Fanconi anemia and RAD50 deficiency: genetic and functional analysis

Dissertation zur Erlangung
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“When we find a reason to live,
the way to do will follow”

(Mahatma Gandhi)

CONTENTS

Preface and thesis outline

Basic research involving human chromosomal instability and cancer susceptibility syndromes is necessary to gain insights into molecular mechanisms which maintain genomic integrity. The focus of this thesis is directed at the molecular, cellular and clinical aspects of two groups of inherited diseases that serve as models for the elucidation of causes and consequences of genetic instability. The genes whose defects cause these diseases are referred to as “caretaker” genes since they appear to function in the recognition and repair of DNA lesions, most notably DNA double strand breaks. Mutational inactivation of these genes results in a sharply increased risk of cancer and contributes to premature aging.

The first of these diseases is Fanconi anemia (FA). Several FA genes have been identified in the last few years, raising questions regarding cellular phenotype, mutation spectra, frequency, function , and genotype-phenotype correlations. At the beginning of this thesis only eight of the currently twelve FA genes had been identified. In close collaboration with the laboratory of Professor Hanenberg (Düsseldorf) we routinely assigned our FA patients to one of the known complementation groups. FA cell lines that could not be assigned to one of the known groups were further classified as defective or proficient in FANCD2 monoubiquitination, a central process in the FA caretaker pathway. This allowed to classify these patient cell lines as upstream or downstream partners within the FA caretaker pathway.

Since defective caretaker genes convey susceptibility to a variety of agents that damage DNA, primary FA fibroblasts belonging to 7 different complementations groups were initially tested for their sensitivity regarding ionizing and UV-irradiation. These results were of special interest in the context of the then newly discovered *FANCD2* gene which turned out to play a central role in the FA/BRCA pathway. As such, the molecular analysis of the *FANCD2* gene represents a major part of this thesis. These studies provided the first comprehensive mutation analysis in the *FANCD2* gene, based on a cohort of 32 FA-D2 patients. I was also able to show that FA-D2 patients, despite their exclusively hypomorphic mutations, are more severely affected than the average FA patient. In order to be activated, FANCD2 must undergo monoubiquitination by the FANCL ubiquitin ligase. It was therefore of great interest to detect the worldwide second only FA patient belonging to complementation group FA-L. As observed with FA-D2 patients, there were no biallelic null-mutations in our FA-L patient, confirming the ontogenetic importance of these evolutionarily conserved genes. FA cell lines who could not be assigned to any of the known complementation groups were subjected to extensive genetic screens in order to detect the corresponding genes. These studies were carried out in cooperation with a number of laboratories in the US, UK, Spain and the Netherlands. As a result of these collaborative projects, I participated in the identification of *FANCJ* and *FANCN* as novel FA genes. *BRIP1/FANCJ* was identified by positional cloning using samples of consanguineous Inuit families under the leadership of Dr. Arleen Auerbach (Rockerfeller University, NY, USA). *FANCN* was found in the lab of Dr. Nazneen Rahman (Sussex, UK) using a candidate gene approach.

In addition to the FA genes, another group of prominent players in the DNA damage response is a group of genes that includes *ATM*, *ATR* and the members of the so-called NMR complex (NBS1/ MRE11/RAD50). Whereas a number of patients with defects in one of the first two members of the NMR complex are known (i.e. Nijmegen breakage syndrome, and ATM-related syndrome) the last part of my thesis work concerns the characterization of the worldwide first patient who was shown to carry biallelic mutations in the RAD50 gene, the third member of the NMR complex. This patient displayed a clinical phenotyp resembling the Nijmegen breakage syndrome (NBS), however without any signs of immunodeficiency. In collaboration with Professor Thilo Dörks group (MHH, Hannover), I carried out complementation analysis, cell cycle and other functional studies in order to characterize the molecular, cellular and phenotypic consequences of human RAD50 deficiency.

The publications reporting the results of my experimental work on the FA and *RAD50* genes are preceeded by copies of two review articles that are meant to introduce the reader to the topic of human caretaker genes, most notably those causing Fanconi anemia. During the time of my thesis, I was witness of a virtual explosion of knowledge concerning the role and function of this group of genes, including their prominent role in the protection against bone marrow failure, leukaemia and solid tumors.

CHAPTER 1

Fanconi anemia: consequences of genetic instability

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Abstract

Fanconi anemia (FA) is a rare recessive disease that reflects the cellular and phenotypic consequences of genetic instability: growth retardation, congenital malformations, bone marrow failure, high risk of neoplasia, and premature aging. At the cellular level, manifestations of genetic instability include chromosomal breakage, cell cycle disturbance, and increased somatic mutation rates. FA cells are exquisitely sensitive towards oxygen and alkylating drugs such as mitomycin C or dieboxybutane, pointing to a function of FA genes in the defense against reactive oxygen species and other DNA damaging agents. FA is caused by biallelic mutations in at least 11 different genes which appear to function in the maintenance of genomic stability. Eight of the FA genes form a nuclear core complex with a catalytic function involving ubiquitination of the central FANCD2 protein. The posttranslational modification of FANCD2 promotes its accumulation in nuclear foci, together with known DNA maintenance proteins such as BRCA1, BRCA2, and the RAD51 recombinase. Biallelic mutations in *BRCA2* cause a severe FA-like phenotype, as do biallelic mutations in *FANCD2*. In fact, only leaky or hypomorphic mutations in this central group of FA genes appear to be compatible with live birth and survival. The newly discovered *FANCJ (=BRIP1)* and *FANCM* (=*Hef*) genes correspond to known DNA-maintenance genes (helicase resp. helicase-associated endonuclease for fork-structured DNA). These genes provide the most convincing evidence to date of a direct involvement of FA genes in DNA repair functions associated with the resolution of DNA crosslinks and stalled replication forks. Even though genetic instability caused by mutational inactivation of the *FANC* genes has detrimental effects for the majority of FA patients, around 20% of patients appear to benefit from genetic instability since genetic instability also increases the chance of somatic reversion of their constitutional mutations. Intragenic crossover, gene conversion, back mutation and compensating mutations in *cis* have all been observed in revertant, and, consequently, mosaic FA-patients, leading to improved bone marrow function. There probably is no other experiment of nature in our species in which causes and consequences of genetic instability, including the role of reactive oxygen species, can be better documented and explored than in Fanconi anemia.

Introduction

In recent years, the maintenance of genome integrity has emerged as a major determinant of organismic longevity and cellular viability [1]. Genetic instability increases the risk of neoplastic transformation and contributes to premature aging [2–4]. The importance of a stable genome for healthy aging is exemplified by numerous genetic instability syndromes which are caused by defective caretaker genes [5–7]. Classical examples are the chromosome breakage syndromes such as Bloom syndrome, Werner syndrome, Ataxia telangiectasia, and Fanconi anemia. Instability of telomeres due to defective telomerase or telomerase-associated dyskerin causes Dyskeratosis congenita, a premature aging syndrome with pigmentation changes, leukoplakia and aplastic anemia [8]. The least specific type of nuclear or chromatin instability is caused by mutations in the lamin A/C gene which encodes the inner lamina of the nuclear membrane. Destabilization of the nuclear membrane by defective lamin A/C leads to global disturbance of gene expression affecting many cell types. The resulting clinical phenotypes are referred as “laminopathies” and include various myopathies, lipodystrophies, neuropathies and, most prominently, the Hutchinson-Gilford juvenile progeria syndrome in which many organ systems and cell types exhibit the detrimental effects of nuclear instability [9]. The present chapter deals with one of the most enigmatic human

chromosome breakage syndromes, Fanconi anemia (FA), a rare recessive disease whose molecular basis has been elucidated in recent years [10–13]. FA serves as a paradigm for the far reaching and mostly detrimental effects of genetic instability, including congenital malformations, neoplasia and premature aging. FA genes appear to be centrally important in maintaining a stable genome. At the same time, FA serves as an example of rare beneficial effects of genetic instability, i.e. increased likelihood of somatic reversion. Such self-corrections of disease causing mutations ("natural gene therapy") have been described in only a handful of human diseases [14]. FA is a prime example of the different types of molecular events that lead to the restoration of heterozygosity, and thereby normal function, of the self-corrected cell types in individuals constitutionally homozygous or compound heterozygous for disease causing gene mutations.

The Fanconi anemia phenotype

Fanconi anemia (FA) is a chromosome instability disorder characterized by diverse clinical features including developmental abnormalities, progressive bone marrow failure and increased risk of neoplasia [15] (see also Table 1). The first case report was published in 1927 by the Swiss paediatrician Guido Fanconi. He described a hereditary form of progressive aplastic anemia in three children with specific somatic abnormalities. Since then, more than 1300 FA patients belonging to all ethnic groups have been diagnosed [16]. It is estimated that 1 in 200.000 people are affected (i.e. homozygotes or compound heterozygotes), and the carrier (heterozygote) frequency is estimated at 1 in 300 [17]. Higher frequencies of patients reflecting carrier frequencies >1% are encountered in certain populations due to founder and inbreeding effects [17–19].

Average life expectancy of FA patients is around 20 years, and patients reaching the age of 50 are exceedingly rare [20]. 70% of the patients show developmental defects including short stature, radial ray defects, pigmentary changes and urogenital malformations [21]. Endocrine abnormalities such as hyperinsulinemia, growth hormone insufficiency, and hypothyroidism occur in more than 80% of classical FA patients [22]. Bone marrow failure and high risk of myelodysplastic syndrome, myelogenous leukemia and squamous cell carcinomas account for the reduced life expectancy [23]. The classical clinical course is marked by childhood onset of bone marrow failure which first manifests as low platelet counts, complicated by transfusion-dependent anemia and irreversible

Table 1. Phenotypic features of Fanconi anemia

Clinical phenotype	Cellular phenotype
1) Developmental Abnormalities • pre- and postnatal growth retardation • microcephaly • microphtalmia • skeletal malformations (radial ray • defects) • malformation of inner organs (kidney and heart) and auditory system • hypogonadism and reduced fertility • hyperpigmentation, hypopigmentation, café au le spots) **2) Progressive bone marrow failure** **3) Cancer predisposition (AML, squamous cell carcinomas)**	**1) Spontanous chromosomal instability** **2) Spontanous arrest in the G2 phase of the cell cycle** **3) Hypersensitivity against DNA crosslinking agents such as mitomycin C, diepoxybutane, cisplatin, psoralen and nitrogen mustard** **4) Hypersensitivity against oxygen**

pancytopenia in the first two decades of life. However, clinical course and clinical phenotype are highly variable both within and between families. Revertant mosaicism may account for some of this variability [24, 25], and "mild" mutations may account for a number of patients that are diagnosed incidentally or as adults when they present with MDS, AML or squamous cell carcinomas [26]. Treatment is mostly supportive and includes blood product substitution and stimulation of bone marrow cell growth by androgens, hydrocortisone and cytokines; the only curative treatment is bone marrow transplantation, but a high proportion of successfully transplanted patients develop solid cancers later in life [27].

The FA cellular phenotype

The FA cellular phenotype reflects the underlying genetic instability which can be assessed by a variety of assays. The most frequently applied tests are listed in Table 2. Under ambient-air cell culture conditions FA cells show elevated spontaneous chromosomal breakage rates with a predominance of chromatid-type lesions [28] which probably reflect the innate hypersensitivity of FA cells towards oxygen [29]. Glycophorin assays provide direct proof of ongoing and increased somatic mutation [30]. Another hallmark feature of FA cells is their hypersensitivity towards bifunctional alkylating and crosslinking agents such as mitomycin C (MMC), diepoxybutane (DEB) or nitrogen mustard (NM) [31, 32]. In response to these agents FA cells show elevated rates of chromatid breaks and chromatid exchanges (cf. Fig. 1a). As a consequence of damaged and/or misrepaired DNA, FA cells accumulate with 4N DNA content at the S/G_2 phase border of the cell cycle [33–36]. The characteristic cell cycle disturbance can be used as a diagnostic test ([37]; Fig. 1b).

Table 2. Laboratory tests for the confirmation of FA

Test System	Procedure	Advantage/Disadvantage
Chromosome breakage test	72 h blood lymphocyte culture in the presence of DEB, MMC, or NM	high sensitivity; reliable detection of mosaicism, but experience required and labor intensive
Cell cycle test	single or dual parameter flowcytometry; measures G2-phase accumulations	fast and accurate screening test, but detection of mosaicism is difficult
FANCD2 (posttranslational modification)	immunoblot using antibodies detects FANCD2-S (native) and monoubiquitinated isoform (FANCD2-L)	identifies patients with defects in FANCD2 and in FA genes necessary for FANCD2 monoubiquitination (FA core complex genes); requires cycling cells; difficult with fibroblasts
DNA-test (genomic DNA)	prescreening with DHPLC, exon scanning sequencing;	works well with FA genes except FANCD2 (pseudogenes) and FANCA (frequent large deletions; detection requires quantitative techniques)
DNA-test (cDNA)	cDNA-sequencing of RT-PCR products	detects multiple types of mutations; requires high quality mRNA, detects expressed genes only; misses large deletions
Glycophorin-test	analysis of somatic mutation frequency using RBC	provides evidence for ongoing mutation; requires heterozygosity for the MN blood group

Unlike any other genetic instability syndrome, FA cells are hypersensitive towards oxygen [29] and grow poorly in conventional cell culture incubators with ambient oxygen tension. However, *in vitro* growth and cloning of FA cells can be restored to near normal under hypoxic cell culture conditions [38]. In contrast to ATM, FA-cells are not markedly hypersensitive towards ionizing radiation under (physiological) low-oxygen conditions, nor do they show consistent hypersensitivity towards UV irradiation [39]. This may be different when FA cells are exposed to ambient oxygen tension and assessed via the single cell electrophoresis (comet) assay [40]. The unique combination of cellular resistance and cellular sensitivities sets FA apart from the other genetic instability syndromes. Hypersensitivity to crosslinking agents and oxygen points towards an important role

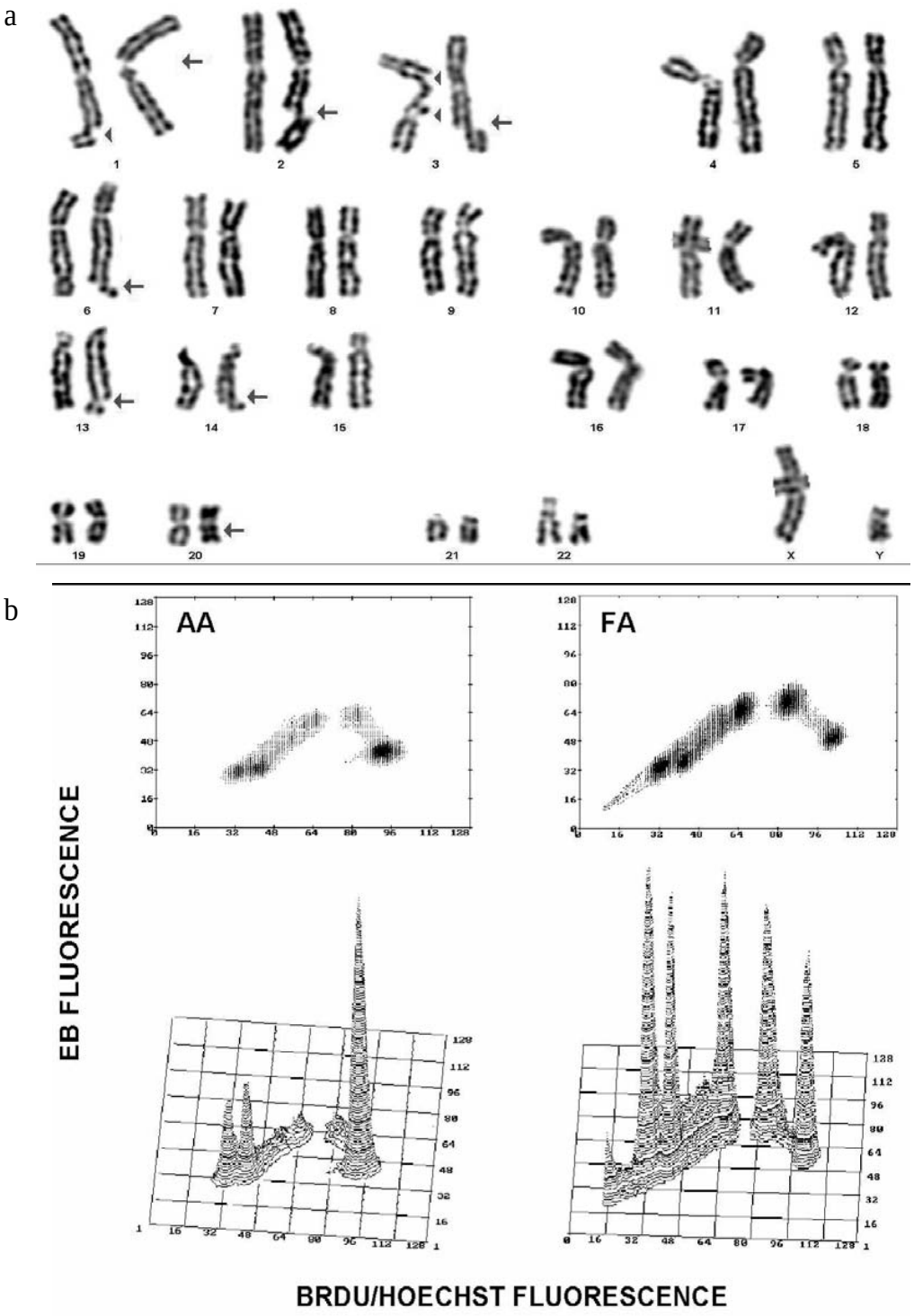

Fig. 1. (a) Chromatid-type chromosome lesions (arrows/arrowheads) as cytogenetic hallmarks of spontaneous and induced chromosome fragility in Fanconi anemia (FA). Blood lymphocyte karyotype stained with Giemsa. (b) Cell cycle alterations as flowcytometric hallmarks of Fanconi anemia. The upper panels show bivariate flowcytograms of 72-h peripheral blood mononuclear blood cell cultures grown in the presence of a lectin mitogen (PHA) and the base analog bromodeoxyuridine (BrdU). Double staining with ethidium bromide (X-axis) and the Hoechst 33258 dye (Y-axis) resolves the distribution of cells throughout the G1, S and G2/M phases of three consecutive cell cycles. In contrast to non-genetic aplastic anemia (AA), cells from patients with Fanconi anemia (FA) accumulate in the G2-phases of consecutive cell cycles. The three-dimensional representation of the bivariate cytogram data (bottom panels) emphasizes the cell cycle differences between the non-genetic and the genetic form of aplastic anemia. For details of the cell cycle testing see [37].

of FA genes in the defence of nuclear stability and integrity. Together with genes such as *XP, ATM, NBS1, RAD50, MRE11, BRCA1, BRCA2* and *p53*, the FA family of genes has been appropriately included in the category of "caretaker" or "guardian of the genome" genes [10].

Genetic heterogeneity as a hallmark of Fanconi anemia

So far, 12 complementation groups (FA-A, B, C, D1, D2, E, F, G, I, J, L and M) have been defined via somatic cell fusion [41] and 11 of the corresponding genes (denoted as *FANC-A, B,*

Table 3. Fanconi anemia (FA) complementation groups and genes

Complementation Group	Frequency in FA *	Gene nomenclature	Exons	Protein size [kDa]	mono-Ub of FANCD2
FA-A	**66**	***FANCA***	**43**	**163**	**no**
FA-B	**1**	***FANCB/ FAAP95***	**10**	**95**	**no**
FA-C	**10**	***FANCC***	**14**	**63**	**no**
FA-D1	**3**	***BRCA2/ FANCD1***	**27**	**384**	**yes**
FA-D2	**3**	***FANCD2***	**44**	**155 (162)**	**-**
FA-E	**2**	***FANCE***	**10**	**58**	**no**
FA-F	**2**	***FANCF***	**1**	**42**	**no**
FA-G	**9**	***FANCG***	**14**	**68**	**no**
FA-I	**2**	**-**	**-**	**-**	**no**
FA-J	**2**	***FANCJ/ BRIP1/ BACH1***	**20**	**140**	**yes**
FA-L	**<1**	***FANCL***	**14**	**43**	**no**
FA-M	**<1**	***FANCM***	**23**	**230**	**no**

* Relative prevalence of FA complementation groups according to Levitus et al. [41].

C, D1, D2, E, F, G, J, L, M) have been identified (Table 3; [42–46]). Expression cloning led to the identification of FANC-A, C, E, F, and G, whereas a positional cloning strategy was used for the isolation of *FANCA*, *-D2*, and *-J*. Three FA-genes (*FANCB*, *-L* and *-M*) have been defined via biochemical isolation of proteins, and the *FANCD1* gene was found via a classical candidate gene approach. Interestingly, both the *FANCJ* and *FANCM* genes were first suggested as genuine FA genes

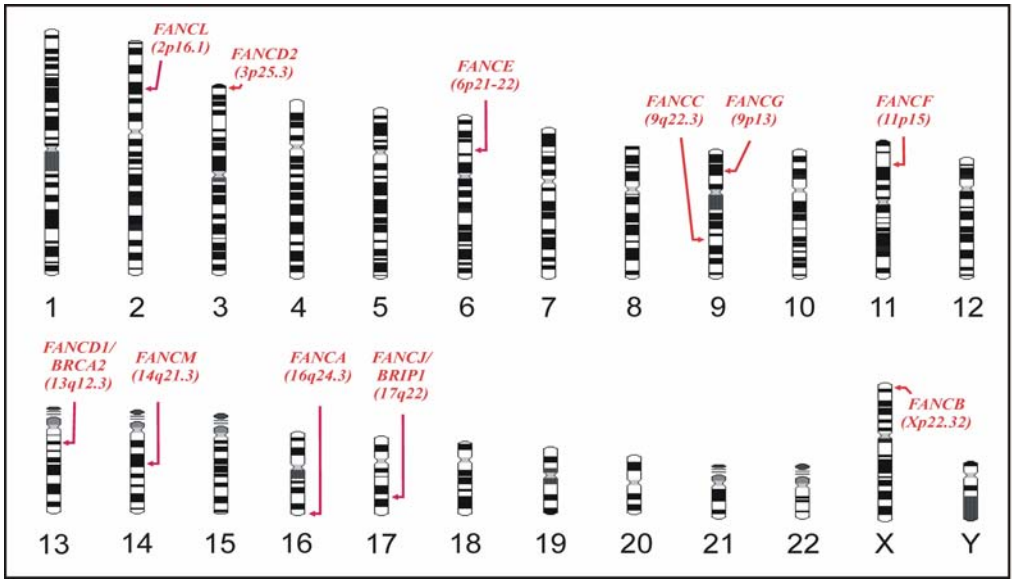

Fig. 2. Human karyotype map with locations of the 11 Fanconi anemia genes known to date. FA-genes are denoted with the prefix "FANC" and alphabetical letters. Note that there are two types of *FANCD* genes: *FANCD1* (corresponding to *BRCA2*) and *FANCD2*. Chromosome 9 is the only chromosome harboring two FA-genes (*FANCG* and *FANCC*), and *FANCB* is the only X-chromosomal FA-gene.

by the MMC sensitivity of their mutant orthologs in the chicken DT40 cell line [46, 47]. FANCB is the only X-linked FA gene, all others are located on autosomal chromosomes (Fig. 2). Five FA genes were found to correspond to previously identified genes: *FANCG* to *XRCC9, FANCD1* to *BRCA2, FANCL* to *PHF9, FANCJ* to *BRIP1/BACH1,* and *FANCM* to *Hef*. This identity strongly supports the caretaker role of the FA genes, since XRCC9, BRCA2, BRIP1 and Hef are known to be involved in DNA-maintenance pathways. Biallelic mutations in *BRCA2/FANCD1* cause an unusually severe type of disease with early childhood leukemia and solid tumors preceding onset of bone marrow failure. Most of these patients will never be diagnosed as FA since they succumb to their malignancies prior to onset of pancytopenia. Some authors therefore consider patients with biallelic mutations in *FANCD1/BRCA2* "FA-like" rather than genuine FA [48, 49]. *Brca2* knock-out mice are embryonic lethals, and it appears that only hypomorphic mutations in *FANCD1/BRCA2* are compatible with survival in humans [50, 51].

Complementation studies using somatic cell fusion or retroviral transduction have assigned around 60% of FA patients to complementation group A, 8–10% each to groups C and G, and 6% to group D2. The other complementation groups account for less than 2% of the patients (cf. Table 3; [41, 52, 53]). With the exception of *FANCD1/BRCA2, FANCD2, FANCL, FANCJ/BRIP1* and *FANCM/Hef*, the other FA genes have arisen rather recently in vertebrate evolution [10]. *FANCL* was shown to evolutionarily co-exist with *FANCD2* in several species, and it is the only FA gene with proven enzymatic (ubiquitin E3 ligase) activity [54]. The FANCL protein is part of the FA core complex and contains three WD40 repeats known as sites of protein protein interaction. At the C terminus FANCL has a PHD zinc finger motif with presumptive E3 ligase activity. Meetei et al. demonstrated that FANCL is able to ubiquitinate itself *in vitro* and that lack of FANCL results in defective FANCD2 ubiquitination [55]. The crucial role of FANCL in the FA/BRCA-pathway is highlighted by the fact that there are only two FA-L patients known to date, both with hypomorphic mutations (chapter 4). Because of the lack of evolutionary homologies among the other FA genes, and because of a notable absence of signature motifs at the DNA and protein levels, it remains unclear whether, in addition to their role in the FA core complex (see below), the FA genes serve other specific functions. However, some of the FA genes are highly expressed in spermatogenesis and oogenesis [56], and the location of *FANCC* and *FANCG* on human chromosome 9 within regions that are orthologous to the avian Z chromosome implies a meiotic function (Nanda et al, unpublished observations, 2005). An important role of FA genes in gametogenesis is further suggested by impaired fertility of male FA patients, failure of spermatogenesis in FA knock-out mice [57] and physical association of some of the FA proteins with the synaptonemal complex [58]. A plethora of putative special functions have been ascribed to the *FANCC* gene, including interaction with NADPH cytochrome P450 reductase, maintenance of a DNA damage-induced G_2 checkpoint [59], and regulation of genes involved in differentiation and inflammation [60]. Most importantly, it is now firmly established that the proteins encoded by at least 9 of the FA genes participate in the formation of a nuclear protein complex (*FA core complex*) which is assembled in response to DNA damage [61, 62].

