in an increased sensitivity to DNA damaging agents as seen by methyl methanesulfonate (MMS) treatment sensitivity assays in yeast [78]. A loss of FANCM has shown a loss of DNA damage inducible monoubiquitination of FANCD2 and many groups have suggested its role as a member of the CC; however, evidence is directing its role upstream of the CC suggesting it acts as a platform to recruit proteins to DNA. Additional evidence shows the FANCM-FAAP24 subcomplex has also been associated with interactions of the BLM complex [30, 74]. The role of FANCM in both Bloom syndrome and FA explains the similarities of the BLM and FA patients' high predisposition to cancer. Additionally the FANCM-FAAP24 subcomplex has also been implicated in ataxia telangiectasia and Rad3-related protein (ATR), a protein kinase associated with cell cycle arrest and checkpoint signalling independently from the rest of the CC proteins, through binding HCLK2 [79].

6. Discussion

The FA pathway has rapidly expanded over the last 15 years to a current count of 15 proteins. The number of FA proteins reflects the complicated nature of understanding the FA pathway, particularly the CC, which consists of over half of the FA proteins. Ascertaining functions for the FA proteins have been exceptionally challenging due to the lack of information divulged from the primary amino acid sequences. Extensive efforts have been made by researchers to define the roles of the individual CC proteins within the CC and to understand the need for such a large CC. However, there are still many remaining questions.

(1) How does the CC support FANCLs E3 ligase activity? Is there a requirement for other CC proteins to localize FANCL to the nucleus? Or do the other CC proteins act as a structural scaffold for the monoubiquitination event?

(2) Do the subcomplexes come together to form a full CC? If so how and where does the assembly take place? And are there independent roles for the subcomplexes?

(3) What are the molecular and stoichiometric details of the CC? The requirement for all CC proteins for the monoubiquitination event is clear from patients with defects in the CC. Therefore, understanding the molecular details of the protein interactions that occur in the CC is key, because therapeutics could be designed to restore or diminish these interactions and furthermore tailored to the different FA complementation groups. The combination of biochemical, biophysical, clinical, and cell work will in time answer these questions.

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