Molecular function of the FA family of genes

Since the discovery of the first FA gene (*FANCC)* in 1992 it took nearly 10 years until the identification of the *FANCD2* gene provided a functional link between the FA- and the BRCA caretaker pathways [58, 63]. In response to DNA damage, eight of the known (FANC-A, B, C, E, F, G, L, and M), and a single as yet unidentified FA protein (FAAP 100) assemble into a nuclear complex (*FA core complex*) whose central function appears to be the monoubiquitination of FANCD2 at amino acid residue K561 [55, 64, 65]. Complex formation begins with FANCA and FANCG multimerization, particularly in response to oxidative stress [66]. In addition, both FANCG and FANCF appear to play a key role in the assembly process. The FANCL protein has ubiquitin ligase activity and is thought to act as catalytic subunit of the FA core complex. Monoubiquination

of FANCD2 can be used as a functional test of the integrity of the FA core complex proteins [67]. As shown in Fig. 3, a defect in one of the core complex genes causes failure of monoubiquination of FANCD2 such that immunoblotting detects only the smaller of the FANCD2 isoforms (S band).

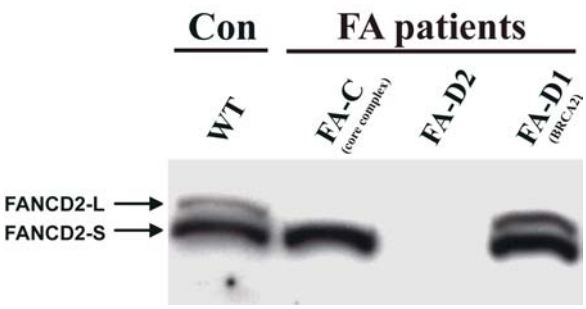

Fig. 3. Westernblots showing examples of unmodified (FANCD2-S) and monoubiquitinated (FANCD2-L) isoforms of the human FA-D2 protein. From left to right: following mitomycin C induction, healthy donors (WT) show both the S and the L band, indicating proficient function of the FA core complex proteins. In contrast, defective core complex proteins (e.g. FA-C) are unable to ubiquitinate FANCD2 (second lane). Immunoblotting detects little or no FANCD2 protein in patients carrying biallelic mutations in the *FANCD2* gene (third lane), whereas patients with defects in FA-genes downstream of *FANCD2* (such as *FANCD1/BRCA2*) display both the L and the S band owing to normal function of the core complex proteins (lane four).

Monoubiquitination of FANCD2 reflects functionally intact core complex genes and yields the larger isoform (FANCD2-L). The larger isoform FANCD2-L (corresponding to an activated FANCD2 protein) is targeted to nuclear foci containing BRCA1, BRCA2/FANCD1 and RAD51 known to be involved in recombinational DNA repair [13, 68–70]. Figure 4 illustrates the current view of how FA proteins react in response to DNA damage, emphasizing the role of the FA-D2 protein.

FANCD2 as a central effector of the FA/BRCA network

Several lines of evidence support a key role of FANCD2 in the FA/BRCA caretaker network. First, FANCD2 interacts with members of the FA core complex and with BRCA2, a known player of homology directed DNA repair. Second, as already mentioned, next to *FANCL, J* and *M*, *FANCD2* is a highly conserved FA gene with orthologs in organisms like *Arabidopsis thalia*, *Caenorhabditis elegans*, *drosophila melanogaster* and the zebrafish [63, 71, 72]. *Fancd2*-deficient zebrafish embryos exhibit features reminiscent of the human FA phenotype, including shortened body length, microcephaly and microphthalmia. These observations emphasize the essential role of an intact FANCD2 gene during embryogenesis [72]. Third, the activated form of FANCD2 (FANCD2-L) is targeted to sites of DNA-damage and colocalizes with BRCA1, BRCA2, RAD51, PCNA, ATR and probably others. FANCD2-L directly associates with chromatin at the site of DNA repair and is deubiquitinated by USP1 [73]. Depletion of the *Fancd2* ortholog in *C. elegans* causes sensitivity to crosslinking agents and drop in progeny survival, implying an essential role in the DNA damage response [74]. Fourth, there are no FA-D2 patients known to date who carry biallelic null mutations which indicates that only hypomorphic mutations in *FANCD2* are compatible with survival [75]. This observation renders the importance of *FANCD2* equivalent to that of *FANCD1/BRCA2* for which biallelic null mutations appear to be lethal in humans [50]. Like FANCD2, FANCD1/BRCA2 is involved in the stabilization of replication forks [76], and both FANCD1/BRCA2 and FANCD2 are DNA-binding proteins with a structure specific affinity to branch points and free DNA ends [77].

In addition to FANCD1 and FANCD2, the recently discovered DEAH-box containing DNA helicase (= FANCJ) and the helicase-associated endonuclease for fork-structured DNA Hef (= FANCM) appear to interact directly with DNA [45]. In contrast to most of the other FA core complex genes, there is compelling evidence for the direct involvement of these newly discovered FA genes in DNA-repair functions: like the genes underlying Werner, Bloom, Rothmund-Thomson and Rapadilino genetic instability syndromes, FANCJ/BRIP1 is a member of the RecQ DEAH helicase family that unwind DNA in 5′ > 3′ direction. The highly conserved RecQ-type helicases

are capable of unwinding Holliday junctions formed during homologous replication or during the resolution of stalled replication forks [44]. Likewise, FANCM/Hef has sequence similiarities to known human DNA repair proteins such as ERCC4 and XPF, binds directly to intermediates of DNA replication, and may act as an engine that translocates the FA core complex along DNA [45]. Together with FANCD1/BRCA2 and FANCD2, the discovery of FANCJ/BRIP1 and FANCM/Hef provides the strongest evidence yet for a direct involvement of FA proteins in DNA repair functions associated with the resolution of DNA crosslinks and stalled replication forks.

Monoubiquitination is not the only modification sustained by the FANCD2 protein in response to DNA damage (Fig. 4). FANCD2 is also one of the many targets of phosphorylation of the DNA damage sensors ATM and ATR [78, 79]. Phosphorylation of FANCD2 at Ser222 by ATM (cf. Fig. 4) is necessary for the activation of the intra-S phase checkpoint [79, 80]. ATR mediated phosphorylation of FANCD2 in response to crosslinks or stalled replication forks depends on the intact FA core complex and the NBS1 protein [79]. These observations suggest that FANCD2 maintains genomic stability by the avoidance of cell cycle progression in situations of DNA replication stress due

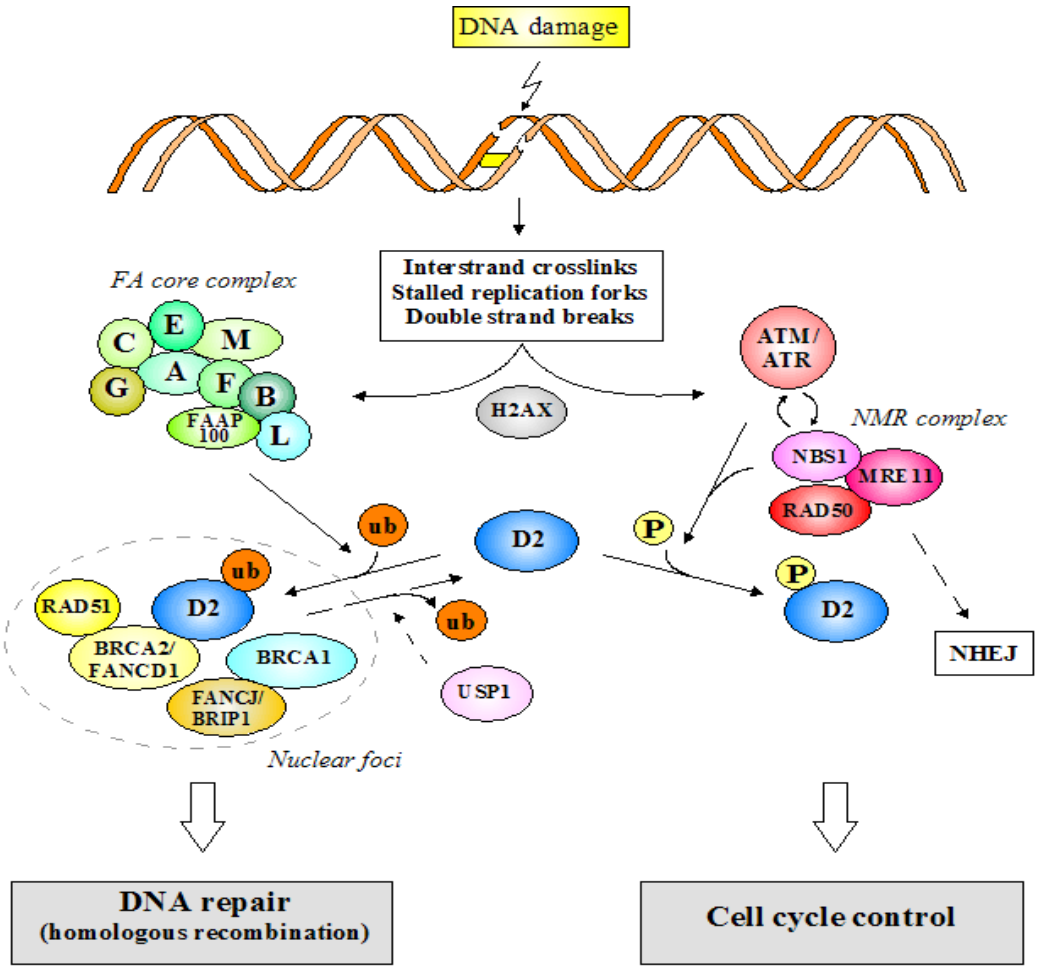

Fig. 4. Central role of the FANCD2 (D2) protein in the FA/BRCA network of genes. In response to DNA-interstrand crosslinks, stalled replication forks and/or double strand breaks, the FA gene products form a nuclear complex consisting of at least 8 separate FA proteins. The FANCL protein acts as the putative catalytic subunit of the core complex that monoubiquinates the FANCD2 protein at K561. The ubiquinated L-form of FANCD2 colocalizes in nuclear foci together with the products of the BRCA1, FANCJ/BACH1, BRCA2/FANCD1 and RAD51 genes that are known to contribute to homology directed DNA repair. Also shown is an alternative way of modification and activation of the FANCD2 via phosphorylation by the DNA damage sensors ATM/ATR working together with the NBS1/MRE11/RAD50 protein complex. Phosphorylation of FANCD2 at S222 presumably leads to activation of an intra-S phase cell cycle checkpoint.

to damaged DNA and/or stalled replication forks [81]. Additionally, via the BRCA2 connection, FANCD2 is directly involved in recombinational DNA repair [82, 13]. A number of recent studies use the FA-deficient chicken cell line DT40 to investigate the role of FA genes in crosslink repair (e.g. [46, 47, 70, 83]. An important result of these studies is that the FA genes, in concert with other caretakers such as BLM, appear to promote homologous recombination, translesion synthesis and mutational repair of endogenously generated abasic sites. However, as with all non-human and transformed cell lines, caution is warranted in extrapolating data obtained from transformed cell lines. A case in point is the very popular, p53 deficient DT40 chicken cell line which, if Fancd2 deficient, shows a twofold elevation of spontaneous SCEs [83] which is not seen in primary human FANCD2 cells.

The FA-cancer connection

Severe anemia and pancytopenia due to bone marrow failure are life threatening clinical features of FA. Additionally, the disease is associated with a high risk of neoplasia, most frequently acute myeloid leukaemia (AML) and squamous cell carcinoma, but many other types of neoplasia have been described [16, 21]. Inactivation of the FA/BRCA pathway might be an early step in developing sporadic malignancies, especially in cancer types that occur frequently in Fanconi anemia. Acquired dysfunction (deletion or reduced expression) of the *FANCA* gene has been described in patients with acute myelogenous leukemia [84, 85], and mutations of *FANCC* and *FANCG* have been found in a subset of pancreatic cancers [86]. Even though germline and somatic mutations in *FANCC* and *FANCG* may contribute to the occurrence of pancreatic cancer, the cancers with mutations in these FA genes arise in an apparent sporadic fashion rather as familiar cancer types. *FANCC* and *FANCG* mutations may therefore have a relatively low penetrance for the pancreatic cancer phenotype [87]. The most consistent dysfunction of FA genes found in a variety of human neoplasias involves the inactivation of the *FANCF* gene due to methylation of its CpG islands. Hypermethylation of *FANCF* has been observed in several kinds of tumors, including cervical cancer [88], ovarian cancer [89, 90], AML [85], head and neck squamous cell carcinomas (HNSCC), non-small-cell lung cancers (NSCLC) [91], and a bladder carcinoma cell line [92]. The interpretation of these findings as to their causal links to tumor initiation remains tenous, since *FANCF* is located on chromosome 11p15 within a hot-spot region for hypermethylation. It remains to be seen whether silencing of *FANCF* expression is a specific process or reflects the general changes in methylation pattern observed in many types of cancers and leukemias. Given the likely importance of the FA/BRCA pathway in the maintenance of genomic stability, it is rather surprising that apart from the above mentioned sporadic cases there appears to be no widespread involvement of the FA genes in tumorigenesis. This may indicate that functional FA genes are essential for the formation and survival of malignant cells.

Fanconi anemia proteins: players within networks

Even though the very existence of the FA core complex, its modification of FANCD2, and the subsequent formation of nuclear foci suggests a linear biological pathway leading from DNA damage to DNA repair, recent evidence favors the view that the FA proteins are part of an interchangeable network of multiple proteins involved in DNA replication, DNA recombination, and DNA repair [13, 93, 94]. For example, yeast two hybrid studies provide evidence for a consistent interaction between FA proteins and a number of proteins known to be involved in chromatin assembly and chromatin remodeling [95–97]. Changes in chromatin structure must precede any repair process in order to permit repair proteins to gain access to damaged DNA. Multisubunit protein complexes with partially overlapping and shared functions are required for the disruption and reformation of nucleosomal arrays during transcription, replication and repair [98]. Wild-type FANCC protein and the interacting protein FAZF have been shown to colocalize in nuclear repair foci and may be involved in transcriptional repression via chromatin remodeling [95]. Biochemical fractionation and

immunocytochemical studies provide convincing evidence for a close physical association between FA proteins, nuclear matrix and interphase chromatin [65, 99, 100]. This association is enhanced by exposure to MMC but is abolished during the metaphase stage [101].

As shown in Fig. 5, the FA family of genes can be placed in the center of a network of genome maintenance genes [102] with close interconnections to the DNA damage sensors *ATM/ATR*, the *NMR* complex genes, the Werner and Bloom helicases and the *BRCA1* and *BRCA2* tumor suppressor genes [7, 103]. Moreover, interactions with Trp53 have been observed both in murine knock-out and human FA-cell models [104–106]. As indicated by the shaded sector, biallelic mutations in *FANCD1/BRCA2* and *FANCJ/BRIP1* (a BRCA1 interacting protein) cause FA-like phenotypes which emphasizes the close relationship of these (downstream) genes with the FA core complex members. Since a number of FA patients have not been assigned to any of the known FA genes, it would come as no surprise if some of these unclassified FA-patients would turn out to carry biallelic mutations in *BRCA1* itself, or in any of the numerous BRCA1 interacting proteins [107]. In the context of the striking oxygen hypersensitivity phenotype of FA cells, it might be relevant that BRCA1 has been shown to induce antioxidant gene expression and resistance to oxidative stress [108]. Moreover, both BRCA1 and BRCA2 have been reported as instrumental in transcription coupled repair of oxidative DNA-lesions in human cells, even though this needs to be confirmed by additional studies [109].

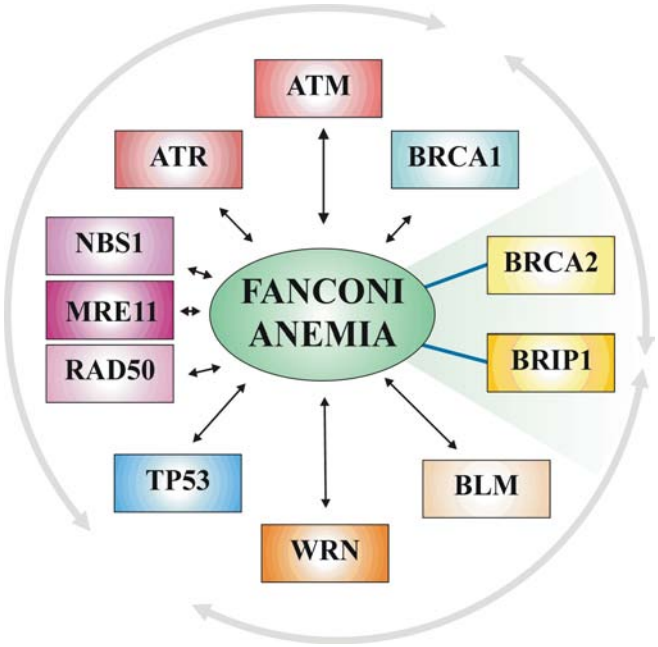

Fig. 5. Hypothetical concept of Fanconi anemia genes participating in a multifunctional network of caretaker genes, including the DNA-damage sensors *ATM*, *ATR*, *NBS1*, *MRE11*, *RAD50* and *TP53*, the DNA repair-helicases *WRN*, *BLM* and *BRIP1/BACH1*, and the cancer genes *BRCA1* and *BRCA2* involved in homology-directed DNA repair. The shaded sector emphasizes that biallelic mutations in *BRCA2* and *BRIP1/BACH1* cause a FA-like phenotype. Modified after Surralles et al. [7].

Genetic instability and oxidative stress: a causal connection?

One can hardly avoid the conclusion that FA cells lack sufficient protection against the damaging effects of reactive oxygen species [110–112]. Pagano and coworkers make a persuasive case that many of the clinical and cellular abnormalities observed in FA, including congenital malformations, pigmentation changes, bone marrow failure and insulin resistance, may result from macromolecular damage caused by elevated levels of oxidative stress (cf. Table 4). As early as 1981, Joenje and coworkers detected a striking correlation between rates of chromosome breakage and oxygen tension in FA cells [29]. A subsequent study showed that growth and cloning efficiency of FA fibroblasts is severely impaired under ambient oxygen (20% v/v), but turns to near normal under hypoxic (5% v/v) cell culture conditions [38]. Saito and coworkers confirmed these observations and concluded that hypersensitivity towards oxygen is a “uniform but secondary defect” in FA-cells which might, however, contribute to bone marrow failure [113]. At least in the murine *Fancc* knock-out model there is evidence for enhanced oxidant-mediated apoptosis of FA hematopoietic stem or progenitor cells [114], and repeated cycles of hypoxia-reoxygenation induce premature senescence in FA hematopoietic cells [115]. Testing the cell cycle behavior of FA-lymphoid cell lines under various oxygen tensions, Poot et al. suggested

that oxygen sensitivity in FA cells might be mediated by the amount of iron in the culture media, implicating a reduced capacity of FA cells to deal with (reactive oxygen) products of Fenton-type reactions [116]. Evidence for mutagenic activity of oxygen in FA cells was provided by passaging indicator plasmids through FA cells grown at different oxygen tensions [117]. The proponents of the "oxidative stress theory in FA" believe that oxidative stress characterizes the metabolic situation of several types of patient blood cells, both *in vitro* and *in vivo* [111, 118]. Overexpression of the antioxidant thioredoxin apparently mitigates the clastogenic effects of MMC or DEB in FA fibroblasts [119]. A similar effect has previously been reported with DL-alpha-tocopherol (a potent lipophilic antioxidant) [120]. The hypersensitivity of FA cells towards MMC or DEB may be caused by the property of these agents (that need to be metabolically activated) to generate oxygen radicals under *in vitro* conditions [121, 122]. Several studies have implicated the *FANCC* gene in antioxidant defence functions [111, 123], and specifically in the repair of oxidatively damaged DNA [124]. A direct role of FANCC in the protection against oxidative damage is supported by a murine model in which both the genes for Cu/Zn superoxide dismutase and *Fancc* have been inactivated [125]. In addition to liver abnormalities, and in contrast to single FA gene knock-outs, these mice exhibit the FA-phenotype of impaired hematopoiesis as if inactivation of Fancc in the absence of the scavenger SOD would lead to bone marrow failure, possibly due to the overwhelming effects of endogenously generated reactive oxygen species.

If FA genes play a major role in the defense against endogenous oxidative damage, one should expect to find increased baseline levels of oxygen-related DNA base modifications such as 8-OHdG in FA patients. However, such evidence is lacking [126, 127] or controversial [128, 129]. What remains unclear at this point is whether the *in vitro* hypersensitivity toward oxygen causes greater accumulations of DNA lesions in FA as opposed to non-FA cells, or whether the removal of oxidative lesions is less effective or somehow impaired in cells which carry mutations in one of the FA genes. There is compelling evidence, however, that oxidative stress and/or oxidative damage activates the FA/BRCA pathway by multimerization and interaction of FANCA, FANCG and FANCC, transport into the nucleus, and FA core complex formation [68]. An attractive hypothesis posits that FA proteins act as sensors of the cellular redox status and translate this information to other members of the caretaker gene network [13]. It is quite obvious that warm-blooded and long-lived mammals that generate high levels of reactive oxygen species during normal cellular functions (i.e. oxidative phosphorylation) require special protection against the "time bomb" within [130]. Although oxidized bases, AP sites and DNA single-strand breaks are removed by DNA excision repair [131], the evolutionarily recent family of FA proteins might serve the purpose of removal, via

Table 4. The FA-oxygen sensitivity connection

O X Y G E N S E N S I T I V I T Y	
Oxidative stress parameters:	**Function of FA proteins in redox-related pathways:**
↑ *8-OHdG* *Luminol-dependent chemiluminescence* *SOD-sensitive clastogenic factor* *TNFα*	***A) FANCC:*** ↑ *Gluthathione S-transferase (GSTP1)* ↓ *NADPH cytochrome P-450 reductase electron transport in microsomal membrane* *Transcriptional regulation of NFκB, COX2, HSP70*
↓ *Catalase activity* *Thioredoxin expression* *Cell growth in 0_2 rich atmosphere*	***B) FANCG:*** ↑ *CYP2E1* *8-OHdG after H_20_2- or MMC exposure*
Oxydative stress-related cytoskeleton abnormalities *Iron-sensitive G_2 arrest*	

recombinational repair, of double strand breaks as the most deleterious end-products of oxidative DNA damage. With defective FA genes cells suffer the consequences of oxidative stress without sufficient protection, manifesting as chromosomal instability and early neoplasia.

Somatic reversion as a (positive) consequence of genetic instability

In recessive diseases, somatic reversion of one of the two inherited mutations restores heterozygosity in the descendants of the reverted cell [14, 132]. Depending on the mechanism of somatic reversion, the function of the affected cell lineage may be partly or completely restored. Complete restoration of a cellular phenotype to wildtype usually results from mechanisms such as intragenic recombination, back-mutation (reverse point mutation), or gene conversion [14, 133]. Partial restoration of protein function has been observed with so-called compensating or second site mutations [134]. Such second site mutations in *cis* leave the constitutional mutation unchanged but alter the downstream DNA sequence via insertion, deletion or point mutation. As a consequence of the compensating mutation a protein with at least partial function is produced [134].

Revertant mosaicism is a rather frequent phenomenon in the autosomal recessive genetic instability syndromes, particularly Bloom syndrome and Fanconi anemia [24, 25, 133, 135, 136]. In FA, intragenic recombination, gene conversion and compensatory second site mutations have been reported in lymphoblastoid cell lines of four FA-C patients, and cytogenetic evidence for mosaicism in additional five unclassified patients [24, 134]. Five compound heterozygous FA-A patients have been reported to date who developed mosaicism as a consequence of a putative back mutation or gene conversion [25, 133]. In addition to FA-C and FA-A patients, four FA-D2 patients were reported as mosaics [137], proving that somatic reversion also affects the central *FANCD2* gene.

Mechanisms of somatic reversion observed in FA patients

Two FA-A patients with homozyogous mutations were shown to harbor compensatory mutations in their peripheral blood mononuclear cells [134]. An unusual compensatory mutation involving the loss of the natural splice acceptor of exon 7 and the use of the next AG for splicing leading to restoration of the open reading frame was found in a heterozygous FANCL patient (Kalb et al, manuscript in preparation) In addition, the case history of a patient reported by Gross et al. demonstrates that compensatory second site mutations can also arise during the *in vitro* cultivation of lymphoblastoid cells, explaining the conversion of these cells from MMC-sensitivity to MMC-resistance [25]. Intragenic crossover represents a reversion mechanism that requires compound heterozygosity with mutually distant locations of the paternal and the maternal mutations. It has been described as the predominant mechanism of reversion in Bloom syndrome [136] and in the lymphoblastoid cell line of occasional FA-C patients [24]. Gene conversion or back mutation was postulated as the mechanism of reversion in four FA-A compound heterozygous patients described by Gross et al [25]. In each of these patients, reversion led to the restoration of precisely the wildtype sequence. How can this apparent non-randomness be reconciled with the stochastic nature of mutations? One obvious explanation would be that selection creates a proliferative advantage for cells with complete rather than only partial restoration of protein function. In addition, there might be constraints imposed by DNA-structure which would favor the restoration of the original sequence. Back mutation combined with selection therefore might explain the surprisingly uniform pattern of reversion in the four reported mosaic FA-A patients. Another possibility of course would be gene conversion. Gene conversion requires an intact DNA-strand opposite to the mutation, a condition which is fulfilled during the postreplicative phase of the cell cycle when sister chromatids are paired. However, gene conversion through sister chromatid pairing does not work with constitutional mutations since both sister chromatids carry the respective mutation. In this situation, gene conversion requires some sort of somatic pairing between homologous chromosomes. Since the *FANCA* gene is located on human chromosome 16 that harbors a large block of heterochromatin known to promote somatic pairing [138], gene conversion via somatic pairing might occur in FA-

A patients who are compound heterozygotes for point mutations or small deletions. It would not, however, work in cases where the second allele involves a large deletion.

The clinical course of FA is highly variable and may be determined in part by complementation group and mutation type [139, 140]. Whether revertant mosaicism leads to clinical improvement depends on when and where the reversion occurred during evolution of the various bone marrow cell lineages [24, 133]. The best prospects for clinical improvement probably exist if the reversion takes place in a hematopoietic stem cell as evidenced by reversal of the FA-phenotype in all descendant blood cell lineages [133, 141]. Regarding the prospects of future gene therapy, the evidence for *in vivo* selective advantage of spontaneously reverted stem cell progeny in mosaic patients is encouraging indeed. At the same time, like few other examples, this phenomenon illustrates the interplay between genomic instability and cellular selection which may be at the root of cancer and aging.

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CHAPTER 2

Fanconi Anemia Genes: Structure, Mutations, and Genotype-Phenotype Correlations

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Abstract
This article is meant to introduce the reader to the FA/BRCA pathway, the currently known FA genes, and their mutations. We review structure and mutational profiles of the 11 FA genes identified to date. The FA genes display striking variability of size, molecular composition and mutational load which partly reflect their genomic structure and functional importance. Three genes (*FANCA, FANCC and FANCG*) account for over 80% of all FA patients worldwide, and there are only single or very few patients with biallelic mutations in the more recently discovered FA genes. *FANCA* as the most frequently affected gene displays the entire spectrum of genetic alterations, including at least 32% large deletions resulting from *Alu*-mediated recombination. In contrast, *FANCD2* harbors comparable amounts of *Alu* repeats but displays only few deletions. Viability of FA-D2 patients appears to depend on the presence of hypomorphic mutations and residual protein. Gene frequencies in excess of 1:100 have been observed, mainly for mutated *FANCA* and *FANCC* genes, in special populations and there are a number of founder mutations. With the exception of complementation group FA-D1 whose patients overwhelmingly present with malignancies in early childhood, most FA genes lack strict genotype-phenotype correlations. Rather, the particular type of mutation in a given gene appears to determine the severity of congenital malformations, the age of onset of bone marrow failure, and the length of survival. FA patients who reach adulthood may carry mild mutations or may have developed somatic reversions. Prospective studies are needed for a more definitive exploration of genotype-phenotype correlations.

Introduction

Around 80% of all patients suffering from Fanconi anemia (FA) belong to one of three prevalent complementation groups (FA-A, FA-C and FA-G). According to the International Fanconi Anemia Registry (IFAR), 57% of the FA patients have mutations in *FANCA*, 15% in *FANCC* and 9% in *FANCG*, followed by 4% in *FANCD1* and 3% in *FANCD2* [1]. Mutations in the other FA genes are rare, and only single patients each have been described for *FANCL* and *FANCM*. The estimated overall incidence of FA is 1 in 200.000 with an estimated heterozygous carrier frequency of 1 in 300 [2]. However, much higher patient and carrier frequencies are encountered in certain populations due to founder and inbreeding effects (see below).

FA-complementation groups were defined via somatic cell fusion [3,4], but a variety of strategies were applied for the identification of the underlying genes. Expression cloning was used for the identification of *FANCA* [5], *FANCC* [6], *FANCE* [7], *FANCF* [8] and *FANCG* [9], while positional cloning independently led to the identification of *FANCA* [The Fanconi anaemia/ breast cancer consortium, 10], but also to the identification of *FANCD2* [11] and *FANCJ* [12,13]. *FANCD1* [14] and *FANCJ* [15,16] were identified by a classical candidate gene approach. Biochemical isolation of the respective proteins led to the identification of *FANCB* [17], *FANCL* [18] and *FANCM* [19]. The identification of the first FA genes (*FANCC, FANCA* and *FANCG)* provided only few hints to their molecular function. Except for *FANCG*, which is identical with *XRCC9*, the initially discovered FA genes shared neither homology to other proteins nor to known functional domains. This changed when Howlett and coworkers [14] reported that the breast cancer associated protein 2 (*BRCA2*) is defective in FA patients belonging to complementation group D1. This finding implicated the definitive involvement of at least some of the FA proteins in DNA repair via homologous recombination. In addition, some of the subsequently discovered genes contain protein domains with defined biochemical functions. For example, FANCL or PHF9/Pog has a RING domain with

ubiquitin E3 ligase activity [18] – and the latest two FA proteins, FANCJ (identical to BRIP1/BACH1) and FANCM (homolog to Hef) have both DNA helicase domains. FA proteins participate in a complex molecular crosstalk with a large number of other proteins involved in the regulation and execution of DNA maintenance functions [20].

The FA/BRCA pathway

As a specific but not exclusive function, FA genes are thought to play an important role in the removal of DNA interstrand crosslinks (ICL). Cells of all FA subgroups display a characteristic hypersensitivity against DNA damage induced by bifunctional alkylating agents implying cooperation of FA proteins in a common pathway, which is referred to as *FA/BRCA pathway*. The current model of FA protein function (cf. figure 1) provides a direct link to recombinational types of DNA repair and translesion synthesis [21-23]. As shown in figure 1, specific types of DNA damage result in the assembly of at least 8 FA proteins (FANC-A, B, C, E, F, G, L and M) into a nuclear multisubunit complex (*FA core complex*) that is required for FANCD2 monoubiquitinaton at lysine 561 [24]. A member of the complex, the RING finger containing protein FANCL, provides the presumptive ubiquitin E3 ligase activity [18]. An extended form of the FA core complex is the so called "*BRAFT complex*" in which other proteins like BLM helicase, topoisomerase III alpha and replication protein A (RPA) participate [25], underlining the close association if not partial overlap of DNA replication and DNA repair functions.

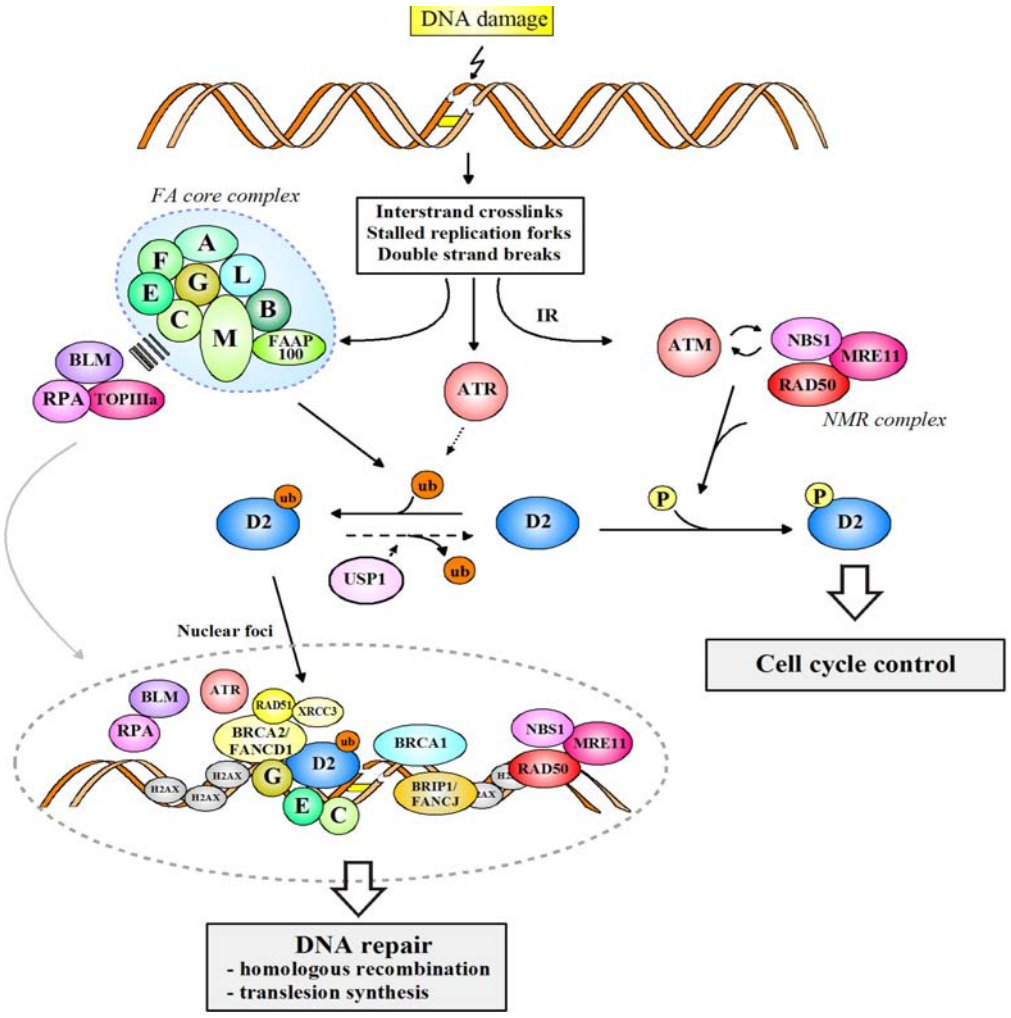

Figure 1. Schematic illustration of the FA/BRCA pathway.

A central step in the FA/BRCA pathway is the monoubiquitination of FANCD2. This post-translational modification causes a dynamic nuclear re-localisation of FANCD2 from a state of diffuse nuclear distribution into discrete nuclear foci containing many other DNA repair proteins [24,26]. Colocalization with FANCD2 was found for a number of proteins involved in DNA damage signalling including ATR kinase [27], components of homologous recombination (RAD51, BRCA1, BRCA2) [28], proteins of the non homologous end joining pathway such as NBS1 [29,30], the BLM helicase, and partially other FA proteins (FANCE, FANCC, FANCJ) [1,31]. FANCD2 is able to bind directly to DNA with a structure specific affinity [32] similar to that observed for FANCJ/ BRIP1 and FANCD1/BRCA2 – the only known FA proteins that are not required for FANCD2 monoubiquitination.

In addition to the proteins of the FA core complex, the ATR kinase is required for DNA damage induced monoubiquitination and relocation of FANCD2 [27]. ATR-mediated activation of FANCD2 takes place in response to DNA-ICLs or stalled replication forks. It appears to depend on the intact FA core complex but also on NBS1, linking FA to the non-homologous end-joining (NHEJ) pathway of DNA repair [29,33]. Depletion of the ATR-interacting protein, ATRIP, in *Xenopus* egg extracts results in defective chromatin binding of xFANCD2 [34]. ATR functions either by direct phosphorylation of FANCD2, as indicated by *in vitro* experiments, or indirectly by phosphorylation of components participating or regulating the FA/BRCA pathway [27].

As shown in figure 1, following exposure to ionizing radiation, FANCD2 is phosphorylated at serine 222 in an ATM kinase dependent manner which activates an intra S phase checkpoint [35]. Interestingly, ATM mediated phosphorylation depends on NBS1, implying a regulatory connection between the NBS1/MRE11/RAD50 complex and the FA proteins [30]. More detailed descriptions and discussions of the increasingly complex and manyfold interactions of the Fanconi anemia proteins can be found in series of recent reviews [20,22,23,36].

FA genes

The FA genes are scattered widely throughout the human genome and vary considerably in both size and structure, as summarized in table 1. Sequence comparisons indicate that many of the FA genes must have arisen fairly recently in evolution, lacking homologs beyond the vertebrate kingdom. However, there are also some FA genes/proteins that are more ancient and highly conserved. For example, FANCM is closely related to the archael protein Hef (helicase-associated endonuclease for fork-structured DNA) [19], and FANCJ belongs to the group of recQ-like helicases that share highly conserved domain structures. In addition, homologs have been found for FANCD2 in *D. melanogaster*, *C. elegans* and *A. thalia* [11], and for FANCL in *D. melanogaster* and *A. gambiae* [18]. This might reflect a basic function of the conserved FA proteins, most likely in the area of

Table 1. Summary of Fanconi anemia (FA) complementation groups and corresponding genes

Complementation Group	Frequency in FA [%]*	Gene	Chromosme location	Exons	Protein size [kDa]	Protein domains	Identification
FA-A	57	*FANCA*	16q24.3	43	163	partial leuzine zipper, NES, NLS	Lo Ten Fo et al. (1996) [5]
FA-B	0.3	*FANCB/ FAAP95*	Xp22.3	10	95	NLS	Meetei et al. (2004) [17]
FA-C	15	*FANCC*	9q22.3	15	63	-	Strathdee et al. (1992) [6]
FA-D1	4	*BRCA2/ FANCD1*	13q12.3	27	384	BRC repeats, RAD51-/ DSS1-/ssDNA binding, NLS	Howlett et al. (2002) [14]
FA-D2	3	*FANCD2*	3p25.3	44	155 (162)		Timmers et al. (2001) [11]
FA-E	1	*FANCE*	6p21-22	10	58	-	de Winter et al. (2000) [7]
FA-F	2	*FANCF*	11p15	1	42	homology to ROM	de Winter et al. (2000) [8]
FA-G	9	*FANCG/ XRCC9*	9p13	14	68	Tetratricopeptide repeat	de Winter et al. (1998) [9]
FA-I	rare	-	-	-	-		-
FA-J	1.6	*FANCJ/ BRIP1/ BACH1*	17q22	20	140	DEAH-helicase, BRCA1-binding	Levran et al. (2005) [12] / Levitus et al. (2005) [13]
FA-L	0.1	*FANCL/ PHF9*	2p16.1	14	43	WD40 repeats, PHD finger	Meetei et al. (2003) [18]
FA-M	rare	*FANCM/ Hef*	14q21.3	23	250	homology to helicase and endonuclease domains	Meetei et al. (2005) [19]

* based on the International Fanconi Anemia Registry (IFAR), reviewed in [1]

DNA repair [37], which has been complemented by the recruitment of the other FA genes later in evolution. Since oxidative stress and oxidative damage appear to activate the FA/BRCA pathway, an attractive hypothesis posits that warm-blooded and long-lived species require the extended FA family of genes as additional protection against DNA damage caused by reactive oxygen species.

Figure 2 provides a graphic summary of the known FA genes, depicting relative size and intron/exon structure. In addition, numbers and types of mutations are indicated above and below the respective genes. As such, figure 2 illustrates an impressive degree of size and structural variation among the FA genes, both at the genomic and the cDNA levels. One of the smallest genomic regions, comprising less than 5 kb and only a single exon, contains the entire *FANCF* gene, whereas *FANCC* and *FANCJ* are relatively large genes spanning 218 kb and 180 kb, albeit with widely differing intron/exon structures.
The occurrence of genetic alterations in a given gene depends on a variety of factors related to gene structure, including the presence of repetitive elements and base pair composition such as CpG dinucleotides. Functional factors such as expression during ontogenesis or organ specificity of course influence the spectrum of mutations that is permissive and thus viable in our species. Figure 2 illustrates and impressive degree of variation among the mutational spectra of the 11 known FA genes. Size cleary is not correlated with the number of mutations in a given gene. A multitude of mutations have been described in relatively small or medium sized genes like *FANCG* and *FANCA*, while genes with large transcripts like *FANCM* and *FANCD1* appear to be affected by relatively few mutations. With respect to type, quantity and distribution of mutations, *FANCA* clearly stands out. It is the only FA gene with a high proportion of large deletions, amounting to around 32% [38,39] of all observed mutations in *FANCA*. The prevalence of large deletions has been correlated with the presence of numerous *Alu*-type sequences, promoting *Alu*-mediated recombination [40]. With the advent of the MLPA technique, it is very likely that the proportion of deletions detected in FANCA will increase in the future. As illustrated by the example shown in figure 3, MLPA uncovers deletions that escape detection with standard techniques of mutation analysis.

FANCD2, a gene nearly of the same size and structure as *FANCA* also contains a high number of repetitive elements including many *Alu* repeats but so far we know of only two relatively small deletions involving a single exon. This suggests that the kind of mutations e.g. large deletions that are compatible with survival in *FANCA* are not permissive in FANCD2. The mutational record of the FA genes indeed suggests that evolutionarily conserved genes like *FANCD2*, *FANCL* or *FANCM* may be more important than, for example, the more recent *FANCA* or *FANCG* genes which seem to be highly tolerant for mutations. Since FANCL is thought to serve as catalytic subunit of the FA core complex and as such responsible for the monoubiquitination of FANCD2, this central function might well explain the relative paucity of patients and mutations. Much more in depth analysis of FA gene structure and function will be required to clarify whether the less frequently affected genes have indeed more important functions, or whether their underrepresentation has other, possibly structural reasons. A case in point is the highly conserved *FANCD2* gene. All FA-D2 patients examined to date have at least one hypomorphic or leaky mutation and residual levels of protein which clearly implicates lethality of biallelic null mutations. However, patients with biallelic mutations in the evolutionarily novel genes *FANCE* and *FANCB* also are exceedingly rare, suggesting that mutational inactivation of these genes might not be as permissive as mutational inactivation of FANCA, FANCC or FANCG. To add to the complexity, several functions other than its participatation in the FA core complex have been suggested for *FANCA*, and yet defects in this gene are impressively numerous. Biallelic null mutations in *FANCA* are compatible with survival such that in terms of functional importance *FANCA* must be clearly ranked below *FANCD2*.

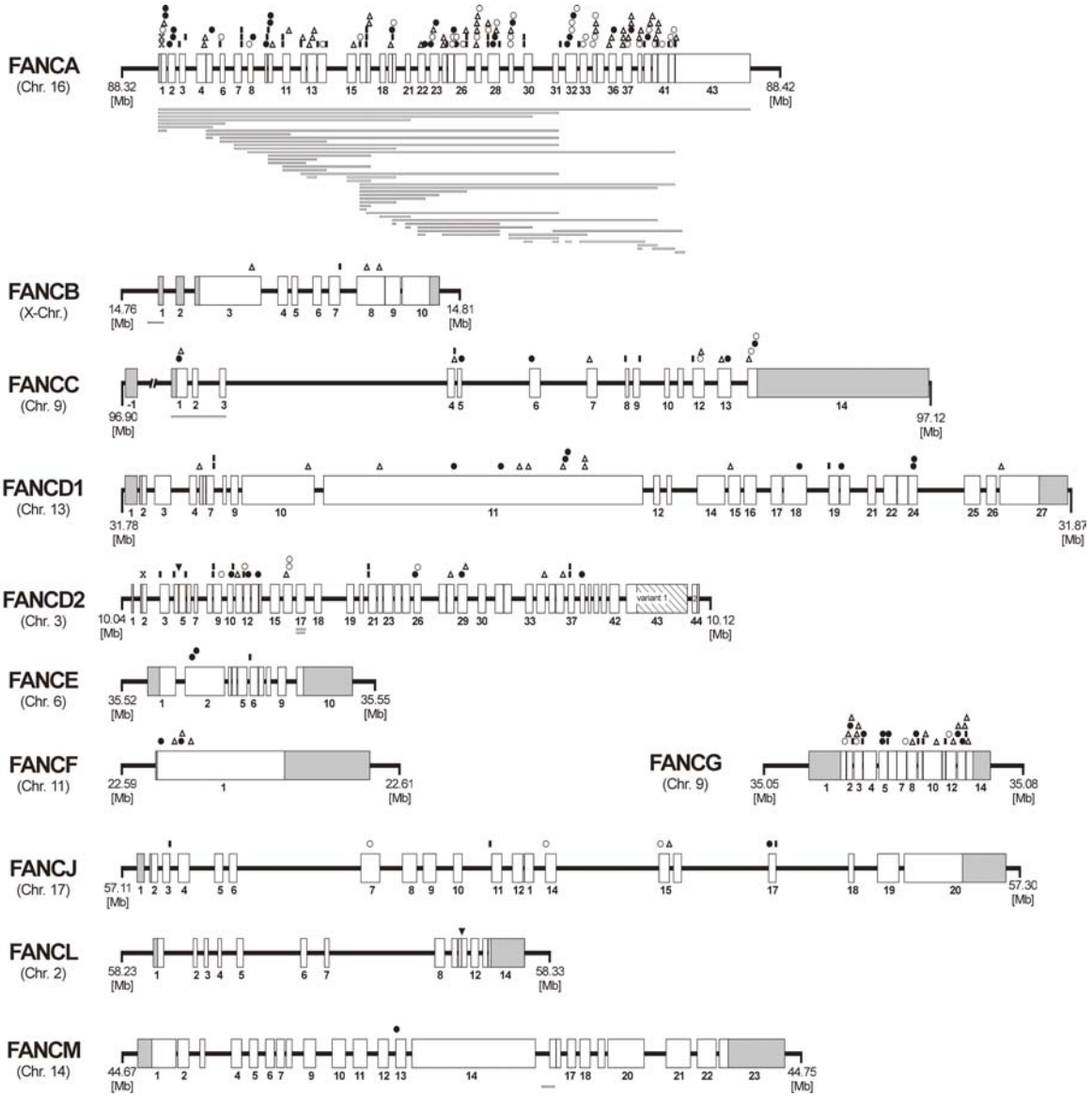

Figure 2. Relative size, exon/intron structure and mutational spectra of the known FA genes. In order to accommodate and compare the distribution of mutations, exons were drawn 50 fold larger than corresponding introns. Mutational chances are shown above and below the respective genes. Key to symbols: ○ missense mutation, ● nonsense mutation, ▲ small deletion/insertion or duplication, ▌ aberrant splicing/splice site mutation ▼ large insertion, ▬ large deletion (unpublished data and references for: *FANCA* [43], *FANCB* [17,47], *FANCC* [2,6,86], *FANCD1* [50], *FANCD2* [11], FANCE [7], *FANCF* [8], *FANCG* [84], *FANCJ* [12,13], *FANCL* [18], *FANCM* [19] and IFAR (Rockefeller University))

Annotations of individual FA genes (in alphabetical order)

The FANCA protein contains a leucine zipper-like motif, two overlapping bipartite nuclear localization signals (NLS) [41] and five functional leucine-rich nuclear export sequences (NES) that contribute to CRM-dependent nuclear export [42]. Since the first identification in 1996 more than 200 different mutations of all possible types have been described (see figure 1) (reviewed in reference [43]). Large deletions are notably frequent in *FANCA* with a striking overlap of the affected exons. Even though the exact break points have not been determined in all cases, a number of reports indicate a direct correlation between the presence of *Alu* repeats and deletion breakpoints [38,44,45]. Availability and widespread use of the multiplex ligation mediated probe amplification assay (MLPA) is likely to increase the yield of detected deletion-type mutations in *FANCA*. In addition to large deletions, small deletions and insertions/ duplications are also prevalent. These

changes often are associated with short direct repeats, homonucleotide tracts, and hotspot consensus sequences like CpG dinucleotides [46]. Base substitutions account for less than half of the mutations in *FANCA* and cause premature termination, amino acid exchanges, or affecting translation initiation or normal exon recognition.

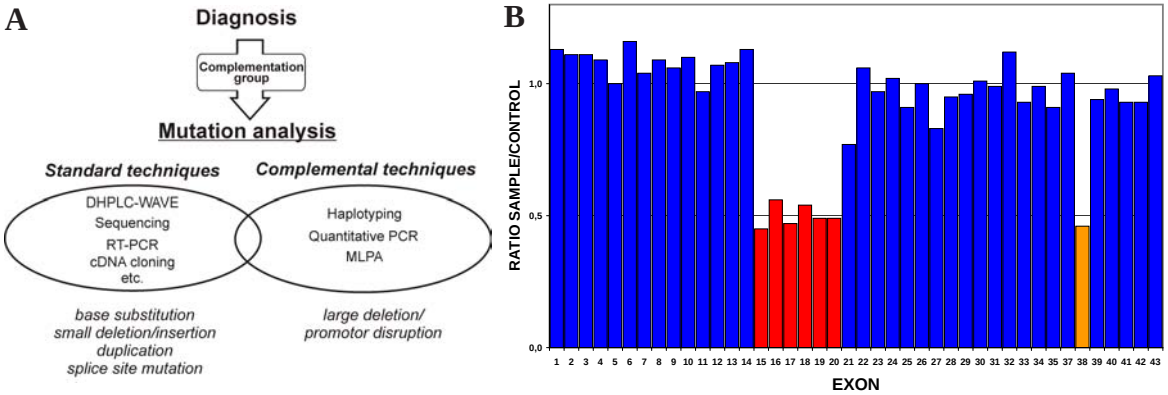

Figure 3. Mutation analysis in Fanconi anemia. A) Following to diagnosis the assignment of patient cells to one of the FA complementation groups enables direct mutation analysis in the underlying gene. B) Fast and efficient detection of large deletion in the *FANCA* gene by MLPA. The presented patient have a heterozygous deletion of exon 15 to 20 (red) and a small deletion in exon 38 (c.3788_3790delTCT) (orange), which may effect the binding site. Both mutation are indicated by the reduction of the ration sample/control to 0.5.

FANCB is the only X-linked FA gene. Approximately 60% of the genes located at Xp22.3 exhibit biallelic expression in females, which however is not the case for *FANCB* (NCBI, Omim, *300515) To date, mutations in *FANCB* have been found in 5 male FA patients [17,47] including a deletion affecting the promotor region and portions of the 5´ untranslated regions (exon 1), three microdeletions/-insertions, and a single splice site mutation. The analysis of three healthy female *FANCB* carriers revealed the preferential inactivation of the X-chromosome carrying the mutated allele in both peripheral blood cells and fibroblasts, suggesting a proliferative advantage for cells with the wildtype allele early in embryogenesis [17].

FANCC was the first FA gene, identified in 1992 [6]. It codes for two alternative transcripts differing in usage of the first untranslated exon, denoted as -1 or -1a [40]. At the genomic level, *FANCC* is one of the larger FA genes spanning more than 200 kb, but coding for a protein with a molecular weight of only 63 kDA. Two putative p53 binding sites of unknown functional significance have been described in *FANCC* [48]. Mutations in *FANCC* are responsible for 10-15% of all FA cases, but surprisingly few private mutations have been reported. As shown in figure 2, the mutational spectrum is heterogenous, and changes are scattered widely throughout the gene with the exception of a possible cluster in the C-terminal region. So far, only a single large deletion has been detected in *FANCC* (Rischewski et al , submitted).

FANCD1 is identical to the gene coding for the breast cancer associated protein 2 (*BRCA2*) [14], whose monoallelic mutations predispose to breast, ovarian, pancreatic and possibly other cancers. Biallelic mutations in *FANCD1/BRCA2* result in a severe form of FA with a high and very early risk for the development of malignancy [49-51]. At the mRNA level, *FANCD1*/*BRCA2* is the largest of the FA genes with an open reading frame of 10254 nucleotides. There are numerous functional domains which have been extensively reviewed [52,53]. Crucial for FANCD1/BRCA2 function are 8 BRC repeats, a helicase domain followed by 3 oligonucleotide/-saccharide binding folds (OB1, OB2, OB3), and a tower domain inserted in OB2. The FANCDD1/BRCA2 protein shows structure specific DNA binding activity and interacts directly with several other proteins some of which have been implicated in DNA repair like RAD51, FANCG and FANCD2 [54-56]. FANCD1/BRCA2

appears to function in multiple and diverse cellular processes including stabilization of stalled replication forks, homologuous recombination (via interaction with the RAD51 recombinase), and regulation of cytokinesis [57]. Mutations in *FANCD1/BRCA2* are distributed over the entire gene, but monoallelic preservation of a functional BRC-repeat domain has been observed for the majority of mutations in FA-D1 patients. Compared to the prevalence of gene carriers in different populations, the frequency of FA-D1 patients appears low, suggesting non-viability of most biallelic types of mutations [50,58].

FANCD2 is expressed in two alternatively spliced variants, one with a large continuous exon 43, and one with an additional exon 44 [11]. *FANCD2* is a highly conserved gene which is flanked by two distinct pseudogene regions *FANCD2-P1* and *FANCD2-P2* showing homologies to the middle part of the gene. FANCD2 protein is recruited to chromatin after DNA damage-induced monoubiquitination at lysine 561 [24,59] where it colocalizes with other proteins involved in DNA repair including BRCA2, RAD51 and BRCA1 [54,55]. Purified FANCD2 has DNA-binding activity with structure-specific affinity to branch points and free DNA ends such as Holliday junctions and DNA double strand breaks [32], supporting its active participation in DNA repair pathways. Based on the analysis of more than 30 patients and disregarding recurrent mutations, there is a non-random distribution of mutations with preferential involvement of the N-terminal region (cf. figure 1). In fact, more than 50% of the mutations observed in *FANCD2* affect splicing, a figure that is much higher than in any of the other FA genes. Residual levels of FANCD2 protein were detected in all patient-derived cell lines, which underline the functional importance of FANCD2 (thesis chapter 4).

FANCE was identified by expression cloning and mutation analysis of three families assigned to complementation group FA-E [7]. Except for the original families, no further mutations of FA-E patients have been reported.

FANCF consists of only a single exon. The encoded protein shares homologies to the prokaryotic RNA-binding protein (ROM) [8], which is without known significance [60]. To date, only 5 nonsense mutations and microdeletions have been found in FA-F patients [8]. These alterations appear to cluster in the 5´ portion of the gene. However, *FANCF* has gained special prominence as a gene that is frequently silenced by hypermethylation of its promotor region in various types of malignancies [61-63].

FANCG is identical to *XRCC9* which was identified in Chinese hamster cells as a gene involved in DNA repair [9,64]. FANCG has seven tetratricopeptide repeat motifs (TRPs) [65] thought to function as a scaffold for mediating protein-protein interaction. The molecular function of FANCG is not restricted to the FA core complex, since it has been shown to interact with BRCA2 and XRCC3 directly and independently of other FA proteins [66]. Mutations in FANCG are of all types, with the exception of deletions. They are scattered throughout the gene, with possible preference for the N- and C-terminal regions (cf. figure 1).

FANCJ is identical to the BRCA1 interacting protein 1 (BRIP1), also denoted as BRCA1-associated C-terminal helicase 1 (BACH1) [12,13]. FANCJ/BRIP1 was originally found as a BRCA1 interacting protein containing two functional domains, a DNA-dependent ATPase and a 5´ to 3´ DNA helicase [67]. BRIP1 has *in vitro* DNA unwinding activity with a high preference for forked duplex substrates and apparently executes efficient strand displacement from a D-loop substrate representing a likely intermediate in DNA repair via homologous recombination [68]. On the genomic level, *FANCJ* belongs to the larger FA genes, but only 8 different mutations have been described to date with most of the affected patients being homozygous for c.2392T>C (R798X) [12,13].

FANCL was identified by mass spectroscopy of a previously isolated 43-kDa FA-associated polypeptide [18]. Sequence analysis revealed identity to the WD40-repeat containing PHD finger protein-9 (PHF9), which is highly conserved. FANCL is thought to serve as the catalytic subunit of the FA core complex during monoubiquitination of FANCD2. Consistent with this important role in the FA/BRCA pathway, there is only a single patient known to date whose disease is caused by a homo- or hemizygous insertion of 177 bp into a pyrimidine-rich sequence between intron 10 and exon 11, leading to skipping of exon 11.

FANCM also belongs to the highly conserved FA genes [19]. It shows similarities to the archael protein *Hef* which displays a helicase and an endnuclease domain. However, the biological relevance of these domains for the current function of FANCM has yet to be established. Using specific antibodies, lack of FANCM was discovered in a single cell line from an patient previously excluded from most of the known complementation groups [19]. Genetic work up revealed compound heterozygous mutations, a maternal nonsense mutation in exon 13 and a paternal deletion within exon 15.

Table 2. Recurrent/ founder mutations in Fanconi anemia

Gene	Mutation	Ethnicity	Allel frequency	Other populations	Reference
FANCA	c.295C>T	Spanish Gypsy	1/64-1/70	Portuguese	[69]
	c.890_893del	Tunisian Jewish	rare	-	[80]
	c.894_1359del	Irish			[74]
	c.894_1626del	South African	n.d.	-	[71]
	c.1007_3066del	South African	n.d.	German (Western Ruhr), French Huguenots ancestry	[71]
	c.1115_1118del	variable	n.d.	-	[46]
	c.2574C>G	Indian Jewish	1/53	-	[80]
	c.2172_2173dupG	Maroccan Jewish	1/200	-	[80]
	c.3398delA	South African	n.d.	-	[71]
	c.3788-3790del	Brazilian	n.d.		[46,81]
	c.4275delT	Maroccan Jewish	rare	-	[80]
FANCC	c.67delG (c.322delG)	Northern European	n.d	-	[2]
	c.456+4A>T (c.711+4A>T)	Ashkenazi Jewish	1/89	Sephardic Jews (with Ashkenazi ancestry)	[2,82]
	c.456+4A>T (c.711+4A>T)	Japanese	n.d		[79]
FANCD2	c.1948-16T>G	Turkish	n.d.	Turkish North Americans, Czech	unpuplished data
	c.1948-6C>A	German	n.d.	North American	unpublished data
	c.2444G>A	Spanish	n.d.	Italian, North American	unpuplished data
FANCG	c.307+1G>C	Japanese		Korean	[83] [84]
	c.313G>T	German	n.d.	-	[85]
	c.637_643del	black South African	1/35-1/475	Bantu-speakers: Swaziland, Mazambique, Malawi	[72]
	c.1066C>T	Japanese/Korean	<1/156	Korean	[83]
	c.1077-2A>G	Portuguese-Brazilian	n.d.	Portuguese	[84]
	c.1183_1192del	German	n.d.	-	[85]
	c.1480+1G>C	French-Acadian	n.d.	-	[84]
	c.1649delC	Turkish	n.d.	-	[85]
	c.1794_1803del	Northern European	n.d.	-	[84]
BRIP1/FANCJ	c.2392C>T	Inuit	n.d.	different ethnics	[12]

Founder mutations and ethnically associated mutations

A number of FA causing mutations are recurrently prevalent in certain populations due to founder or inbreeding effects. Examples of such “marker” mutations are shown in table 2. A prominent example is the *FANCA* mutation c.295C>T in the Spanish Gypsy population where it is encountered with a carrier frequency of 1/64 to 1/70. The occurrence of the mutation is restricted to the Iberian peninsula, and it presumably emerged less than 600 years ago in a Gypsy family that migrated to

Spain [69]. Carrier frequencies higher than 1/100 have also been observed in the Ashkenazi Jewish population for the *FANCC* mutation c.456+4A>T (previously IVS4 +4A>T or c.711+4A>T) [2]. This mutation must have originated in the Israelite population that left Palestine during the Roman Empire and settled in Europe, since it is not encountered among Sephardic Jews [70].

A classical founder effect was demonstrated for the large intragenic deletion of exons 12-31 in the *FANCA* gene which has been shown to account for 60% of FA chromosomes in 46 unrelated Afrikaner FA patients [71]. This mutation was apparently introduced to the Cape region of South Africa at the end of the 17th century by a French Huguenot couple. In the black South African population, including individuals from Swaziland, Mozambique, and Malawi, 82% of all FA patients were found to carry the deletion c.637_643delTACCGCC in *FANCG* [72]. The distribution of this mutation in diverse geographic regions and tribes was used to date the origin to the arrival of the Bantu speakers in South Africa around 400 AD.

In regions of high historical migration like Europe or North America some mutations were found recurrently among patients of the same ethnic origin (cf. table 2). However, due the obvious heterogeneity of the populations there is little or no increased allele frequency, even if haplotype analysis suggests a common ancestry. Notable exceptions are mutations found in geographically isolated regions. In Italy, for example, two mutations in *FANCA*, c.790C>T and c.3559insG were detected in otherwise unrelated families within the Campania region [73]. Founder mutations or mutations sharing the same haplotype have been reported for several FA complementation groups (FA-A, C, D2, G and J) among different ethnic backgrounds, and independent of the overall mutation frequencies of the FA genes.

Not all recurrent mutations can of course be sufficiently explained by founder effects but may represent examples of gene regions vulnerable to genetic change (“hotspots”). A case in point is the mutation c.2392C>T in *FANCJ*, which was detected in a few unrelated Inuit patients that share the same haplotype. However, the same mutation was also found in patients from diverse populations and ethnical backgrounds, where it was correlated with at least three different haplotypes [12]. The combination of different haplotypes and the rarity of FA-J patients imply an independent recurrence of the mutation c.2392C>T.

Genotype-Phenotype correlations

Clinical course and severity of FA vary strongly between and even within families, suggesting relatively weak if any genotype-phenotype correlations. In particular, the onset of haematological abnormalities and longterm survival appear to depend on additional factors such as number and severity of infections, nutritional status, and access to and quality of medical care. Any definite conclusion about genotype-phenotype correlations must await the results of prospective studies which are not available to date. A retrospective study comparing the haematological profiles of complementation groups FA-A,-C and –G failed to reveal statistically significant differences in the onset of aplastic anemia [74]. However, the manifestation of cytopenia was more severe in patients belonging to complementation group FA-G. In addition, FA-G patients may develop AML or MDS earlier than patients of the other complementation groups. Congenital abnormalities were comparable among FA-A and FA-G patients, but less severe in FA-C patients. The clinical data of patients belonging to complementation group D2 suggests certain differences to other FA groups (thesis chapter 4). Compared to other patients listed in the IFAR database, FA-D2 patients exhibit a higher average frequency of congenital abnormalities. Their clinical course is additionally marked by an early average onset of bone marrow failure and early dependency on hematopoietic stem cell transplantation compared to FA patients register in the IFAR. It should be noted, however, that the patient cohort contained a number of patients with identical mutations which complicates

the statistical comparison. An undoubtedly severe phenotype is caused by biallelic mutations in *FANCD1/BRCA2*. Many of these patients succumb to their malignancies (nephroblastoma, Wilms-tumor, medulloblastoma and leukemias) prior to the onset of pancytopenia and thus may never be diagnosed as FA [49,50]. Because of the unusually severe and early onset of neoplasia in FA-D1 patients, their phenotype appears to be distinctive and may be more appropriately labelled FA-like rather than genuine FA [49,75].

Genotype-phenotype correlations are further complicated by the obvious heterogeneity of the mutational spectrum within each FA gene, the private character of mutations and, of course, the high prevalence of compound heterozygosity increasing the possibility of somatic reversion. Any missense change involving a non-conserved position within the gene should be formally tested for its functional significance. The availability of site directed mutagenesis, retroviral vectors with high transduction efficiencies, and MMC-sensitivity as a functional marker of complementation facilitate the performance of such "confirmatory" tests. Adachi and coworkers [76] have published an exemplatory study in which the functional significance of a large number of *FANCA* mutations has been tested. In terms of cost and labor, such confirmatory studies tend to exceed the capacity of the average diagnostic laboratory and might best be performed by specialized centres. Notwithstanding these difficulties, empirical studies have already shown that some mutations can be correlated with distinct phenotypes. The mutation c.1007_3066del in *FANCA*, for example, is associated with an increased rate of AML or MDS and a higher frequency of severe congenital abnormalities [74]. In contrast, the *FANCC* c.67delG alteration is associated with milder hematologic symptoms and a lower rate of somatic abnormalities when compared to a mean of other FA patients. Another example of a mild mutation is the missense mutation c.2444G>A in *FANCD2* which seems to be associated with a relatively mild clinical course (thesis chapter 4).

A mild clinical course may either be due to mutations that retain some degree of protein function or to revertant mosaicism. Mild mutations and/or revertant mosaicism will be preferentially found among older FA patients or patients who have escaped the diagnosis of FA because of lack of clinical symptoms. Such an example has been described by Huck et al. [77] whose patient lacked somatic abnormalities and was diagnosed as FA, at the remarkable age of 49 years, only because of an unusually severe response to chemotherapy for bilateral breast cancer. Concerning somatic reversion, there are numerous examples of reversions in the *FANCA*, *FANCC* and *FANCD2* genes. Somatic reversions preferentially involve compound heterozygous patients and, depending on the cell lineages affected, may lead to improved blood counts and a mild clinical course (see chapter by Hoehn et al, 2006).

An additional level of complexity is introduced by the genetic background of the respective patients or patient group. A case in point is the mutation c.456+4A>T in *FANCC* which shows a high prevalence in patients of both Ashkenazi Jewish and Japanese origin [78,79]. The Ashkenazi Jewish patients are severely affected whereas the Japanese patients have a much milder phenotype both in terms of hematologic onset and pattern of congenital abnormalities.

In summary, there is wide variation of phenotypes and clinical course among the most prevalent FA subgroups FA-A,-C and –G. The cumulative record of genotype-phenotype correlations clearly indicates that the nature of the underlying mutation is more important than the underlying complementation group. An exception to this rule are biallelic mutations in *BRCA2/FANCD1* which cause unusually severe phenotypes with early childhood leukemia and solid tumors, setting FA-D1 patients apart from the majority of FA patients.

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CHAPTER 3

Lack of sensitivity of primary Fanconi anemia fibroblasts towards UV and ionizing irradiation

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Abstract

Clinical observations and theoretical considerations suggest some degree of radiosensitivity in Fanconi anemia (FA), but experimental evidence remains controversial. We tested the sensitivity of primary skin fibroblast cultures from all known FA complementation groups towards ionizing radiation (IR) and ultra-violet (UV) light using conventional cell growth and colony formation assays. In contrast to previous studies, and because FA fibroblasts grow and clone poorly at ambient oxygen, we performed our sensitivity tests under hypoxic cell culture conditions. Fibroblast strains from healthy donors served as negative, and those from patients with ataxia telangiectasia (AT) and Cockayne syndrome (CS) as positive controls. We observed interstrain variation but no systematic difference of the IR response between FA and non-FA control fibroblasts. Following UV exposure, only complementation group A, G and D2 strains displayed colony formation EC_{50} values intermediate between negative and positive controls. Because of considerable interstrain variation, minor alterations of the IR and UV response of individual FA strains should be interpreted with caution and should not be taken as evidence for genotype-specific sensitivities of primary FA fibroblasts. Altogether, our data indicate neither systematic nor major IR or UV sensitivities of primary FA fibroblast cultures of any complementation group grown under hypoxic cell culture conditions.

1. Introduction

Fanconi anemia (FA) is an autosomal recessive disease caused by mutations in at least seven different genes [1]. Like the *ATM, WRN* and *XP* genes, the FA family of genes belongs to the group of caretaker genes whose presumptive function is to safeguard the genome against endogenous and exogenous DNA damage. Mutations in FA genes cause genomic instability, cell cycle alterations, bone marrow failure and neoplasia. Allogenic bone marrow transplantation (BMT) is the treatment of choice for patients with a matching sibling donor, and the success rate using HLA-compatible unrelated donors is improving [2]. While it is well established that FA fibroblasts are highly sensitive towards DNA-crosslinking agents such as diepoxybutane, mitomycin C (MMC) and nitrogen mustard, it remains controversial whether FA cells are sensitive towards ionizing radiation (IR; reviewed recently in [3]). Within the context of conditioning regimens prior to BMT, there has been the longstanding clinical impression of increased radiosensitivity of FA patients because of prominent skin reactions [4][. In contrast, no excess radiation toxicity was observed after 6 Gy single dose total body irradiation in a recent study of FA patients in a dose escalation trial [5].

In vitro studies with FA cells have also yielded conflicting results, with some studies claiming enhanced and others normal sensitivity towards IR (for review, see [3]). To explain this discrepancy it is conceivable that the various types of assays used to assess radiation sensitivity measure different facets of the radiation response. In addition, radiosensitivity may vary as a function of complementation group as the FA phenotype can be caused by mutations in any one of multiple FA genes. A study by Carreau et al. [6] tested FA lymphoblasts and suggested increased sensitivity, to the radiomimetic drug bleomycin, of FA complementation groups D2 (EUFA202), E (EUFA410), F (EUFA121), G (EUFA143, EUFA316) and H (EUFA173). These results were partly at variance with the re-assignment of the FA-H line to group FA-A [7] that did not show increased sensitivity. Other hints regarding potential X-ray (or bleomycin) and UV sensitivity of FA cells came from the Chinese hamster ovary (CHO) mutant lines UV40 [8] and NM3 [9], both of which have genetic defects in the Chinese hamster homologue of the human *FANCG/XRCC9* gene. Differences in genetic

Abbreviations used: FA, Fanconi anemia; AT, ataxia telangiectasia; CS, Cockayne syndrome; IR, ionizing radiation; UV, ultra-violet; EC_{50}, effective level of a treatment that yields 50% inhibition of the growth or cloning rate relative to untreated cells; BMT, bone marrow transplantation; MMC, mitomycin C; MEM, minimal essential medium, PD, population doublings; n.d., not detected

background, and the possibility of additional mutations in rodent cell lines derived by chemical mutagenesis may explain these obvious variations in the sensitivity spectrum of FA- and FA-like cells.

Recently, the *FANCD2* gene has been identified as a putative key player in the FA gene network [10]. Both IR and UV exposures were shown to activate a time- and dose-dependent conversion, via monoubiquination, of the FANCD2-S to the FANCD2-L protein leading to increased numbers of FANCD2 foci in irradiated HeLa cells [10]. Moreover, in response to IR, FA cells derived from multiple complementation groups failed to show the FANCD2-S to L conversion. These studies therefore suggest a biochemical basis for the putative radiation sensitivity of FA cells and implicate the participation of FA proteins in DNA repair pathways.

In order to further clarify the issue of *in vitro* radio-, and UV-, sensitivity of human FA cells, we undertook a systematic study employing primary fibroblast cultures of all known FA complementation groups. A technical aspect which sets the current study apart from previous studies is the fact that cell growth and colony formation following ionizing and UV irradiation was determined at hypoxic rather than ambient oxygen cell culture conditions. Close to physiologic, hypoxic culture conditions were chosen because of the known oxygen dependence of chromosomal aberrations in FA cells [11], and because FA fibroblasts grow and clone poorly at ambient oxygen, whereas near normal proliferation and colony formation can be achieved under hypoxic culture conditions [12].

2. Materials and methods

2.1. Cells

Primary FA fibroblasts were subcultures of reference strains established by the participating laboratories with informed consent from patients undergoing diagnostic evaluation for FA. Assignment to a given complementation group was by complementation via somatic cell hybrids [13], and/or detection of one or two mutant alleles in a given FA gene. Multiple independent strains were obtained for the complementation groups FA-A, FA-C and FA-G, whereas only single strains were available for the groups FA-B, FA-D1, FA-E, FA-F and FA-H (which has been reassigned to FA-A [7]) Two strains were established from siblings belonging to group FA-D2. Two types of control cells were used: Negative controls from healthy laboratory volunteers and positive controls from patients with known sensitivity towards UV and IR. The following 36 strains were investigated in this study (with the corresponding FA mutations shown in parentheses [14]): Complementation group FA-A: EUFA432 (*FANCA* 3639delT/3639delT), EUFA598 (*FANCA* exon43del/exon43del), EUFA517 (*FANCA* IVS40+1del18bp/n.d.), EUFA880 (*FANCA* 2493insC/n.d.); group FA-B: HSC230; group FA-C: 77RD325 (*FANCC* 322delG/1806insA), EUFA001 (*FANCC* 322delG/322delG), VU811 (*FANCC* 322delG/322delG), VU911 (*FANCC* 322delG/322delG), F97/16 (*FANCC* 322delG/n.d.), F99/194 (*FANCC*: 322delG/n.d.); group FA-D1: EUFA423; group FA-D2: VU202 and VU008 (both *FANCD2* 904C→T/958C→T); group FA-E: EUFA622 (*FANCE* IVS5−8g→a/IVS5−8g→a); group FA-F: EUFA121 (*FANCF* 16C→T/349-395del); group FA-G: FA1BER (*FANCG* IVS2+1g→a/346-347delCA), FA15BER (*FANCG* IVS13−1g→c/IVS13−1g→c), EUFA316 (*FANCG* 313G→T/1183-1192del), F99/112 (*FANCG* 313G→T/n.d.); former group FA-H, reassigned to FA-A [7]: EUFA173 (*FANCA* 2852G→A/exon17-31del). Control fibroblast strains from healthy individuals were: F86/15; F88/75; F96/1; F96/35; F98/JRR; F99/9; and F99/GRS. Positive controls with proven hypersensitivity towards UV radiation, derived from patients with Cockayne syndrome (CS) included GM00739, GM01428 and GM01098 (NIGMS Human Genetic Mutant Cell Repository, Camden, NJ, U.S.A.). Fibroblast strains with proven increased sensitivity towards IR (strains F95/20, F95/47, F96/35SYM, F96/49SND, and F98/72KSA were established in the participating laboratories from patients with ataxia telangiectasia (AT). Aliquots of each culture were frozen at

in vitro passages 2-3, and were re-thawed for the present series of experiments. All strains tested negative for mycoplasma at the time of study.

2.2. Reagents and Equipment

For cell culture, NUNC plasticware (Gibco BRL, Karlsruhe, Germany) was used. Minimum essential medium (MEM) with Earle's salts (cat. no. 41500), pre-tested fetal bovine serum (FBS; cat. no. 10270), phosphate-buffered saline (PBS; cat. no. 14200), and trypsin-EDTA (cat. no. 35400) were all from the same supplier (Gibco BRL) and used throughout the study to maintain consistent culture conditions.

Other reagents included MMC (Mitomycin 2, medac, Hamburg, Germany) and the Mycoplasma PCR ELISA kit (Roche, Mannheim, Germany).

IR was by a ^{60}Co source. Radiation energy was 1.033 MeV and distributed evenly through 1 cm acrylic glass covering the culture flasks The focus-source distance was approximately 80 cm in order to yield a dose rate of about 1 Gy/min. Dosimetry ensured exact dosage application. UV radiation of 254 nm wavelength was from a UV lamp with variable aperture and exposure timer (Type 3.260 002; Schuett, Goettingen, Germany). Dosage was adjusted by means of an UV-C meter (Kuehnast, Waechtersbach, Germany).

2.3. Cell culture and irradiation procedures

Cells were maintained at 37 °C and 5% CO_2 in Earle's minimal essential medium (MEM) supplemented with 16% FBS in high humidity incubators equipped with CO_2 and O_2 sensors (Heraeus, Hanau, Germany). Air was replaced by nitrogen in order to obtain a final oxygen concentration of 3% (v/v). Fibroblasts were grown in 25-cm^2 tissue culture grade flasks until they reached subconfluency (monolayer of spindle shaped cells without visible mitotic activity and without crowding). At this stage, the cells were detached using 1X trypsin-EDTA in PBS. Aliquots of the single-cell suspensions were counted in a Fuchs-Rosenthal chamber. Density of the cell suspension was adjusted to 50,000/ml in MEM.

For exposure to IR, volumes containing 55,000 cells each (1.1 ml) were transferred into seven 1.8-ml plastic cryotubes. While one vial was left untreated, the other six were exposed to γ-radiation at 0.5, 1.0, 1.5, 2.5, 4.0, and 8.0 Gy. Immediately thereafter, cell suspensions were diluted by the factor 10 (55,000 cells per 11 ml of medium). Aliquots of 3 x 0.1 ml from each of the seven vials corresponding to 3 x 500 cells were seeded in three Petri dishes of 6 cm diameter for colony formation studies. The remainder, 10.7 ml containing 53,500 fibroblasts, was plated in a 25-cm^2 tissue culture flask (at about 2,000 cells per cm^2) and incubated for the 5 day growth study After 5 days, these cultures were suspended by trypsinization and the total number of cells per culture flask (n) was determined using a Fuchs-Rosenthal chamber. The number of population doublings achieved after 5 days (PD) and was calculated using the function PD = log(n:53,500):log2.

Colony formation was evaluated by plating triplicate aliquots of 500 fibroblasts each in three 60-mm Petri dishes containing a total of 5 ml culture medium. There were two medium changes on days five and ten during the 12- to 14-day incubation period. These conditions were chosen in accordance with studies by Pomp et al. [15] showing the influence of cell density on the outcome of colony forming assays. At days 12 to 14 after seeding, colonies were simultaneously fixed and stained using 1% crystal violet in 20% ethanol for 3 min. The number of colonies was assessed visually by counting and marking the colonies on a light table projector. All colonies visible by eye were counted by two independent investigators. 80 to 90% of these colonies consisted of more than 30 cells per colony. The agreement between the two independent colony counts was better than 95%.

To study the effect of UV irradiation, aliquots of 53,500 cells were plated in a single, and triplicate aliquots of 500 fibroblasts each were seeded in three 60-mm diameter dishes containing 5 ml of culture medium. Cells were allowed to settle down over night. before the lid and the culture

medium were removed such that cells were exposed for the duration of the irradiation process with no medium over them. This procedure was necessary because culture medium and polystyrol plasticware absorb almost all UV light and prevent its effective application to the cells. After UV exposure, growth medium was restored and changed, for the colony formation assay, at days five and ten. Evaluation of cell growth and colony formation following UV light exposure was as described for the IR experiments.

2.4. Statistics

Experimentally determined cell and colony counts after irradiation were expressed relative to those of non-irradiated cultures. EC_{50} values were computed by curve fitting to the corresponding dose-response graphs. Wilson's t-test was used to compare fluctuations between FA and control strains.

3. Results

Table 1 displays average EC_{50} values following IR of cultures representing FA complementation groups A, C and G for which multiple independent strains were tested. The difference between normal control and A-T cells is significant in both types of assay (cell growth and colony formation). In contrast, there is no significant difference between the growth and cloning parameters of all three categories of FA cultures and the (negative) controls. This finding also holds for most comparisons between FA and control cultures that were exposed to UV radiation (Table 2). However, the mean EC_{50} values for colony formation of the FA-A and FA-G, but not the FA-C strains were intermediate between those of the negative and positive (CS) control cells. Does this observation indicate increased UV sensitivity of fibroblasts belonging to the FA-A and FA-G complementation groups?

3.1. Sensitivity towards UV irradiation

The question of differential UV sensitivity as a function of complementation group can be answered by inspection of figure 1A which displays colony formation as a function of UV dosage for all tested strains. Differences in EC_{50} values are reflected by the shape of the dose-response curves indicating a slightly higher UV sensitivity of the FA-A and FA-G strains compared to the negative controls. This holds also for a single FA-C strain, but there is considerable interstrain variation within all three groups. In contrast, the positive control strains with known UV sensitivity

Table 1. Sensitivity of FA and control skin fibroblast cultures to IR, expressed as EC_{50} values

Group	Cell Growth			Colony Formation		
	n	EC_{50}± 1SD[Gy]	t	n	EC_{50}± 1SD[Gy]	t
Normal ('negative') controls	4	4.33± 1.22		5	1.51± 0.31	
Positive controls (AT)	3	1.45± 0.51	0.023	3	0.51± 0.07	0.002
FA-A	3	3.71± 0.58	0.527	4	1.82± 0.48	0.325
FA-C	4	4.89± 2.53	0.741	3	1.59± 0.40	0.775
FA-G	4	3.67± 0.47	0.070	4	1.55± 0.27	0.836

Mean ± 1SD of EC_{50} values were computed by fitting to dose-response curves reflecting five-day cell growth in mass cultures and 12- to 14-day colony growth. AT = Ataxia telangiectasia; FA-A, FA-C and FA-G = Fanconi anemia complementation groups A, C and G.

Table 2. Sensitivity of FA and control skin fibroblast cultures to UV light, expressed as EC50 values.

Group	Cell Growth			Colony Formation		
	n	EC_{50}± 1SD[J/m^2]	t	n	EC_{50}± 1SD[J/m^2]	t
Normal ('negative') controls	3	11.06± 1.32		3	8.19± 0.92	
Positive controls (CS)	3	1.45± 0.49	0.001	3	1.35± 0.24	0.001
FA-A	3	11.10± 1.07	0.976	4	5.77± 1.06	0.045
FA-C	4	11.26± 2.54	0.922	6	8.42± 2.46	0.893
FA-G	4	10.05± 1.94	0.539	4	5.98± 0.87	0.040

Mean ± 1SD of EC_{50} values were computed by fitting to dose-response curves reflecting five-day cell growth in mass cultures and 12- to 14-day colony growth. CS = Cockayne syndrome; FA-A, FA-C and FA-G = Fanconi anemia complementation groups A, C and G.

(CS) are clearly set apart from FA- and negative control cultures. There is no obvious systematic difference in the UV response between FA fibroblasts and those from healthy donors, and all of the FA strains, but none of the CS cells were able to form colonies at exposures above 4 J/m^2. Figure 1B depicts the corresponding data for the five-day cell growth experiment in mass cultures. As in the colony assay data, there is considerable interstrain variation, but there is no systematic difference between any of the FA groups of strains and the negative controls.

3.2. Sensitivity towards IR

As shown in figure 2A, there is a certain amount of interstrain variation within each complementation group as a function of IR dosage, but the degree of this variation is very similar among all tested groups. In contrast, there is a systematic difference between the dose-response curves of AT and all the other (FA and negative control) strains. Both interstrain variation and the difference between FA and AT strains are even more pronounced when five-day cell growth data is taken as the endpoint (figure 2B). A single FA-G strain appears to be slightly IR-sensitive, albeit much less than the positive (AT) control curves. AT strains completely cease to grow beyond 2.5 Gy, whereas relative growth of the exceptional FA-G strain still amounts to 30% at 4 Gy.

3.3. Radiation response of FA-D2 fibroblasts

Since the recently identified *FANCD2* gene appears to play a central role in the function of the FA proteins [1], and since (SV-40 transformed) FA-D2 fibroblasts have been reported to be sensitive to IR [10], we paid special attention to fibroblast cultures derived from two siblings belonging to FA complementation group D2. Figure 3 compares the growth and cloning performance of the two D2 strains with the control group means. There was no difference of the IR response between the FA-D2 strains and the non-FA controls. Following exposure to UV light, colony formation of the FA-D2 strains declined more steeply than the controls, whereas the five-day cell count assays behaved like those of the non-FA controls. Since the outcome of the colony assay following UV exposure was at variance with the five day cell count data, the colony formation experiment was repeated three times with identical results.

4. Discussion

In analogy to the sensitivity of bacteria to antibiotics, we here refer to radiosensitivity as any enhanced cytotoxic, cytostatic or anti-proliferative reaction to IR (or UV light). Other types of assays assess the reaction to IR (or UV) as short-term biochemical effects such as DNA fragmentation,

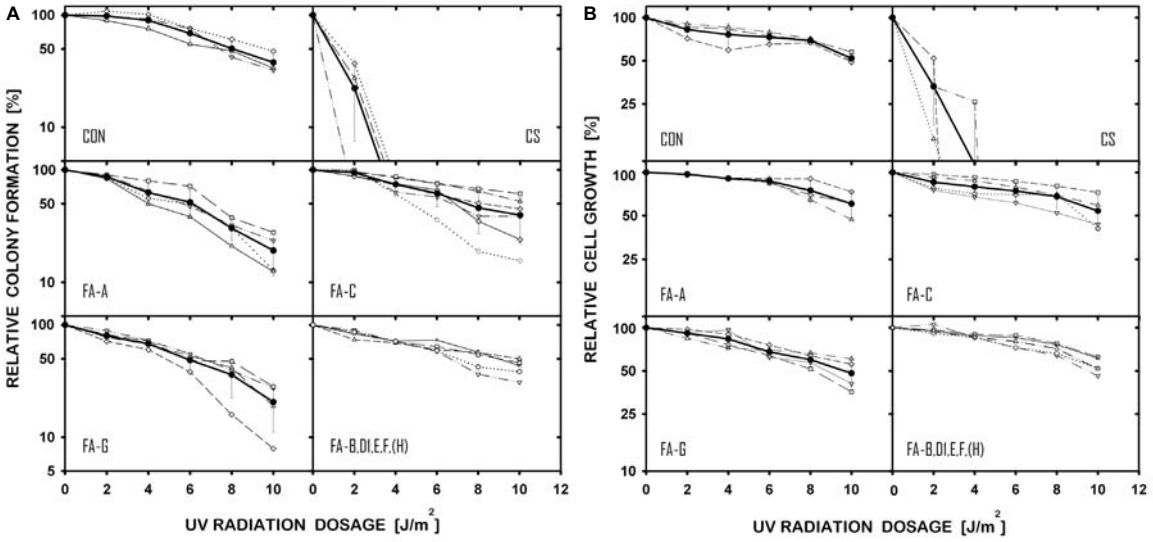

Figure 1. A) UV-induced reduction of colony formation. Various types of fibroblasts were exposed to increasing doses of UV light; CON = fibroblast cultures derived from clinically normal individuals *('negative controls')*; CS = fibroblast cultures derived from patients with Cockayne syndrome (*'positive controls'*); FA-A, FA-C, FA-G, and FA-B,D1,E,F,(H) = fibroblast cultures of FA complementation groups A-G(H), where H has been reassigned to A [7]. Solid lines with dark circle symbols denote mean ± 1SD derived from 3 to 6 single curves each (light lines). For complementation groups with only one available strain each, the following symbols are used: circle: FA-B, inverted triangle: FA-D1, cross: FA-E, square: FA-F, upward triangle: FA-(H**). B)** UV-induced reduction of 5 day cell growth. Various types of fibroblasts were exposed to increasing doses of UV light. Abbreviations, and the symbols used for FA-B,D1,E,F,(H), are as in figure 1A.

activation of FANCD2-L protein, and the formation of nuclear foci [3,10]. The conditions employed in the present study mainly reflect long-term cellular effects of IR (or UV light). Using multiple primary fibroblast cultures from all known FA complementation groups and comparing their UV and IR response relative to positive and negative controls, we arrive at the following conclusions:

1. With AT cells serving as positive controls, there is no evidence for a systematic IR sensitivity of FA fibroblasts of any complementation group under the conditions of our assays. Rather, FA

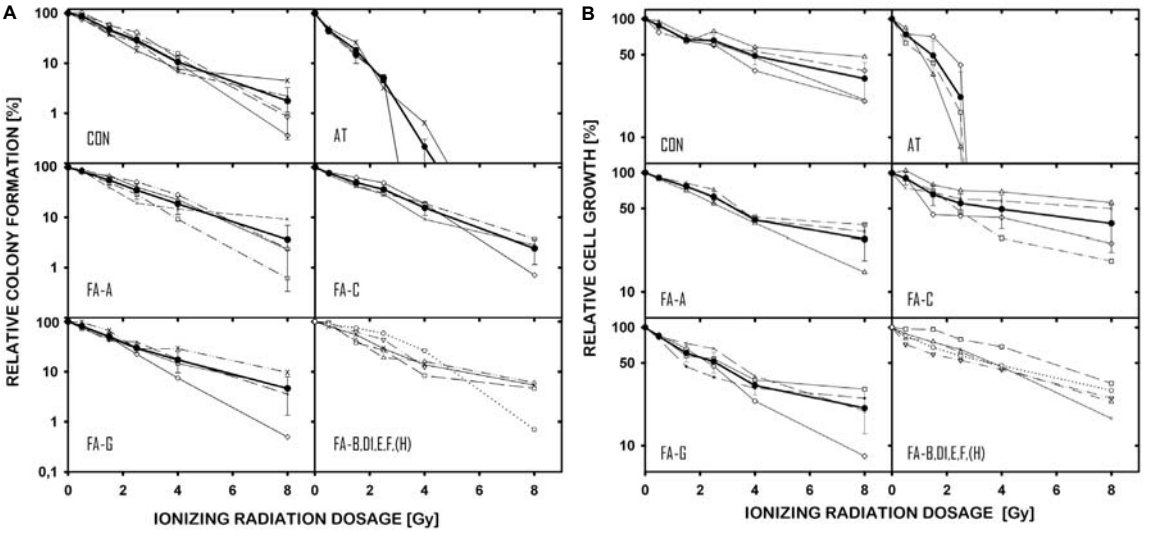

Figure 2. A) IR-induced reduction of colony formation. Various groups of fibroblast cultures were exposed to increasing doses of IR. AT = fibroblast cultures derived from patients with ataxia telangiectasia (*"positive controls"*). Other abbreviations, and symbols used for FA-B,D1,E,F,(H), strains are as in figure 1A. B) IR-induced reduction of cell growth. Various types of fibroblasts were exposed to increasing doses of IR. AT = fibroblast cultures derived from patients with ataxia telangiectasia (*"positive controls"*). Other abbreviations, and symbols used for FA-B,D1,E,F,(H), are as in figure 1A.

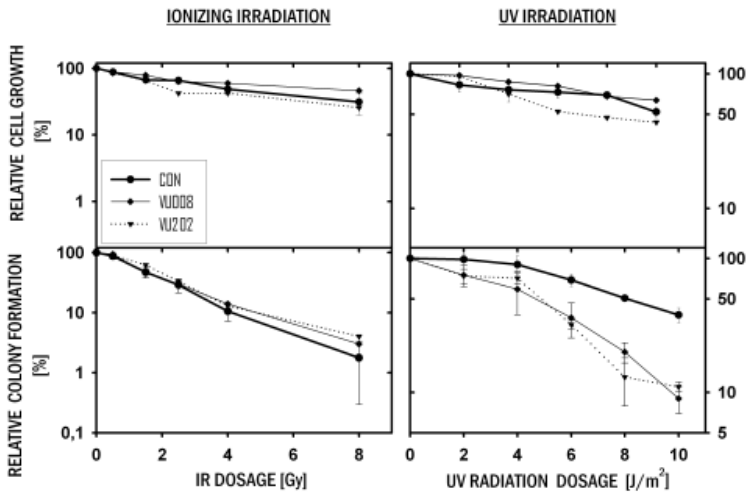

Figure 3. IR- and UV-induced reductions of cell growth and colony formation of FA-D2 strains. Relative numbers of cells in fibroblast mass cultures (top) and relative numbers of colonies after dilute plating (bottom) in response to the initial exposure of cells to increasing doses of IR (left hand panels) and UV light (right hand panels). FA-D2 = fibroblast cultures derived from patients belonging to complementation group D2. FA-D2 strains are denoted by thin dotted resp. solid lines connecting inverted triangle and diamond symbols. The dark and solid lines connecting solid circles denote means ± 1SD of 4 control strains derived from healthy donors.

fibroblasts exhibit the same dose-response relationship and the same degree of interstrain variation as non-FA control cultures derived from healthy donors. A considerable range of the radiation response of human diploid fibroblast cultures appears to be the rule rather than the exception in this type of study [16,17]. Differences related to cell type, culture history, culture conditions, nutrient supply, and donor genotype may account for such heterogeneity [18-20].

Likewise, our study provides no evidence for increased radiosensitivity of FA-D2 fibroblast cultures derived from two sibling donors. This finding seems to be at variance with the original report on the function of the *FANCD2* gene where SV40-transformed skin fibroblast-derived lines were found to react abnormally to IR [10]. The apparent discrepancy is unexpected, since transformation of human diploid fibroblasts using either SV40 T-antigen or the HPV16 E6 gene usually results in enhanced rather than decreased radioresistance [21-24]. One possible explanation might be that this is the first study in which cell growth and colony formation was assessed under hypoxic culture conditions that mimic *in vivo* oxygen tension. Cell cycle progression of primary, but not transformed FA cells is known to be sensitive to ambient oxygen [11,12,25]], and many studies make a case for a protective function of the FA gene products against reactive oxygen species [26]. Since damage induced by IR is thought to occur in part via formation of reactive oxygen intermediates [27], hypoxic cell culture conditions might prevent or lessen the burden of such damage in FA cells. This would imply that there is no gross and systematic deficiency of the recognition and/or repair of IR-induced damage in FA cells as long as these cells are kept under culture conditions that are hypoxic relative to ambient air but physiologic with respect to the *in vivo* state.

2. With CS cells serving as positive controls, there is no consistent evidence for increased UV sensitivity of FA fibroblasts. This is uniformly true if five-day cell growth in mass cultures is taken as the endpoint. Compared to non-FA control cultures, FA-A and FA-G strains show reduced colony formation at higher doses of UV exposure. Since *FANCG/XRCC9* is the human homologue of the gene defective in the UV-sensitive Chinese hamster ovary cell lines UV40 and NM3 [8,9,28], the marginal UV-sensitivity of our FA-G strains is interesting. However, the degree of UV sensitivity displayed by our FA-G group of strains is in no way close to the sensitivity displayed by the positive control (CS) fibroblasts. In addition, there is considerable interstrain variation, with individual strains within the FA-A and FA-G groups showing a completely non-FA control-like response. A close to normal UV response of FA cells is consistent with the fact that the major DNA lesion following UV irradiation consists of DNA adducts that are removed by nucleotide excision repair, a process that has been shown to function normally in FA cells [29]. Nevertheless, it may be of interest that yeast two-hybrid studies have shown a much more prominent interaction between the products of *FANCA* and *FANCG* genes compared to other FA gene products [30] such that the common radiation response of these two complementation groups may reflect their close functional relationship.

3. Whereas our study provides no evidence for increased IR sensitivity of FA-D2 primary fibroblast cultures, our two FA-D2 strains were found to be more UV-sensitive than strains from non-FA donors with regard to their colony forming ability. It must be pointed out, however, that the UV-induced decline in colony numbers of this group of strains is far less severe than that of cells with the CS genotype. In order to establish firmly whether the observed dose-dependent reduction in colony formation following UV exposure might be a consistent and characteristic property of FA-D2 cells, additional strains from different FA-D2 donors with other genetic backgrounds need to be tested. We clearly cannot exclude the possibility that the unusual behavior of our FA-D2 strains might be a special characteristic of the genetic background of these particular strains that were derived from sibling donors. Moreover, the question remains why the reduced colony survival following UV exposure of our FA-D2 strains was not paralleled by their corresponding cell growth data. We can only speculate that the hundredfold difference in plating density, by some mechanism such as metabolic cooperation and/or bystander effect [31], may have contributed to these discrepant results.

A certain degree of variability among cell lines is a common experience of similar studies and might reflect subtle differences in genetic background, growth fraction, and cell culture conditions. A case in point is the study by Carreau and colleagues [6] who tested the drug sensitivity spectra of FA lymphoblast lines and who found a similar degree of variation as described in the present study but few systematic differences. In particular, there was no difference in the UV response of lymphoblast cell lines originating from various complementation groups. However, Carreau et al. [6] found increased sensitivity of some of their cell lines to the radiomimetic drug bleomycin. It is quite clear that the behavior towards clastogens can vary as a function of cell type. Differences in radiosensitivity between FA lymphocytes and FA fibroblasts were noted in previous studies [32], and a recent study [33] describes consistent differences between FA fibroblasts and lymphoblasts in the way that these cells repair DNA double-strand breaks. In addition, FA lymphoblasts are known to effectively undergo apoptosis in response to DNA-crosslinking agents whereas FA fibroblasts have been shown to be delayed in the 4c stage of the cell cycle and eventually may recover [34]. In addition, it has yet to be tested whether the reported radiosensitivity of FA lymphoblast cell lines [6,35] persists under hypoxic cell culture conditions. Since fibroblast cultures derived from FA knock-out mice of the complementation groups A, C and G are not radiosensitive [36], it seems that mesenchymal cell types from both mouse and man agree with respect to their *in vitro* response towards IR.

Radiation therapy studies generally report a positive correlation between *in vivo* and *in vitro* radiosensitivity [37]. However, at present the risks of radiation therapy in FA patients are not clear [38]. When testing the *in vitro* behavior of patient cells towards clastogens one must be aware of the fact that the (artificial) *in vitro* situation might not be representative of the (physiologic) *in vivo* state. This complicates the “predictive” use of *in vitro* sensitivity assays. A very instructive case in point is the pronounced discrepancy between *in vivo* and *in vitro* radiosensitivity that has been reported by Marcoua et al. [39]. While their FA patient showed a severe tissue reaction during radiation therapy for tonsillar carcinoma, there was no evidence for *in vitro* radiosensitivity using conventional assays with the patient’s fibroblast cultures. As mentioned above, it is conceivable that different cell types use different mechanisms to respond to DNA damage which could in part account for differences when comparing *in vivo* with *in vitro* exposures.

In summary, with the possible exception of minor alterations in the UV response of FA-A, FA-G and FA-D2 cells, our study provides no evidence for systematic differences of the radiation response as a function of FA complementation group, nor do we find evidence for increased radiosensitivity of FA-fibroblasts of any complementation group compared to fibroblasts from healthy donors. This is true also for the single FA-D1 strain included in our testing which has been recently shown to carry biallelic mutations in the *BRCA2* gene [40], and this is true for the IR response of our two strains belonging to complementation group FA-D2 which acts downstream of and divergent from the other

members of the FA protein complex [10,41]. It must be stressed, however, that our results were obtained under hypoxic cell culture conditions, and using fibroblast strains with major proven IR- and UV-sensitivities (AT and CS cells) as positive controls. Since each of the positive controls represents an extreme degree of sensitivity, minor degrees of sensitivity, such as observed with some of our UV-irradiated strains, cannot be excluded since such minor sensitivites cannot be reliably separated from the background variation of our cell culture assays.

Acknowledgments

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CHAPTER 4

Molecular and clinical spectrum of the central Fanconi anemia gene *FANCD2*: a series of 32 patients

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Blood, submitted

Abstract

FANCD2 is an evolutionary conserved Fanconi anemia (FA) gene which plays a central role in DNA double-strand type damage responses. Using complementation assays and immunoblotting, a consortium of American and European groups assigned 29 FA patients from 23 families and 3 additional unrelated patients to complementation group FA-D2. This amounts to 3 to 6% of FA patients registered in various datasets. Malformations are frequent in FA-D2 patients and hematological manifestations appear earlier and progress more rapidly when compared to patients from all other FA groups combined, as represented by the International Fanconi Anemia Registry. *FANCD2* is flanked by two pseudogenes. Mutation analysis revealed the expected total of 64 mutated alleles, 33 of which result in aberrant splicing patterns. Many mutations are recurrent and have ethnic associations and shared alleles. There were no biallelic null mutations, and residual FANCD2 protein of both isotypes was observed in all patient cell lines available. These analyses suggest that unlike in a reported mouse model, total absence of FANCD2 is incompatible with survival in humans and that hypomorphic *FANCD2* mutations generally lead to a relatively severe form of FA. This highlights the indispensible role of FANCD2.

Introduction

Fanconi anemia (FA) is a rare genome instability disorder with the variable presence of congenital malformations, progressive bone marrow failure, predisposition to malignancies, and cellular hypersensitivity towards DNA-interstrand crosslinking (ICL) agents [1]. There are at least twelve complementation groups (FA-A, B, C, D1, D2, E, F, G, I, J, L and M), each of which is associated with biallelic or hemizygous mutations in a distinct gene [2]. To date, eleven of the underlying genes have been identified, denoted as *FANCA, B, C, D1/BRCA2, D2, E, F, G/XRCC9, J/BRIP1, L/PHF9 and M/HEF* [3-5]. Eight of the FA proteins (FANCA, B, C, E, F, G, L and M) and other components assemble in a nuclear complex, the FA core complex, that is required for the monoubiquitination of FANCD2 at amino acid residue K561 [6,7]. Monoubiquitination occurs in response to DNA damage and during the S phase of the cell cycle [7,8]. The monoubiquitinated FANCD2 isoform (FANCD2-L) is targeted to nuclear foci containing proteins such as BRCA1, BRCA2 and RAD51 that are involved in DNA damage signaling and recombinational repair [9-12]. The precise role of FANCD2 in the maintenance of genome stability remains unknown, but FANCD2-deficient DT40 cells show defects in homologous recombination (HR)-mediated DNA double-strand break (DSB) repair, translesion synthesis and gene conversion [9,13,14]. Therefore, FANCD2 is thought to play a central role in the maintenance of genome stability [9,14,15].

The human and murine *FancD*2 genes show a higher degree of homology than most of the other FA genes [16]. *FancD2* knock-out mice suffer from perinatal lethality, microphthalmia and early epithelial cancers [17], but it remains controversial whether the murine FA-D2 phenotype is more severe than the corresponding murine knock-outs of the other FA genes [17,18]. FancD2 is required for survival after DNA damage in *C. elegans* [19], and FancD2-deficient zebrafish embryos display severe developmental defects due to increased apoptosis underlining the importance of *FancD2* function during vertebrate ontogenesis [20]. Finally, knock-down of drosophila *FancD2* causes pupal lethality [21]. In humans, it has been estimated that complementation group FA-D2 accounts for less than 1% [22] to 3% [23] of all FA patients. With a size of *FANCD2* matching roughly that of *FANCA*, the relatively lower abundance of FA-D2 than FA-A patients suggests that, akin to the FA-D1 (BRCA2-deficient) subtype [24], only a narrow spectrum of *FANCD2* mutations may be viable in humans. The presence of *FANCD2* pseudogenes complicating mutation analysis may explain why there has been no other report of human *FANCD2* mutations since the original

description [25]. As a concerted effort among seven laboratories we present the first comprehensive mutation profile of the *FANCD2* gene. We show that the FA phenotype resulting from FANCD2 deficiency is relatively severe and, in contrast to all other FA genes, (1) the mutation spectrum of *FANCD2* is dominated by splicing mutations, and (2) residual FANCD2 protein exists in all tested cell lines from FA-D2 patients, suggesting lethality of biallelic null mutations.

Patients, materials and methods

Diagnostic procedures

Anti-coagulated peripheral blood and skin biopsy samples were referred to the participating laboratories for diagnostic testing. Confirmation of the diagnosis of FA, subtyping and mutation analysis were performed with informed consent according to the Declaration of Helsinki. The study was approved by the institutional review boards of the participating centers. The clinical suspicion of FA was confirmed by the detection of cellular hypersensitivity to DNA-crosslinking agents following published procedures [26-30]. In cases with hematopoietic mosaicism, skin fibroblasts were used to confirm the diagnosis of FA.

Patient statistics

A total of 29 FA-D2 patients (no. 1-29) with adequate clinical data were included in the present genotype-phenotype study. Five of them with hematopoietic mosaicism were excluded from clinical follow-up studies. Three additional FA-D2 patients (no. 30-32) with incomplete clinical data are not part of the phenotype analysis but results concerning their mutations are shown as indicated in the text, tables and figures.

For calculations of cumulative incidence, the following three end points were evaluated: time to bone marrow failure (BMF; hematological onset, defined as cell count of at least one lineage constantly below normal range), BMF to hematological stem cell transplantation (HSCT), and time to HSCT. Kaplan-Meier estimates were computed for the length of overall survival. Birth was taken as the date of FA onset for all these calculations. Comparisons were made to a body of FA patients in the IFAR as previously reported [31] by means of log-rank test statistics. Multivariate and competing-risk analyses were not possible due to the limited number of informative patients.

Cell culture

Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) were established using cyclosporin A as previously described [32]. All blood-derived cell cultures were maintained in RPMI 1640 medium with GlutaMAX (Gibco) supplemented with 15% fetal bovine serum (FBS; Sigma). Fibroblast strains were established using standard cell culture procedures and propagated in Eagle's MEM with GlutaMAX (Gibco) and 15% FBS. All cultures were kept in high humidity incubators in an atmosphere of 5% (v/v) CO_2 and, in case of fibroblasts, 5% (v/v) O_2 by replacing ambient air with nitrogen [33]. In some cases, RNA stabilization was achieved by cycloheximide (CHX) added to cell cultures at a final concentration of 250 µg/ml 4.5 h prior to RNA isolation.

Retroviral complementation

For construction of the D2-IRES-neo retroviral expression vector S11FD2IN, the D2-IRES-puro plasmid pMMP-FANCD2 [25] was cut using Sal I. The ends were blunted and the fragment containing the *FANCD2* ORF was cut out with EcoRI and ligated into S11IN cut with BamHI, blunted and also cut again with EcoRI (Figure 1A and Supplementary Figure S1). S11 vectors are based on the spleen focus forming virus and are derived from the GR plasmid [34]. Sequencing of the retroviral plasmid S11FD2IN revealed three reported polymorphisms in the *FANCD2* ORF, c.1122A>G, c.1509C>T,

c.2141C>T [25] and another conservative base substitution, c.3978C>T. Stable retroviral packaging cells were generated by infection of PG13 cells with selection in G418 (Sigma) as previously described [35]. Retroviral transduction of cultured cells follwed published protocols [36,37]. Selection of transduced cells was in G418 (Sigma) at a final concentration of 0.8 to 1.2 mg/ml for about 10 days. Transduced cells were analyzed for their sensitivity to MMC using flow cytometry to assess survival rates and cell cycle arrest [37,38]. In addition, enhanced green fluorescent protein (*EGFP*) and *FANCA* cDNAs were separately cloned into the vector S11IN (S11EGIN and S11FAIN; Figure 1A) for transduction of the cells under study, with EGFP serving to monitor complete selection and FANCA serving as negative complementation control.

Immunoblotting

FANCD2 immunoblotting was performed as first described [7] with minor modifications. Detection was by the chemiluminescence technique using standard ECL reagent (Amersham) or SuperSignalWestFemto (Pierce).

Mutation characterization

Total RNA was purified using the Micro RNA Isolation Kit (Stratagene) or the Micro-to-Midi Total RNA Purification System (Invitrogen). For cDNA studies, isolated RNA was reverse transcribed using SuperScript II or SuperScript III RNase H^- Reverse Transcriptase and oligo$(dT)_{20}$ (both Invitrogen) according to the manufacturers instructions. *FANCD2* cDNA was PCR-amplified in 5 overlapping fragments using Phusion (Finnzyme) or Pfx (Invitrogen) DNA polymerase. The primers used for cDNA amplification are shown in Supplementary Table S1A, those additionally used for cDNA sequencing are shown in Supplementary Table S1B.

Genomic DNA was prepared using a modified salting-out technique [39]. A total of 15 large amplicons (superamplicons) that are unique to certain regions of the functional *FANCD2* gene were generated to serve as templates in place of genomic DNA. The primers used for superamplifications and the sizes of the superamplicons are shown in Supplementary Table S2A. Genomic primers for the amplification of all *FANCD2* exons and adjacent intron regions and their sizes are displayed in Supplementary Table S2B. Additional genomic mutation-specific primers are given in Supplementary Table S2C.

Automated direct sequencing of the PCR products was performed using ABI-PRISM big-dye terminator chemistry (BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1) according to the recommendations of the manufacturer and an ABI PRISM 310 Genetic Analyzer instrument (all Applied Biosystems).

For haplotyping, four microsatellite markers in the vincinity up- and downstream of *FANCD2* on chromosome 3 were studied. These included D3S1597, a dinucleotide repeat at 9.340 Mb, D3S1038, a dinucleotide repeat at 10.488 Mb, D3S3611, likewise a dinucleotide repeat at 10.529 Mb, and D3S1675, a tetranucleotide repeat at 10.643 Mb. Primers used for microsatellite amplifications are specified in Supplementary Table S3. Two of the forward primers were 5'-Cy5-, the other two 5'-Cy5.5-labeled for multiplex fragment analysis with DNA Sizing standards on a CEQ 8000 Genetic Analysis System (both Beckman Coulter).

In silico analyses

The NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) nucleotide sequences NM 033084 (43 exons) and AF 340183 (44 exons) were used as the human FANCD2 cDNA reference. The genomic reference sequence was ENSG00000144554. Fancd2 sequence information of other species is available at the same website. Genomic FANCD2 sequences were compared by BLAT homology searches (http://genome.ucsc.edu/cgi-bin/hgBlat) and the Ensembl genome browser (http://www.ensembl.org/). FANCD2 protein sequences of different species including homo sapiens were from the Swiss-Prot database (http://www.expasy.org/sprot/). Polypeptide sequences

were compared using the Windows interface Clustal X (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX), version 1.81, for the Clustal W multiple sequence alignment program. Promotor analyses were done using the CpG island explorer (40 ,http://bioinfo.hku.hk/cpgieintro.html) version 1.9 at the settings GC 60%, CpG O/E ratio 0.7 and minimum length 500 nt. Analysis of repetitive elements was done using the Repeatmasker software (http://www.repeatmasker.org). Predicted splice donor performance was calculated using the Splicefinder algorithm (http://www.uni-duesseldorf.de/rna/html/5_ss_mutation_assessment.php). Deduced splice acceptor function was estimated using a maximum entropy model (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html). Regulatory splice sequences were analyzed using the ESE finder (http://rulai.cshl.edu/tools/ESE) and the Rescue-ESE (http://genes.mit.edu/burgelab/rescue-ese).

Results

Assignment to and frequency of group FA-D2

Figure 1B-E demonstrates our strategy for the assignment of cultured FA cells to group FA-D2. A dual screening strategy yielded optimal results. Cell cycle analysis was used to ensure MMC sensitivity of LCLs (Figure 1C, lane 2), while the apparent absence of FANCD2 bands on standard exposure immunoblots suggested their belonging to group D2 (Figure 1B, lane 2). Transduction of putative D2 LCLs with *FANCD2* cDNA using the retroviral vector S11FD2IN restored FANCD2 expression and function as reflected by the emergence of both FANCD2 isoforms (FANCD2-S and -L; Figure 1B, lanes 3); simultaneously, the MMC sensitivity of transduced cells returned to normal control cell levels as evidenced by the reduction of G2 phase cell cycle fractions (Figure 1C, lane 3; Figure 1B and C, lanes 1). Transduction of D2 LCLs using S11EGIN or S11FAIN did not result in the restoration of either FANCD2 isoform nor in a normalization of G2 phase arrest, as exemplified for *FANCA* (using S11FAIN) in Figure 1B and C, lanes 4. In case of suspected hematopoietic mosaicism, cultured fibroblasts were assayed using a corresponding strategy (Figure 1D and E). As shown in Supplementary Table S4, only a few patients were assigned to group FA-D2 by primary mutation analysis, including four affected siblings of four different index patients, and an unrelated deceased patient with only DNA available.

In the North American IFAR collection, 18/630 FA patients were classified as D2. Eight of these were included in the present study. Among the patients referred to the two German labs, 15/243 FA patients were D2. These data suggest that the proportion of complementation group D2 among two larger series of FA patients may be more frequent than previously reported [2,22,23].

Clinical data of FA-D2 patients

Including information from a prenatal case, malformations in the present cohort of 29 FA-D2 patients were of the following types and frequencies (Supplementary Table S5): 25/28 (89%) had microcephaly, 25/29 (86%) (intrauterine) growth retardation, 21/28 (75%) anomalies of skin pigmentation, 21/29 (72%) radial ray defects, 17/28 (61%) microphtalmia, 10/28 (36%) renal anomalies, 9/28 (32%) malformations of the external ear, 9/29 (31%) anomalies of the brain (including 5/29 or 17% with hydrocephalus), 7/28 (25%) hypogonadism or other genital anomalies, 4/28 (14%) anomalies of the heart and 4/28 (14%) malformations of the GI tract. Of note was a high proportion of FA-D2 patients with psychomotor retardation and attention deficit/hyperactivity disorder (8/28, 29%). Dysplasia and dislocation of the hip (6/28, 21%) also was relatively common. In addition to the VACTERL-like association (1/28) and holoprosencephaly (1/28), the Karthagener syndrome (1/28) and the Michelin tire baby syndrome (2/28) were noted as distinct disorders secondary to or associated with FA-D2. It is evident from this data that the overall clinical phenotype of FA-D2 is relatively severe when compared to other complementation groups, except D1. With the exception

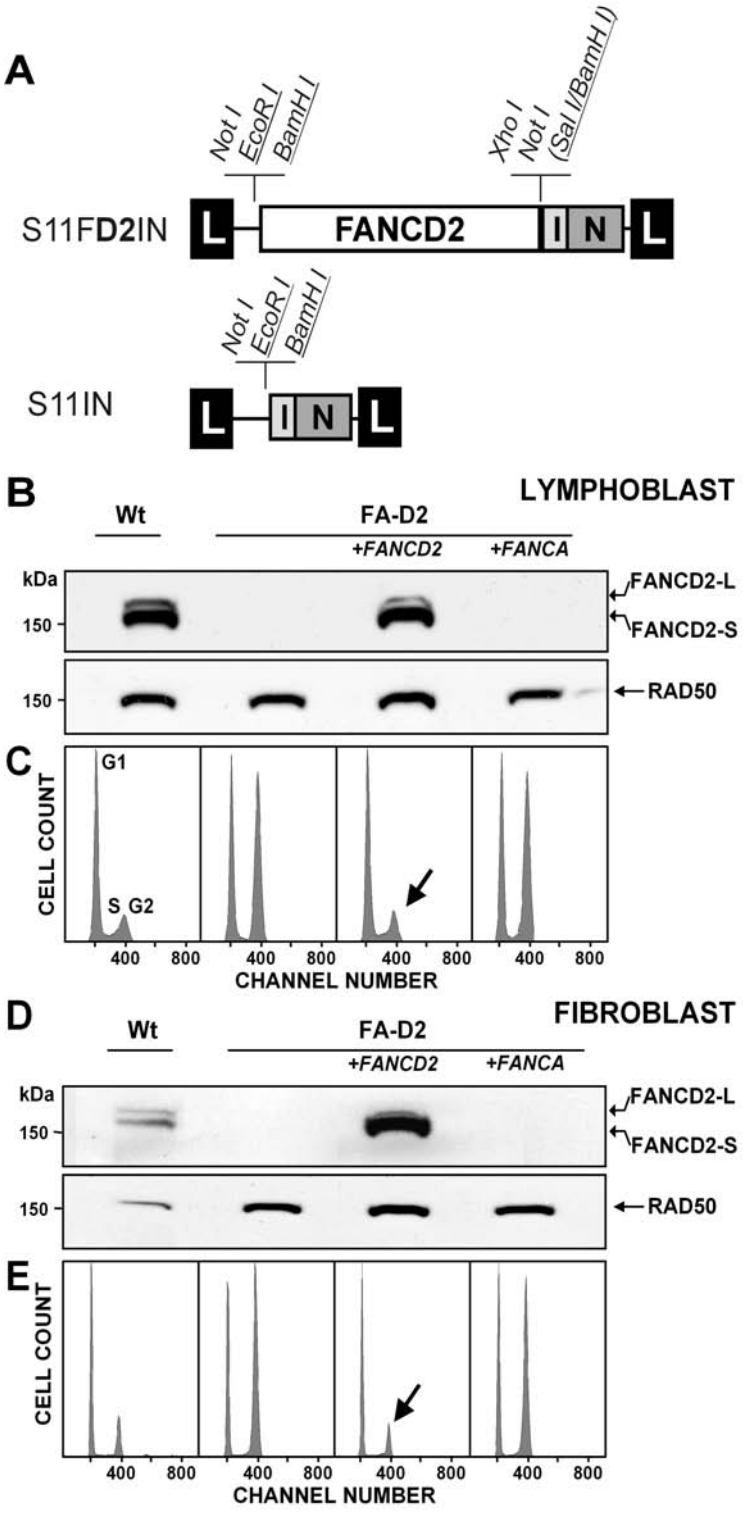

Figure 1. Delineation of group FA-D2. (A) Schematic representation of the retroviral vector construct S11FD2IN expressing *FANCD2* cDNA. The 5' EcoR1 and the 3' Sal I (insert) /BamH I (vector) sites were used for cloning; the two latter were destroyed by blunting. The target vector S11IN without FANCD2 is shown underneath. Abbreviations: L, long terminal repeat; I, internal ribosomal entry site; N, neomycin resistance gene. (B) Assignment to group FA-D2 based on the absence of either FANCD2 band on immunoblots after exposition of the patients' cells to MMC, here shown for a LCL from patient 6 (lane 2). Transduction with *FANCD2* cDNA using S11FD2IN restores both isoforms of FANCD2, S and L (lane 3), similar to a non-transduced normal control (lane 1). Transduction with *FANCA* cDNA in the same vector fails to show such restoration (lane 4). (C) Assignment to group FA-D2 based on cell cycle analysis: After exposition to MMC, the LCL of the same patient shows pronounced G2 phase arrest (56.6%, lane 2, Hoechst 33342 staining). Transduction with *FANCD2* cDNA using S11FD2IN reduces the G2 phase to normal (14.9%, lane 3, arrow), similar to the non-transduced normal control (16.6%, lane 1). Transduction with *FANCA* cDNA in the same vector fails to reverse the G2 phase arrest (53.1%, lane 4). (D) and (E) are analogous to (B) and (C) and show complemention with cultured fibroblasts from patient 10; staining in (E) was with DAPI. G2 phase proportions in (E) are 20.3% (lane 1, control), 61.3% (lane 2, non-transduced FA), 19.9% (lane 3, *FANCD2*-transduced FA) and 58.5% (lane 4, *FANCA*-transduced FA).

of single families, there was no general tendency of families with FA-D2 offspring for increased rates of spontaneous abortions. Among the 28 fully informative FA-D2 patients, there was only a single malignancy (AML) and there was no apparent overrepresentation of tumors or leukemias in the parents or grandparents of the D2 patients in our cohort.

Median age at diagnosis of these FA-D2 patients was 4 y and 5 mo (n=29), median age of transfusion dependency 10 y 10 mo (n=24). Figure 2 compares the progressive hematological course and the outcome of our group of FA-D2 patients to previously reported 754 North American

IFAR patients [41]. BMF in our D2 group (n=24) occurred at an earlier age (median D2 4 y vs. IFAR 6 y 7 mo, p=0.001; Figure 2A), and the period from BMF to HSCT was shorter (D2 n(HSCT)=9, median 5 y 6 mo. vs. IFAR n(HSCT)= 218, median 11 y 4 mo; p<0.08; Figure 2B). HSCT in our D2 patients was earlier than in the IFAR patients of combined groups (median D2 10 y 11 mo vs. IFAR 27 y 11 mo, p<0.01; Figure 2C). Altogether, nine out of 24 FA-D2 patients of our cohort had HSCT. Kaplan-Meier estimates (Figure 2D) suggest that our D2 patients (n=24) may have a shorter overall lifespan as their survival curve falls below that of the IFAR patients after age 9 y; however, the difference of median survival (D2 11 y 4 mo vs. IFAR 24 y 3 mo) was not significant due to only two non-mosaic D2 patients who reached adulthood.

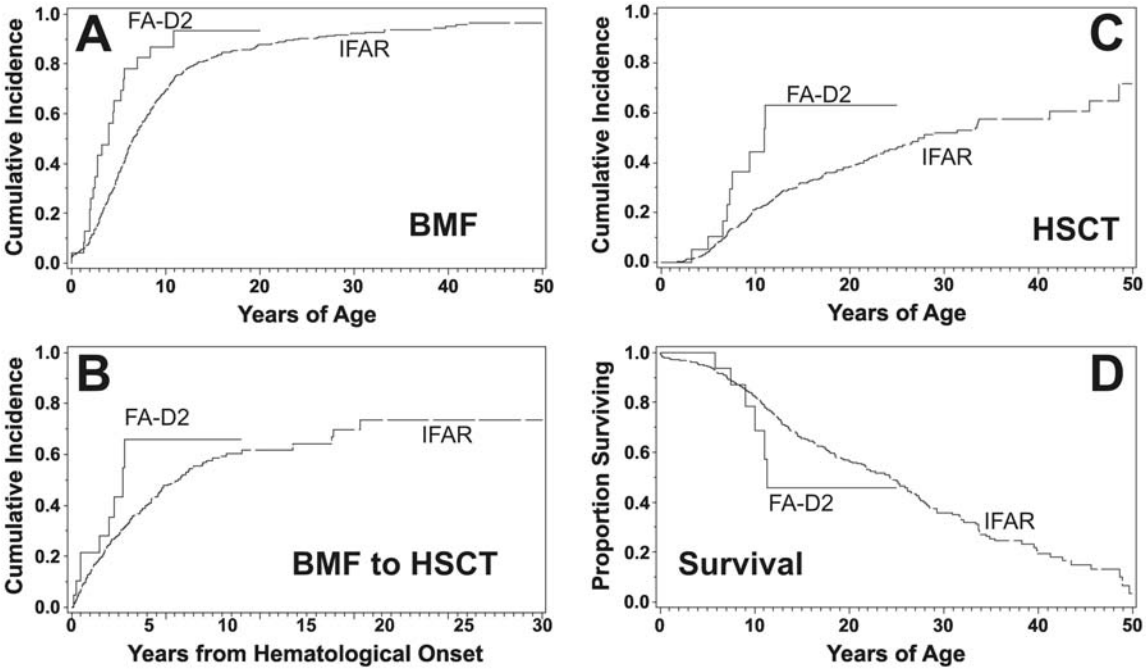

Figure 2. Clinical course of 28 FA-D2 patients in this study. (A) Sequential cumulative incidence of age at diagnosis of FA, transfusion dependency and hematological stem cell transplantation (HSCT) of the D2 patients. (B) Cumulative incidence of bone marrow failure of the FA-D2 patients precedes that of all FA patients in the IFAR. (C) Cumulative incidence of HSCT of the FA-D2 patients likewise antedates that of all FA patients in the IFAR. (D) Kaplan-Meier curves of survival suggest higher death rates of the FA-D2 patients than of all FA patients in the IFAR after 10 years of age.

FANCD2 and the FANCD2 pseudogenes

BLAT searches with the sequence of single *FANCD2* exons and larger genomic fragments identified two pseudogene regions: *FANCD2-P1*, located about 24 kb upstream of *FANCD2* spanning 16 kb and *FANCD2-P2*, located about 1.76 Mb downstream of *FANCD2* spanning 31.9 kb (Figure 3A). *P1* and *P2* are in the same orientation as the functional gene. They are characterized by high homology with certain *FANCD2* exons and have retained ordered arrays of their exon equivalents. On the other hand, the exon replicas of *FANCD2-P1* and *FANCD2-P2* have acquired numerous truncating mutations. Striking sequence similarity of the D2 pseudogenes extends into some intron counterparts, most prominently the regions corresponding to IVS 21 to 26. Thus, *P1* and *P2* reveal recognizable patterns of conserved gene structure (Figure 3B). *FANCD2-P1* is a rough copy of the front portion of *FANCD2* including, with intermittent gaps, the region of exons 1 through 18 (homology with *FANCD2* exons 1, 12 to 16 and the 3´ portion of exon 18). The region upstream of *FANCD2-P1* shares homology with the putative *FANCD2* promotor predicted within the CpG island-containing region of approximately 800 bp upstream of the start codon of the functional gene (http://bioinfo.hku.hk/cpgieintro.html). The corresponding region upstream of *P1* is interrupted by

an *AluY* element. *FANCD2-P2* is an approximate match of the middle portion of *FANCD2* spanning, also with gaps, the region of exons 12 through 28 (homology with *FANCD2* exons 12 to 14 and 17 to 28).

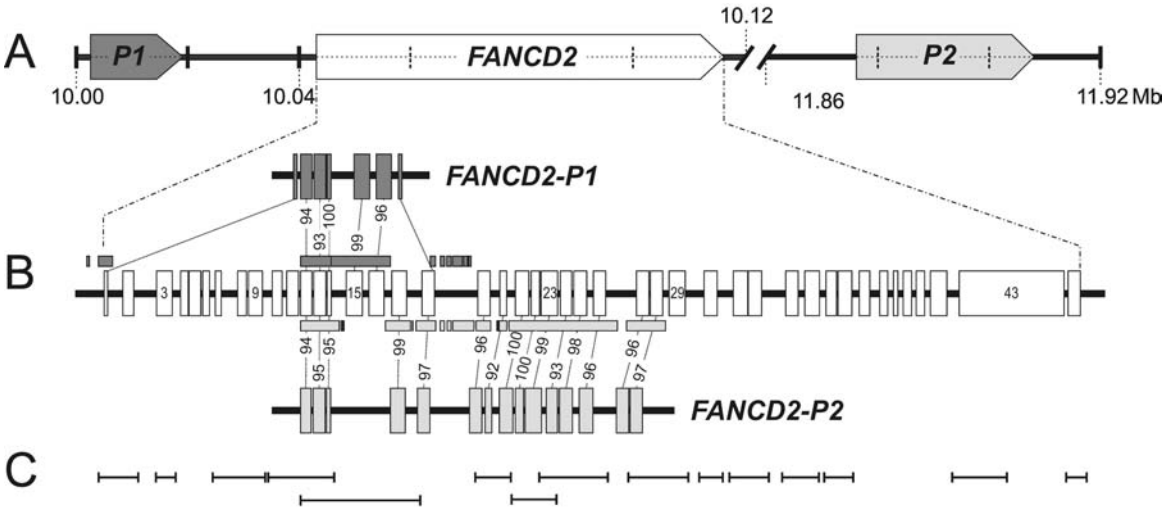

Figure 3. Topography of *FANCD2*, its pseudogenes and the superamplicons. (A) The two pseudogenes, *FANCD2-P1* and *FANCD2-P2*, are located upstream and downstream of the functional *FANCD2* gene, respectively. All three have the same orientation. The scale denotes Mbp on chromosome 11. (B) *FANCD2* exons and their pseudogene equivalents and are connected by dashed lines containing percentages of nucleotide identity. Homology also extends into many introns nearby as indicated by the boxes beyond and below the active gene. (C) Graphical presentation of the positions and sizes of 15 superamplicons relative to the active gene in B. These amplicons represent *FANCD2* exon-exon or exon-intron regions. Unique primer binding sites ensure specific amplification.

Mutations in FANCD2

Unique amplification of the functional *FANCD2* gene using primers excluding pseudogene sequences resulted in 15 superamplicons (Figure 3C) that were used for genomic mutation screens. Studies at the RNA level were implemented to guide the genomic analyses. All mutations identified and their predicted consequences at the protein level are compiled in Table 1. The distribution of the mutations among the individual patients is shown in Supplementary Table S4.

Mutations affecting pre-mRNA splicing

In PBLs, LCLs and cultured fibroblasts from normal controls two species of *FANCD2* cDNAs were consistently detected on sequencing of the regions corresponding to exon 22 and to exons 15 to 17. Sequence analysis revealed low-level skipping of exon 22 (Figure 4A and B) and of exons 15 to 17 (data not shown) consistent with FANCD2 RNA being subject to alternative splicing of these exons. This finding was confirmed by mRNA stabilization via CHX treatments of cultured cells, which resulted in a relative increase of the alternatively spliced mRNA species (Figure 4A and C). Stabilization by CHX also implied inherent instability of the alternatively spliced FANCD2 mRNAs.

Without CHX treatments, cell lines from patients 2, 8, 9, 10, 14, 15 and 20 in our cohort displayed almost equal levels of exon 22 skipping and retention (Figure 4A and D). Patients 3, 4, 5 and 13 showed nearly complete exon 22 skipping, but we consistently observed a small amount of correctly spliced mRNA retaining exon 22 (Figure 4A and E). Genomic sequencing identified a common underlying mutation, the base substitution c.1948-16T>G in IVS21. Homozygosity for this mutation was observed in patients 3, 4, 5 and 13 with nearly complete skipping of exon 22 and also in the deceased patient 25. All of these patients were products of consanguineous matings.

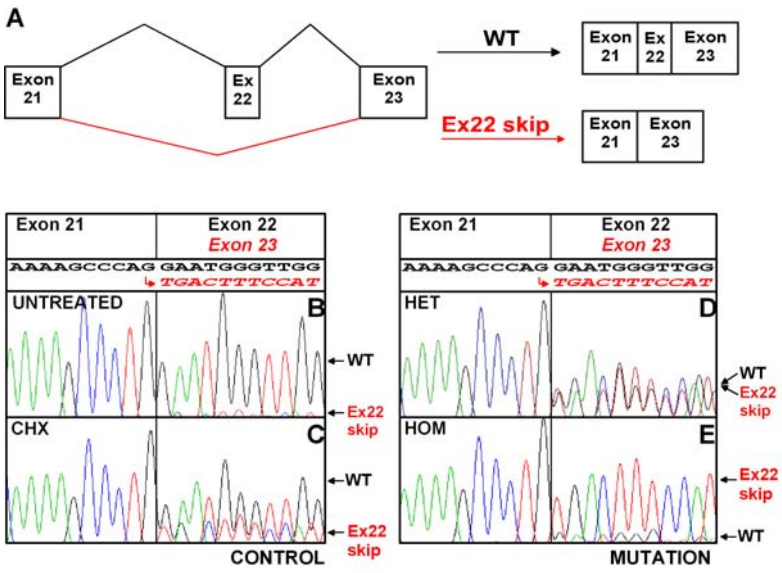

Figure 4. Exon 22 splicing. (A) Schematical depiction of the splicing patterns resulting from exon 22 retention or skipping. (B) cDNA sequencing in normal controls shows abundance of exon 22 sequence following that of exon 21 but also low level underlying sequence readable as exon 23. (C) Treatments of normal control cells with CHX for 4 h prior to cDNA synthesis increase the relative level of sequence with exon 22 skipping. (D) Heterozygotes for splice acceptor mutations in intron 21 show comparable levels of inclusion and exclusion of exon 22 sequence following that of exon 21. (E) Homozygotes for splice acceptor mutations in intron 21 reveal abundance of exon 23 sequence following that of exon 21 but also low level underlying sequence readable as exon 22.

Patients 9 and 10 were compound heterozygous carriers of the same mutation showing balanced levels of exon 22 skipping and retention.

A different base substitution preceding exon 22, c.1948-6C>A, was present on one allele of patients 2, 8, 14, 15 and 20, likewise resulting in similar levels of exon 22 skipping and retention. Both mutations, c.1948-16C>T and 1948-6C>A, are predicted to weaken the splice acceptor recognition of intron 21 by impairing the scores of the 3´splice site relative to wildtype (cf. Supplementary Table S6A). c.1948-16T>G does so by disruption of the pyrimidine-rich tract preceding the splice acceptor in intron 21, whereas c.1948-6C>A leads to the loss of two putative intronic splice enhancer (ISE) motifs in exchange for the creation of a single new one (http://rulai.cshl.edu/tools/ESE).

Three apparently unrelated patients (patients 6, 12 and 30) showed skipping of exon 5 due to insertional mutagenesis of an *Alu* element between positions c.274-57 and c.274-56 into an AT-rich target sequence in IVS4. This *Alu* was identical to the evolutionary young subfamily Yb8 [42,43]. It was lacking its annotated nucleotides 1-35, had integrated in reverse orientation (with its poly-A tail towards the 5´ end of *FANCD2*) and had duplicated the 13-nt sequence c.274-69 to c.274-57 of *FANCD2* IVS4 such that this duplicated sequence flanked the *Alu* repeat on either side. Altogether the insertion was 298 bp long. Integration site, type, length and orientation of the *Alu* and the duplicated FANCD2 intron sequence were identical in all three patients.

Aberrant splicing was also observed for exons 4, 5, 10, 13, 15-17, 28 and 37 in other patients. Patients 28 and 29 showed skipping of exon 4 due to a base substitution in the preceding canonical splice acceptor site (c.206-2A>T). Patients 26 and 27 had a base substitution in exon 5 (c.376A>G) abrogating the recognition of the downstream splice donor. This change led to the inclusion of 13 bp of IVS5 into the transcript by activating a cryptic 5'-splice site in intron 5 (r.377_378ins13; also previously reported [25]). Patient 18 showed skipping of exon 10 due a base substitution in the upstream splice acceptor (c.696-2A>T). Exon 10 skipping was observed in patient 31 who had a substitution of the last minus one base of exon 10 (c.782A>T). In patient 8, we detected a splice acceptor mutation upstream of exon 13 (c.990-1G>A). This change results in the activation of a cryptic splice acceptor 8 bp downstream and exclusion of the corresponding sequence from the mature mRNA. Of particular interest was a 2-bp deletion in exon 16 (c.1321_1322delAG) in patient 18 causing skipping of exons 15 to 17. In this case, aberrant splicing occurs in the same way but at heterozygous levels compared to low-grade alternative splicing in normal controls. Patients 10 and 22 showed inclusion of a 27-bp sequence of intron 28 into mRNA due to a splice

Table 1. Identified FANCD2 mutations and their effects

Location Exon/Intron	Mutation* gDNA	RNA	Consequence* Protein	Patient No.
Exon 2	c.2T>C	r.2T>C	*Failure of normal translation initiation*	32
Intron 3	c.206–2A>T *(IVS3–2A>T)*	r.206_273del68 *(exon 4 skipping)*	p.A69DfsX7	28, 29
Intron 4	c.274–57_–56insinv*AluYb8*nt36_319 +dup c.274–69_–57	r.274_377del104 *(exon 5 skipping)*	p.I92YfsX7	6, 12, 30
Exon 5	c.376A>G	r.376A>G+r.377_378ins13 *(aberrant splicing)*	p.S126RfsX12	26, 27
Exon 9	c.692T>G	r.692T>G	p.L231R	19
Intron 9	c.696–121C>G *(IVS9–121C>G)*	r.695_696ins34 *(exonization)*	p.S232insQNNFX	1
	c.696–2A>T *(IVS9–2A>T)*	r.696_783del88 *(exon 10 skipping)*	p.S232RfsX6	18
Exon 10	c.757C>T	r.757C>T	p.R253X	23, 24
	c.782A>T	r.696_783del88 *(exon 10 skipping)*	p.S232RfsX6	31
Exon 11	c.810_812delGTC	r.810_812delGTC	p.S271del	9
Exon 12	c.904C>T	r.904C>T	p.R302W	7
Intron 12	c.990–1G>A *(IVS12–1G>A)*	r.990del8 *(aberrant splicing)*	p.S330RfsX16	8
Exon 13	c.1092G>A	r.1092G>A	p.W364X	7
Exon 16	c.1321_1322delAG	r.1135_1545del411 *(exon 15-17 skipping)*	p.V379_K515del	18
	c.1367T>G	r.1367T>G	p.L456R	23, 24
	c.1370T>C	r.1370T>C	p.L457P	31
Exon 17	g.22875_23333del459 (c.1414–71_c.1545+256del459)	r.1414_1545del132	p.E472_K515del	28, 29
Intron 21	c.1948–16T>G *(IVS21–16T>G)*	r.1948_2021del74 *(exon 22 skipping)*	p.E650X	3, 4, 5, 9, 10, 13, 25
	c.1948–6C>A *(IVS21–6C>A)*	r.1948_2021del74 *(exon 22 skipping)*	p.E650X	2, 8, 14, 15, 20
Exon 26	c.2404C>T	r.2404C>T	p.Q802X	21
	c.2444G>A	r.2444G>A	p.R815Q	16, 17, 19, 21, 22, 30
Exon 28	c.2660delA	r.2660delA	p.E888RfsX16	20
Intron 28	c.2715+1G>A *(IVS28+1G>A)*	r.2715_2716ins27 *(aberrant splicing)*	p.E906LfsX4	10, 22
Exon 29	c. 2775_2776CC>TT	r. 2775_2776CC>TT	p.R926X	14, 15
	c.2835dupC	r.2835dupC	p.D947RfsX3	11
Exon 34	c.3453_3456delCAAA	r.3453_3456delCAAA	p.N1151KfsX46	12
Exon 36	c.3599delT	r.3599delT	p.I1200KfsX12	2
Exon 37	c.3706C>A	r.3684_3707del24 *(aberrant splicing)*	p.R1228S_F1235del	32
	c.3707G>A	r.3684_3727del44 *(aberrant splicing)*	p.H1229EfsX7	11
Exon 38	c.3803G>A	r.3803G>A	p.W1268X	6, 26, 27

* Nomenclature according to the Human Genome Variation Society (http://hgvs.org/mutnomen/recs)

donor mutation (c.2715+1G>A) and the usage of a cryptic splice donor downstream. Patient 11 had a base substitution in exon 37 (c.3707G>A) that abrogates the normal splice acceptor 25 bp upstream and activates a cryptic one 19 bp downstream of the mutation, resulting in skipping of 44 bp. Interestingly, an adjacent base substitution (c.3706C>A) in patient 32 generates a new splice acceptor that is used instead of the normal one 23 bp upstream, leading to skipping 24 bp. These substitutions affecting adjacent bases resulted in the creation or activation of a new 3´ splice sites within exon 37 while the normal acceptor remains intact. All of these splicing aberrations were

due to heterozygous mutations whereas patient 1 showed a homozygous exonization of an intron 9 fragment due to an intron mutation that creates a new splice donor (c.696-121C>G). Predicted effects of some of these splice mutations are computed in Supplementary Tables S6. Apart from 1321_1322delAG causing skipping of exons 15-17, all mutations affecting splicing in the patients of this study result in a frameshift and subsequent premature termination of translation. More than half, i.e., 30/58 mutation alleles of the 29 fully informative FA-D2 patients, or 33/64 of all identified mutations were splicing mutations. Clearly, the most prevalent effect of *FANCD2* mutations involves aberrant splicing patterns resulting from a diverse spectrum of sequence alterations.

Other mutations

There were five different heterozygous nonsense mutations in nine patients from six families (c.757C>T, siblings 23 and 24; c.1092G>A, patient 7; c.2404C>T, patient 21; c.2775_2776CC>TT, siblings 14 and 15; c.3803G>A, patient 6, siblings 26 and 27; Table 1 and Supplementary Table S4). In addition, we detected five different missense mutations in eleven patients from nine families (c.692T>G, patient 19; c.904C>T, patient 7; c.1367T>G, siblings 23 and 24; c.1370T>C, patient 31; c.2444G>A, siblings 16 and 17, patients 19, 21, 22 and 30). These amino acid substitutions were classified as missense mutations because of their absence from normal controls, due to their absence from FA-D2 patients of our cohort with other biallelic mutations, and because of their occurrence at evolutionary conserved residues. Missense mutations were either compound heterozygous in combination with other types of *FANCD2* mutations or homozygous in consanguineous families. Three unrelated patients had small deletions (c.2660delA, patient 20; c.3453_3456delCAAA, patient 12; c.3599delT, patient 2) resulting in frameshifts. Another small deletion was in frame and affected a single codon (c.810_812delGTC, patient 9). A large genomic deletion (g.22875_23333del459) spanning the entire exon 17 and adjacent 71 bp of intron 16 and 256 bp of intron 17 was found in sibling pair 28 and 29. This deletion resulted in a net loss of 41 aa, but retained the reading frame. There was only a single small frameshift duplication (c.2835dupC, patient 11). In all of our patients, nonsense mutations, deletions and insertions were exclusively affecting single alleles in

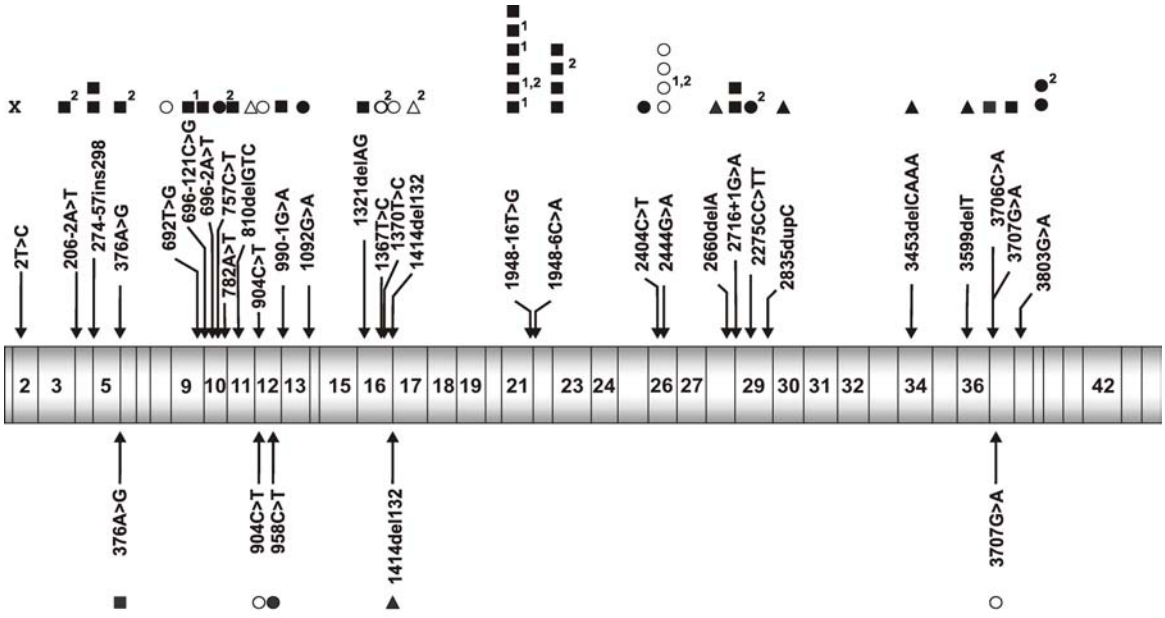

Figure 5. Positions and identity of mutations detected in *FANCD2*. Mutations identified in the present study are shown above, previously reported mutations [25] underneath the schematical display of *FANCD2* cDNA. Solid squares (■) represent mutations resulting in aberrant splicing patterns, solid circles (●) nonsense mutations, open circles (○) missense mutations, solid triangles (▲) frameshift deletions or insertions and open triangles (Δ) *in frame* deletions. [1] denotes homozygous occurrence (2 alleles), [2] affected sibling (relationship bias).

combination with splice or missense mutations. A unique case was a compound heterozygous start codon mutation (c.2T>C) in patient 32.

Figure 5 illustrates the distribution of *FANCD2* mutations that were identified in this study, including those of three FA-D2 patients previously reported [25].

Ethnic associations and shared alleles

Relatively severe birth defects and early hematological onset were observed in three patients (4, 5 and 13) homozygous for the splice mutation c.1948−16T>G with exon 22 skipping. These three patients and two other homozygotes with reverse mosaicism (patients 3 and 25) were from four consanguineous Turkish families. Of two FA-D2 patients compound heterozygous for this mutation, one was also of Turkish origin, the other came from an eastern region of the Czech Republic. The splice mutation c.1948-6C>A, likewise leading to exon 22 skipping, was detected in five patients (patients 2, 8, 14, 15 and 20), including two sisters (patients 14 and 15). These patients came from three families in northern Germany and a German emigrant family in the US (patient 20). They presented with intermediate phenotypic and hematological severity. Relatively mild birth defects and a protracted hematological course into adulthood was observed in two siblings (patients 16 and 17) with the homozygous missense substitution c.2444G>A from a consanguineous Spanish family. Of four compound heterozygotes for this mutation with mild disease manifestations, one had mixed ethnicity (patient 19), one was Hispanic American (patient 21), one had Sicilian (patient 22) and another Spanish and Portuguese ancestry (patient 30). The insertion of an *Alu*Yb8 element was found compound heterozygous in a patient each of German (patient 6), Danish (patient 12), and Spanish/Portuguese FA-D2 (patient 30) descent. We therefore considered the latter mutation as recurrent rather than ethnically associated. All other mutations did not occur in more than two families.

On haplotype analysis, all patients homozygous for the mutation detected in the Turkish population (c.1948-16T>G; patients 3, 4, 5, 13 and 25) were homozygous for markers D3S1597, D3S1938, D3S3611 and D3S1675. The resulting haplotype was shared, in the heterozygous state, with the non-consanguineous compound heterozygous Turkish patient (no. 10). The Czech patient (no. 9) with this mutation had a different haplotype. Lack of homozygotes for the intron 21 mutation prevalent in the German population (c.1948-6C>A; patients 2, 8, 14, 15 and 20) and unavailability of patients' parents precluded construction of a mutation-associated haplotype. However, all patients with this mutation had one or two identical marker(s) at least on one side of their mutated *FANCD2* gene. This finding suggests that c.1948-6C>A is an old mutation with gradual erosion of an ancient haplotype. The consanguineous siblings (patients 16 and 17) homozygous for the mutation prevalent in Spanish or Southern European populations (c.2444G>A) were also homozygous for the set of markers used. Of their common haplotype, the microsatellite markers adjacent to *FANCD2* were shared with a Hispanic patient (no. 21), a patient with Sicilian ancestry (no. 22) and a patient of Spanish/Portuguese descent (no. 30), all compound heterozygotes for this mutation. Additional support for a conserved haplotype came from linkage disequilibrium. All of the patients homo- or heterozygous for the mutation c.2444G>A were also homo- or heterozygous for the polymorphism c.2702G>T (p.G901V). Differential allelic stability in compound heterozygotes, and sequence analysis of the parents indicated that both substitutions were on the same allele. A single patient (no. 19) with the mutation c.2444G>A neither had a shared haplotype nor the polymorphism c.2702G>T. Apart from c.2702G>T that was also observed without association with the mutation c.2444G>A, the only new *FANCD2* polymorphisms detected in our study were c.3978C>T and c.4478A>G in the 3'-UTR, all others have been previously reported [25]. Despite clear ethnical association of the patients with the insertion of an *Alu*Yb8 element in intron 4, it nevertheless seems unlikely that an identical event would have occurred three times independently. Two of these patients (6 and 12) shared all of the four markers studied. Patient 30 with the same mutation had retained a single identical marker adjacent to *FANCD2*. A base substitution in the *Alu* sequence, 260G>A, present

in all three cases but in less than 10% of complete *Alu*Yb8 elements in the human genome (http://genome.ucsc.edu/cgi-bin/hgBlat) further suggested that the *Alu* insertion goes back to a single event and is an ancient rather than a recurrent mutation.

Reverse mosaicism

Among the 29 FA-D2 patients with phenotypic information in this study, five developed reverse mosaicism in the hematopoietic system. Mosaic patients were recognized by the facts that they had levels of both FANCD2-S and -L in protein from LCLs, comparable to normal controls (Figure 6A), that they had low chromosome breakage rates in blood and blood-derived LCLs (Supplementary Table S4) and that they had lost the typical G2 phase arrest of their lymphocytes after exposure to MMC (Figure 6B). Nonetheless, these patients had the characteristic clinical FA phenotype and their cultured fibroblasts had preserved MMC sensitivity, indicated by elevated chromosome breakage and G2 phase blockage (Figure 6B). Molecular studies confirmed these findings. Two patients with heterozygous base substitutions in the coding sequence, resulting in a nonsense (patient 14) and a splice mutation (patient 26), showed reversion to the respective wildtype bases in primary blood cells and LCLs. The mechanism of these reversions is not clear and could involve back mutation, recombination with LOH or recombination with gene conversion. Intragenic mitotic crossover is the likely but not proven mechanism of mosaicism in the sibling of patient 14 (no. 15) who had retained her dinucleotide substitution in her peripheral blood cells. Two patients (3 and 25) with the c.1948-16T>G splice mutation had different second site compensatory mutations nearby. Clinically, 3/5 mosaic patients (3, 14 and 15) in the present cohort experienced a mild or protracted hematological course. The other 2/5 patients (25 and 26) had no apparent benefit from their mosaicism; one of them required HSCT and the other died of intracranial hemorrhage (Supplementary Table S5). The rate of 17% mosaic FA-D2 patients in our study is within the 15% [44] to 20% [45] or 25% [46] range reported for other complementation groups. With a rate comparable to *FANCA*, *FANCD2* appears to be another FA gene particularly prone to reverse mosaicism.

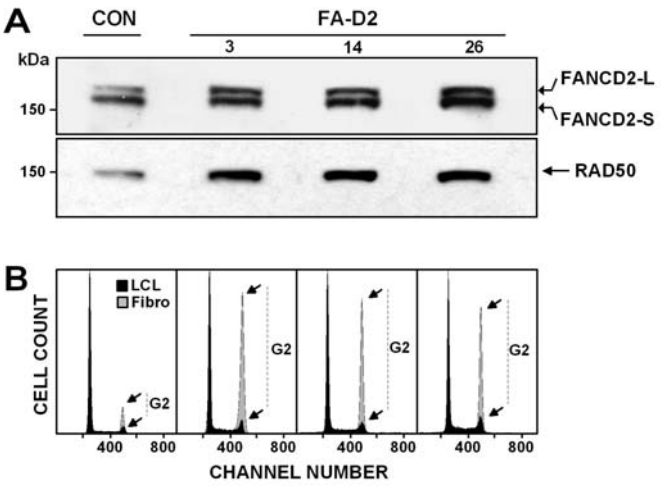

Figure 6. Revertant mosaicism. (A) Blood-derived cells from FA-D2 patients with revertant mosaicism of the hematopoietic system reveal both FANCD2 bands at levels similar to a random normal control (patients 3 and 26, LCLs; patient 14, stimulated PBL) after exposure to MMC. RAD50 was used as loading control. (B) In addition, these LCLs and PBL fail to show G2 phase arrest on flow cytometric cell cycle distributions in response to MMC (black histograms, DAPI stain; CON, 8.0% G2; patient 3, 8.8% G2; patient 14, 8.8% G2; patient 26, 10.4% G2), whereas corresponding cultured FA-D2 fibroblasts retain high G2 phase accumulations, which is in contrast to the non-FA control (superimposed grey histograms; CON, 22.6% G2; patient 3, 53.2% G2; patient 14, 56.0% G2; patient 26, 54.8% G2).

Residual FANCD2 protein

A surprising finding was the presence of residual FANCD2 protein in PBLs and LCLs of every FA-D2 patient tested. Detection of residual protein required overexposure of FANCD2 immunoblots (Figure 6C). Unlike standard exposure that showed no FANCD2 bands in most of the FA-D2 cell lines (cf. Figure 1), both FANCD2-S and FANCD2-L bands were detected when films were exposed overnight. As the study progressed, it became evident that the cell lines initially detected with residual protein were those with the highest levels. When we systematically re-examined all of our FA-D2

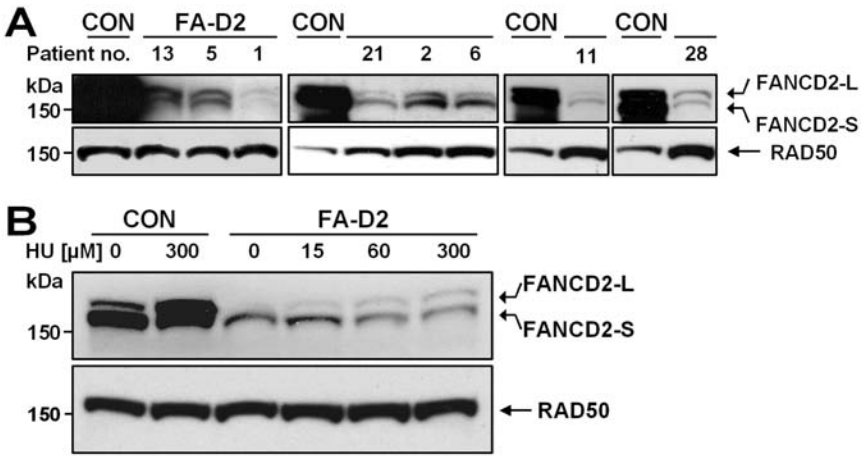

Figure 7. Residual FANCD2 protein. (A) Blood-derived cells from non-revertant FA-D2 patients (examplified 13, 5, 1, 21, 2, 6, 11 and 28) show faint, but conspicuous FANCD2 bands of both species in response to MMC exclusively on overexposed immunoblots as indicated by the very intense FANCD2 signals of the normal controls (patient 13, stimulated PBL; patients 5, 1, 21, 2, 6, 11 and 28, LCLs; loading control RAD50). The individual abundance of residual protein varies considerably at low levels. (B) LCLs were subjected to the indicated concentrations of hydroxyurea (HU) for 16 h. On an overexposed blot, the FANCD2-L band of the residual protein in the LCL from patient 21 increases with the HU concentration in a dose-response reaction. This reaction is similar to that of a normal control LCL discernible by its prominent FANCD2 signals.

lines, 21 LCLs from our 29 fully informative FA-D2 patients had minute but unequivocal amounts of residual protein (Supplementary Table S4). This was also true for CD3/CD28/IL-2 stimulated PBL cultures from patient 13. Primary fibroblasts normally have lower levels of FANCD2 relative to total protein than LCLs, and this might be the reason why detection of residual FANCD2 remained ambiguous in a prenatal case (patient 19) with only fibroblasts available. Because of lack of LCLs, two affected siblings (patients 4 and 17) could not be tested. Finally, five of our patients were mosaic leaving 8/29 patients unconfirmed for residual protein. Given the close to normal amounts of FANCD2 protein in the mosaic patients and the fact that the non-mosaic patients had high chromosome breakage rates and G2 phase arrest, we consider it unlikely that undetected mosaicism accounts for the presence of residual protein in the remainder of our patients. Densitometry suggested reductions of residual FANCD2 protein in the order of 1/100 to 1/1000 relative to wildtype, with the expression differing greatly amongst individual LCLs (Figure 7A). FA-D2 LCLs with the highest levels of residual FANCD2 were used to examine its characteristics on overexposed blots. The intensity of the FANCD2-L band was found to increase as a function of the concentration (Figure 7B) of agents inducing replication fork stalling and the period of treatment (not shown). This time and concentration dependency suggests genuine biochemical activity of the residual FANCD2 protein, implying that most if not all cases of FA-D2 result from functionally hypomorphic mutations.

Discussion

Our results suggest that FA-D2 is a more frequent FA complementation group than previously reported [2,22,23]. The relatively large proportion of Turkish FA-D2 patients in the present study–about 10% of the patients studied in Germany–appears to be due to a founder effect for the *FANCD2* mutation c.1948-16T>G among individuals of Turkish origin. This is similar to the disparity in the frequency of FA-C patients in the IFAR database compared to the European FA population. The proportion of FA-C patients in the IFAR is 15% [47], compared to only 10% in the European dataset [2]. This is due to the relatively high frequency of Ashkenazi Jewish FA patients in the IFAR with the prevalent *FANCC* mutation c.456+4A>G (formerly IVS4+4A>G), comprising 7.5% of all IFAR patients and 50% of their FA-C patients. We calculate roughly that about 6% of FA patients belong to complementation group D2, which is supported by independent studies with a figure of 4/53 ≈ 7.5% [44] and an estimate of 5% [47], as recently reported.

The D2 patients in our cohort displayed anomalies and malformations typical of FA such that

there were no exceptional clinical features that had not previously been observed [48]. However, it is remarkable that not a single D2 patient lacked phenotypical manifestations, whereas the proportion of FA patients without anomalies and malformations is generally estimated as high as 30% [22]. Growth retardation was present in 86% of the present cohort, substantially higher than the 58% [49] and 63% [22] reported. Microcephaly was present in 89% of the FA-D2 cases; in contrast, Faivre et al. [49] found anomalies of the head in only 56%. Anomalies of skin pigmentation were present in 75% of our FA-D2 cohort compared to 71% and 64% [22,49]. 72% of our FA-D2 patients had radial ray defects in contrast to only 47% [49] or 49.1% [48] of all FA patients. 61% of the patients in the present study had microphtalmia, whereas 38% have been reported in other FA patients [22]. As with these rather common phenotypic alterations, FA-D2 patients showed also higher rates of rare FA features such as psychomotor retardation and hyperactivity attention deficit disorder. Psychomotor retardation was present in 29% of our FA-D2 cohort versus 12% or 10% mentally retarded individuals in other studies [22,49]. As many as 31% of our FA-D2 patients had anomalies of the brain, whereas other studies report such alterations in the order of 4.5% [49], 7.7% [48] and 8% [22] of their FA patients. 17% of our D2 patients with brain anomalies had hydrocephalus, in contrast to 4.6% reported [48]. Since several labs contributed to the present study, and since all of our D2 patients came from previously unassigned FA patients, it is unlikely that our rates reflect major biases. A more severe D2 phenotype has also been observed in drosophila comparing *FancD2* and *FancL* knock-down [21]. Given the high frequency of phenotypic alterations, it is not surprising that in 30% of our FA-D2 patients the diagnosis of FA was made by the age of 2 y, and the median of age at diagnosis was 4.5 y which is considerably younger than in other FA patients where in 30% the diagnosis is made before onset of hematological manifestations at the median age of 7.6 y [50]. In addition to an earlier median age of hematological onset (BMF) in our FA patients, there was a shorter median period between BMF and HSCT, earlier HSCT and a tendency towards shorter median survival than all FA in the IFAR [41]. However, due to relatively small numbers and the relative deficit of older patients in our cohort, statistical significance was not reached for all of these end points. HSCT appears to be a therapeutic option also in group FA-D2, as 9/24 non-mosaic patients of our cohort suggest, although ATR-dependent activation of FANCD2 [51,52] could involve additional toxicity of conditioning. Again, compared to studies of other FA patients our data suggests that FA-D2 patients represent a group with frequent but typical congenital anomalies and malformations, and with relatively early hematological manifestations, compared to most other FA complementation groups.

The most prevalent effect of *FANCD2* mutations were aberrant splice patterns caused by single base substitutions in introns and exons. In contrast to the *FANCA* gene, in which large deletions are frequent, there was only a single large genomic deletion detected in the present cohort of FA-D2 patients. This included exon 17 and adjacent genomic sequence similar to an exon 17 deletion previously reported without defined breakpoints [25]. Homologous unequal recombination between repetitive sequence elements is a common cause of gross gene deletions [53] and *Alu* repeats flanking deletion breakpoints have been identified in many human genetic disorders. Accordingly, an above average density of *Alu* elements should promote higher rates of large deletions, as in the *FANCA* gene. It contains 119 *Alu*s occupying 39.3% of the 79096-bp sequence (http://www.ensembl.org, www.repeatmasker.com), and between 32% [54] and 40% [55] of the FA-A patients involve large intragenic deletions. There is a highly significant correlation between the number of breakpoints detected in a given intron and the corresponding number of *Alu* repeats, suggesting *Alu*-mediated recombination as explanation for the high prevalence of deletions in *FANCA* [53]. Similarly, there are 83 *Alu* elements covering 30.1% of the 74853-bp sequence of *FANCD2* which is more than twice the 10 to 15% in the human genome at an average spacing of 4 kb. On this basis, large deletions clearly are underrepresented in our D2 cohort.

Among the FA proteins, FANCD2 is unique since the presence of residual protein and the demonstration of its activation can be accomplished in a single assay. The high quality of FANCD2 monoclonal antibodies, the occurrence of the typical pair of FANCD2-S and -L bands and the

induction of FANCD2-L by characteristic types of DNA damage render FANCD2 immunoblots highly informative. In our cohort of FA-D2 patients, not merely LCLs but also short term PBL cultures from altogether 21/21 fully informative patients studied showed traces of residual FANCD2 protein. Importantly, the residual protein always consisted of both FANCD2 isoforms, and the typical time- and dosage-dependent induction of FANCD2-L was maintained, suggesting a preserved function. Differences in expression levels of residual FANCD2 between individual LCLs might result from variations of conserved splice site recognition, in mRNA and protein stability, and, very clearly, from differences in cell growth. FANCD2 is highly expressed and monoubiquitinated in the S-phase of the cell cycle [8,56]. The proportion of S-phase cells is a function of cell growth such that differences in cell proliferation between individual cell lines account for the wide variation of FANCD2 protein levels and render any quantitative mutation-specific comparisons of residual FANCD2 protein levels close to impossible. The existence of residual protein has previously been described for other FA-D2 patients [7,25,44] but our study confirms residual protein as a consistent and in all likelihood essential feature of FA-D2 patient cells. Somatic reversion as a cause of residual protein levels could be excluded because the diagnosis of Fanconi anemia in all of our D2 patients was based on hypersensitivity towards crosslinking agents, and only five patients developed MMC or DEB resistance in PBLs indicating somatic reversion.

FANCD2 is targeted to chromatin following DNA damage-dependent monoubiquitination where it interacts with the highly conserved C-terminal region of BRCA2 [57]. FANCD2-L promotes BRCA2 loading onto a chromatin complex that is required for effective, but RAD51-independent DNA repair [13,58]. The examination of DT40 cell lines revealed that components of the FA core complex have additional functions in DNA repair pathways which seem to be independent of monoubiquitination and chromatin targeting of FancD2 [59]. However, the common pathway of FANCD2 and FANCD1/BRCA2 appears to be crucial for functional resolution of ICL-induced stalled replication forks and, in order for humans to be viable, may require residual protein activity.

Despite a rather severe phenotype in most of the FA-D2 patients, the vast majority of our FA-D2 patients were found to carry leaky mutations affecting splicing and displayed residual FANCD2 protein of both isotypes in their cell lines. Splicing mutations have become an increasingly successful target for experimental therapeutic approaches. Modified and antisense oligonucleotides have been used to inhibit cryptic exons or to activate regular exons weakened by mutations via targeting of the oligonucleotides to the desired transcript. This approach could eventually lead to effective therapies for the correction of erroneous splicing (reviewed in [59,60]). The tight regulation of FANCD2 expression and activation, and the presence of low-abundant wildtype gene products associated with *FANCD2* mutations should render *FANCD2* an ideal candidate for RNA-reprogramming strategies such as spliceosome-mediated RNA trans-splicing (SMaRT; reviewed in [60,61]).

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Supplementary tables

Supplementary Table S1A. *FANCD2* cDNA amplification primers

PCR Fragment	Designation	Binding Position	Sequence (5´→3´)	Designation	Binding position	Sequence (5´→3´)	PCR Product Size (bp)
1	FA-D2, Fr.1 F	-47 to -27	GCGACGGCTTCTCGGAAGTAA	FA-D2, Fr.1 R	998 to 976	CTGTAACCGTGATGGCAAAACAC	998
2	FA-D2, Fr.2 F	763 to 787	GACCCAAACTTCCTATTGAAGGTTC	FA-D2, Fr.2 R	1996 to 1975	CTACGAAGGCATCCTGGAAATC	1234
3	FA-D2, Fr.3 F	1757 to 1777	CGGCAGACAGAAGTGAATCAC	FA-D2, Fr.3 R	2979 to 2958	GTTCTTGAGAAAGGGGACTCTC	1223
4	FA-D2, Fr.4 F	2804 to 2829	TTCTACATTGTGGACTTGTGACGAAG	FA-D2, Fr.4 R	3942 to 3922	GTCTAGGAGCGGCATACATTG	1139
5	FA-D2, Fr.5(L) F	3761 to 3781	CAGCAGACTCGCAGCAGATTC	FA-D2, Fr.5(L) R	4700 to 4679	GACTCTGTGCTTTGGCTTTCAC	940

Supplementary Table S1B. *FANCD2* cDNA sequencing primers

Designation	Binding Position	Sequence (5´→3´)	Designation	Binding position	Sequence (5´→3´)
sFA-D2, 244 F	244 to 263	ACCCTGAGGAGACACCCTTC	sFA-D2, 367 R	367 to 347	CATCCTGCAGACGCTCACAAG
sFA-D2, 545 F	545 to 566	GGCTTGACAGAGTTGTGGATGG	sFA-D2, 621 R	621 to 600	CAGGTTCTCTGGAGCAATACTG
sFA-D2, 1011 F	1011 to 1033	CAGCGGTCAGAGCTGTATTATTC	sFA-D2, 951 R	951 to 929	CTGTAACCGTGATGGCAAAACAC
sFA-D2, 1308 F	1308 to 1327	GTCGCTGGCTCAGAGTTTGC	sFA-D2, 1158 R	1183 to 1158	TCTGAGTATTGGTGCTATAGATGATG
sFA-D2, 1574 F	1574 to 1596	CCCCTCAGCAAATACGAAAACTC	sFA-D2, 1414 R	1414 to 1396	CCTGCTGGCAGTACGTGTC
sFA-D2, 2142 F	2142 to 2162	GGTGACCTCACAGGAATCAGG	sFA-D2, 1704 R	1704 to 1684	GAATACGGTGCTAGAGAGCTG
sFA-D2, 2381 F	2381 to 2404	GAGAGATTGTAAATGCCTTCTGCC	sFA-D2, 2253 R	2253 to 2232	CTCCTCCAAGTTTCCGTTATGC
sFA-D2, 2679 F	2679 to 2699	TGACCCTACGCCATCTCATAG	sFA-D2, 2526 R	2526 to 2505	GTTTCCAAGAGGAGGGACATAG
sFA-D2, 3268 F	3268 to 3288	GCCCTCCATGTCCTTAGTAGC	sFA-D2, 3346 R	3346 to 3328	GGACGCTCTGGCTGAGTAG
sFA-D2, 3573 F	3573 to 3594	GCACACAGAGAGCATTCTGAAG	sFA-D2, 3674 R	3674 to 3653	GTAGGGAATGTGGAGGAAGATG
sFA-D2, 4049 F	4049 to 4069	ACACGAGACTCACCCAACATG	sFA-D2, 4159 R	4159 to 4139	CCAGCCAGAAAGCCTCTCTAC
sFA-D2, 4303 F	4303 to 4323	GAGTCTGGCACTGATGGTTGC	sFA-D2, 4409 R	4409 to 4387	GGGAATGGAAATGGGCATAGAAG

Supplementary Table S2A. *FANCD2* superamplicon primers

Super-amplicon	Containing Exons	Designation	Sequence (5´→3´)	Designation	Sequence (5´→3´)	PCR Product Size (bp)
I	1, 2	hFANCD2_exon1_F	TATGCCCGGCTAGCACAGAA	hFANCD2_super_1_2_R	GGCCCACAGTTTCCGTTTCT	4346
II	3	hFANCD2_super_3_3_F	GTGTCACGTGTCTGTAATCTC	hFANCD2_super_3_3_R	CTGGGACTACAGACACGTTTT	2323
III	7,8,9	hFANCD2_super_7_14_F	TGGGTTTGGTAGGGTAATGTC	hFANCD2_exon9_R	TACTCATGAAGGGGGGTATCA	4595
IV	10,11,12,13,14	hFANCD2_exon10_F	GCCCAGCTCTGTTCAAACCA	hFANCD2_super_7_14_R	TTAAGACCCAGCGAGGTATTC	5635
V	13,14,15,16,17	FA-D2, sup13-I17 F	CATGGCAGGAACTCCGATCTTG	FA-D2, sup13-I17 F	CTCCCTTAAAAGCTCAAAGCTCAAGTTC	8858
VI	19, 20	hFANCD2_super_19_22_F	ACGTAATCACCCCTGTAATCC	hFANCD2_exon20_R	TGACAGAGCGAGACTCTCTAA	2749
VII	21,22,23	FA-D2, 21_23, F	GCTTCTAGTCACTGTCAGTTCACCAG	FA-D2, 21_23, R	ACGTTGGCCAGAAAGTAATCTCAG	2518
VIII	23,24,25,26	hFANCD2_super_23_29_F	GGCCTTGTGCTAAGTGCTTTT	hFANCD2_exon26_R	TCAGGGATATTGGCCTGAGAT	3252
IX	27,28,29	hFANCD2_exon27_F	GCATTCAGCCATGCTTGGTAA	hFANCD2_super_23_29_R	CACTGCAAACTGCTCACTCAA	3371
X	30	hFANCD2_super_30_32_F	CCAAAGTACTGGGAGTTTGAG	hFANCD2_exon30_R	TACCCAGTGACCCAAACACAA	2186
XI	31,32	hFANCD2_exon31_F	CCATTGCGAACCCTTAGTTTC	hFANCD2_super_30_32_R	ACCCTGGTGGACATACCTTTT	299
XII	33,34	hFANCD2_super_33_36_F	GAGCAATTTAGCCTGTGGTTTT	hFANCD2_exon34_R	TATAGCAAGAGGGCCTATCCA	3457
XIII	35,36	hFANCD2_exon35_F	TTAGACCGGGAACGTCTTAGT	hFANCD2_super_33_36_R	TCTGGGCAACAGAACAAGCAA	2040
XIV	43a	hFANCD2_super_43_44_F	AGGGTCCTGAGACTATATACC	hFANCD2_exon43a_R	AGCATGATCTCGGCTCACCA	2040
XV	44	hFANCD2_exon44_F	CACCCAGAGCAGTAACCTAAA	hFANCD2_super_43_44_R	ACCATCTGGCCGACATGGTA	464

Supplementary Table S2B. *FANCD2* exon primers

Exon	Designation	Sequence (5´→3´)	Designation	Sequence (5´→3´)	PCR Product Size (bp)
1	hFANCD2_exon1_F	TATGCCCGGCTAGCACAGAA	hFANCD2_exon1_R	TCCCATCTCAGGGCAGATGA	324
2	hFANCD2_exon2_F	CCCCTCTGATTTTGGATAGAG	hFANCD2_exon2_R	TCTCTCACATGCCTCACACAT	258
3	hFANCD2_exon3_F	GACACATCAGTTTTCCTCTCAT	hFANCD2_exon3_R	AAGATGGATGGCCCTCTGATT	354
4	hFANCD2_exon4_F	TGGTTTCATCAGGCAAGAAACT	hFANCD2_exon4_R	AATCATTCTAGCCCACTCAACT	253
4/5	FA-D2, exon 4 II F	GAGAAGGAAAACTATGGTAGGAAAC	FA-D2, exon 5 II R	GTGTAAGCTCTGTTTTCCTCAGAG	509
5	hFANCD2_exon5_F	GCTTGTGCCAGCATAACTCTA	hFANCD2_exon5_R	AGCCCCATGAAGTTGGCAAAA	298
6	hFANCD2_exon6_F	GAGCCATCTGCTCATTTCTGT	hFANCD2_exon6_R	GCTGTGCTAAAGCTGCTACAA	341
7	hFANCD2_exon7_F	AATCTCGGCTCACTGCAATCT	hFANCD2_exon7_R	CAGAGAAACCAATAGTTTTCAG	280
8	hFANCD2_exon8_F	TAGTGCAGTGCCGAATGCATA	hFANCD2_exon8_R	AGCTAATGGATGGATGGAAAAG	333
9	hFANCD2_exon9_F	TTCACACGTAGGTAGTCTTTCT	hFANCD2_exon9_R	TACTCATGAAGGGGGGTATCA	323
10	hFANCD2_exon10_F	GCCCAGCTCTGTTCAAACCA	hFANCD2_exon10_R	CATTACTCCCAAGGCAATGAC	229
	FA-D2, exon10, F	GTCTGCCCAGCTCTGTTCAAAC	FA-D2, exon10, R	ATTACTCCCAAGGCAATGACTGACTG	232
11	hFANCD2_exon11_F	GTGGGAAGATGGAGTAAGAGA	hFANCD2_exon11_R	AGCTCCATTCTCTCCTCTGAA	341
	FA-D2, exon11, F	CAGTTCAGTACAAAGTTGAGGTAGTG	FA-D2, exon11, R	CCGGATTAGTCAGTATTCTCAGTTAG	267
12	hFANCD2_exon12_F	TGCCTACCCACTATGAATGAG	hFANCD2_exon12_R	TCTGACAGTGGGATGTCAGAA	211
13	hFANCD2_exon13_F	CAGGAACTCCGATCTTGTAAG	hFANCD2_exon13_R	ATGTGTCCATCTGGCAACCAT	321
	FA-D2, exon 13 F P1+2	CCGATCTTGTAAGTTCTTTTCTGGTACG	FA-D2, exon 13 R P1+2	TGGCAACCATCAGCTATCATTTCCAC	302
14	hFANCD2_exon14_F	CGTGTTTCGCTGATGTGTCAT	hFANCD2_exon14_R	TGGAGGGGGGAGAAAGAAAG	186
15	hFANCD2_exon15a_F	GTGTTTGACCTGGTGATGCTT	hFANCD2_exon15a_R	GGAAGGCCAGTTTGTCAAAGT	325
	hFANCD2_exon15b_F	GTGGAACAAATGAGCATTATCC	hFANCD2_exon15b_R	CTTATTTCTTAGCACCCTGTCAA	204
	FA-D2, exon 15 F uniq	GGAACAAATGAGCATTATCCATTCTGTG	FA-D2, exon 15 R/ P1	CTCAATGGGTTTGAACAATGGACTG	363
16	hFANCD2_exon16_F	AGGGAGGAGAAGTCTGACATT	hFANCD2_exon16_R	TTCCCCTTCAGTGAGTTCCAA	332
	FA-D2, exon 16 F P1	GTCTGACATTCCAAAAGGATAAGCAAC	FA-D2, exon 16 R	CTTGAGACCCAGGTCAGAGTTC	344
17	hFANCD2_exon17_F	GATGGGTTTGGGTTGATTGTG	hFANCD2_exon17_R	GATTAGCCTGTAGGTTAGGTAT	422
	FA-D2, exon 17 F P1+2	CTGGCATATTCCTAAATCTCCTGAAG	FA-D2, exon 17 R	GCCTGTAGGTTAGGTATAAAGAAGTG	472
18	hFANCD2_exon18_F	GGCTATCTATGTGTGTCTCTTT	hFANCD2_exon18_R	CCAGTCTAGGAGACAGAGCT	282
19	hFANCD2_exon19_F	CGATATCCATACCTTCTTTTGC	hFANCD2_exon19_R	ACGATTAGAAGGGAACATGGAA	328
20	hFANCD2_exon20_F	CACACCAACATGGCACATGTA	hFANCD2_exon20_R	TGACAGAGCGAGACTCTCTAA	239
21	hFANCD2_exon21_F	AAAGGGGCGAGTGGAGTTTG	hFANCD2_exon21_R	GAGACAGGGTAGGGCAGAAA	339
22	hFANCD2_exon22_F	ATGCACTCTCTCTTTTCTACTT	hFANCD2_exon22_R	GTAACTTCACCAGTGCAACCAA	279
23	hFANCD2_exon23_F	TTCCCTGTAGCCTTGCGTATT	hFANCD2_exon23_R	ACAAGGAATCTGCCCCATTCT	356
24	hFANCD2_exon24_F	CTCCCTATGTACGTGGAGTAA	hFANCD2_exon24_R	CCCCACATACACCATGTATTG	258
25	hFANCD2_exon25_F	AGGGGAAAGTAAATAGCAAGGA	hFANCD2_exon25_R	GTGGGACATAACAGCTAGAGA	350
26	hFANCD2_exon26_F	GACATCTCTCAGCTCTGGATA	hFANCD2_exon26_R	TCAGGGATATTGGCCTGAGAT	324
27	hFANCD2_exon27_F	GCATTCAGCCATGCTTGGTAA	hFANCD2_exon27_R	CCAATTACTGATGCCATGATAC	324
28	hFANCD2_exon28_F	TCTACCTCTAGGCAGTTTCCA	hFANCD2_exon28_R	GATTACTCCAACGCCTAAGAG	354
	FA-D2, exon 28 F	TCTACCTCTAGGCAGTTTCCA	FA-D2, exon 28 R	GATTACTCCAACGCCTAAGAG	354
29	hFANCD2_exon29_F	CTTGGGCTAGAGGAAGTTGTT	hFANCD2_exon29_R	TCTCCTCAGTGTCACAGTGTT	384
30	hFANCD2_exon30_F	GAGTTCAAGGCTGGAATAGCT	hFANCD2_exon30_R	TACCCAGTGACCCAAACACAA	348
	FA-D2, exon 30 F	CATGAAATGACTAGGACATTCCTG	FA-D2, exon 30 F	GCAAGATGAATATTGTCTGGCAATACG	319
31	hFANCD2_exon31_F	CCATTGCGAACCCTTAGTTTC	hFANCD2_exon31_R	ACCGTGATTCTCAGCAGCTAA	341
32	hFANCD2_exon32_F	CCACCTGGAGAACATTCACAA	hFANCD2_exon32_R	AGTGCCTTGGTGACTGTCAAA	336
33	hFANCD2_exon33_F	CACGCCCGACCTCTCAATTC	hFANCD2_exon33_R	TACTGAAAGACACCCAGGTTAT	340
34	hFANCD2_exon34_F	TTGGGCACGTCATGTGGATTT	hFANCD2_exon34_R	TATAGCAAGAGGGCCTATCCA	349
	FA-D2, exon 34 II F	GGCAATCTTCTTGGGCTTATTACTGAG	FA-D2, exon 34 II R	CAACTTCCAAGTAATCCAAAGTCCACTTC	327
35	hFANCD2_exon35_F	TTAGACCGGGAACGTCTTAGT	hFANCD2_exon35_R	GTCCAGTCTCTGACAAACAAC	300
36	hFANCD2_exon36_F	CCTCTGGTTCTGTTTTATACTG	hFANCD2_exon36_R	GGCCAAGTGGGTCTCAAAAC	398
37	hFANCD2_exon37_F	CTTCCCAGGTAGTTCTAAGCA	hFANCD2_exon37_R	TCTGGGCAACAGAACAAGCAA	277
	FA-D2, exon 37 II F	CATCCTCTTACTAAGGACCCTAGTGAAAG	FA-D2, exon 37 II R	CAGCAACTTCCAAGTAATCCAAAGTCCAC	288
38	hFANCD2_exon38_F	GCACTGGTTGCTACATCTAAG	hFANCD2_exon38_R	AAGCCAGGACACTTGGTTTCT	274
39	hFANCD2_exon39_F	TGCTCAAAGGAGCAGATCTCA	hFANCD2_exon39_R	GCATCCATTGCCTTCCCTAAA	236
40	hFANCD2_exon40_F	CCTTGGGCTGGATGAGACTA	hFANCD2_exon40_R	CAGTCCAATTTGGGGATCTCT	309
41	hFANCD2_exon41_F	GATTGCAAGGGTATCTTGAATC	hFANCD2_exon41_R	CCCCAATAGCAACTGCAGATT	214
42	hFANCD2_exon42_F	AACATACCGTTGGCCCATACT	hFANCD2_exon42_R	GCTTAGGTGACCTTCCTTACA	356
43	hFANCD2_exon43a_F	GTGGCTCATGCTTGTAATCCT	hFANCD2_exon43a_R	AGCATGATCTCGGCTCACCA	366
	hFANCD2_exon43b_F	CTGCCACCTTAGAGAACTGAA	hFANCD2_exon43b_R	TCAGTAGAGATGGGGTTTCAC	358
	hFANCD2_exon43c_F	TAGAATCACTCCTGAGTATCTC	hFANCD2_exon43c_R	CTCAAGCAATCCTCCTACCTT	405
	hFANCD2_exon43d_F	AGTTGGTGGAGCAGAACTTTG	hFANCD2_exon43d_R	CAGCTTCTGACTCTGTGCTTT	367
	hFANCD2_exon43e_F	TCAACCTTCTCCCCTATTACC	hFANCD2_exon43e_R	CTCGAGATACTCAGGAGTGAT	381
	hFANCD2_exon43f_F	GGTATCCATGTTTGCTGTGTTT	hFANCD2_exon43f_R	AGTTCTGCTCCACCAACTTAG	306
44	hFANCD2_exon44_F	CACCCAGAGCAGTAACCTAAA	hFANCD2_exon44_R	GAAAGGCAAACAGCGGATTTC	213
	FA-D2, exon 44 II F	CTAGGAGCTGTATTCCAGAGGTCAC	FA-D2, exon 44 II R	GGATCCTACCAGTAAGAAAGGCAAAC	250

Supplementary Table S2C. *FANCD2* mutation-specific primers

PCR/ Sequencing	Designation	Sequence (5´→3´)
PCR/Seq	FA-D2, exon4-6 F	GAAGGAAAACTATGGTAGGAAACTGGTG
	FA-D2, exon 6 R	CAGATGTATTAGGCTAATAAGCACAG
	FA-D2, exon4-i6 R	CCAGAAGCAGTTTGATGAGACTCTTAG
	FA-D2, exon i4F	GCTTTCCAAAAGAAGCTCTTTCAGAC
	FA-D2, exon4-IVS F	GGAGACACCCTTCCTATCCCAAAG
	FA-D2, exon 5F	GAGTGGGCTAGAATGATTTTTAACAGC
	FA-D2, exon 5 R	CTCTGAGGAAAACAGAGCTTACAC
	D2_AluYb9 F	GCAATCTCGGCTCACTGCAAGCTC
	D2_IVS4/AluYb9, R	GCTGTTAAAAATCATTCTACTTTGGGAGG
PCR	FANCD2_16S	AGAGCTAGGGAGGAGAAGTCTGA
	FANCD2_18A	GAGCTGAGATCGTGCCAACT
Seq	FANCD2_17S	TGGTCAAGTTACACTGGCATATT
	FANCD2_17A	CCATCCTTCAGCAATCACTC
PCR/Seq	D2_P2_21_23 F	GTTTTCTGATACTTGGAAACTACTGGCTTG
	D2_P1_21_23 R	GACACAGAGGTAGCAAAGGATGTTC
Seq	FA-D2, ex21_23, int1	CTATGATGAATTTGCCAACCTGATCC
	FA-D2, ex21_23, int2	GAGGGCTCCTTCACTTAATAACAATC
	FA-D2, ex21_23, int3	GTATTGTTTACCTGCTGGCTGGTTG
PCR	FA-D2_sup_exon26 II F uniq	TAGGGTCACAAGCCTAATCTCCTTT
	FA-D2_sup_exon26 II R uniq	GGCCATGATGAATAATCTTTCTTTTGTTTG

Supplementary Table S3. Microsatellite primers

STR	Genomic Position [Mb]	Sense Primer Sequence (5´→3´)	Antisense Primer Sequence (5´→3´)
D3S1597	9,34	AGTACAAATACACACAAATGTCTC	CAATTCGCAAATCGTTCATTGCT
D3S1038	10,49	AAAGGGGTTCAGGAAACCTG	CCCTCCAGTAAGAGGCTTCCTAG
D3S3611	10,53	GCTACCTCTGCTGAGCATATTC	CACATAGCAAGACTGTTGGGGGC
D3S1675	10,64	GGATAGATGGATGAATGGATGGC	CCTCTCTAACTACCAATTCATCCA

Supplementary Table S4. Laboratory diagnostic data of the 29 cohort FA-D2 patients

Patient number	Kindred Sibling	Cell type of lab diagnosis	G2-phase arrest, G2/GF		Breaks/cell		Technique of complementation group assignment	*FANCD2* mutation		Somatic mosaicism
			Spon	MMC/DEB	Spon	MMC/DEB		Allele 1	Allele 2	
1	1 / I	Lymphocyte	65.7%	n.d.	0.07	4.5 (M) [6.6 (M)]	IB of LCL	c.696–121C>G (exonization)	c.696–121C>G (exonization)	None (residual protein)
2	2 / I	Lymphocyte	54.3%	70.1% (M)	0.09	1.4 (M), 1.5 (D)	IB and RC of LCL	c.1948–6C>A (exon 22 skipping)	c.3599delT	None (residual protein)
3	3 / I	Lymphocyte (prior to mosaicism)	38.6% (prior to mosaicism)	46.6% (M) (prior to mosaicism)	0.04	0.06 (M)	RC of fibroblasts	c.1948–16T>G (exon 22 skipping)	c.1948–16T>G (exon 22 skipping)	1954G>A (exon 22), V652I, reconstitutes exon 22 recognition (blood, BM, LCL)
4	4 / I	Lymphocyte	45.7%	63.6% (M)	n.d.	n.d.	RC of fibroblasts	c.1948–16T>G (exon 22 skipping)	c.1948–16T>G (exon 22 skipping)	None (no LCL)
5	4 / II	Lymphocyte	44.5%	58.9% (M)	n.d.	n.d.	RC of fibroblasts	c.1948–16T>G (exon 22 skipping)	c.1948–16T>G (exon 22 skipping)	None (residual protein)
6	5 / I	Lymphocyte	34.5%	64.7% (M)	0.05	4.7 (M) 5.6 (D)	IB and RC of LCL	c.274–57_–56insinvAluYb8nt36_319 +dup c.274–69_–57 (exon 5 skipping)	c.3803G>A	None (residual protein)
7	6 / I	Lymphocyte	34.8%	51.5% (M)	n.d.	n.d.	IB of LCL	c.904C>T	c.1092G>A	None (residual protein)
8	7 / I	Lymphocyte	45.6%	58.4 (M)	0.06	1.3 (M)	IB of LCL	c.990–1G>A (aberrant splicing)	c.1948–6C>A (exon 22 skipping)	None (residual protein)
9	8 / I	Lymphocyte	65.3%	70.9% (M)	0.12	n.d.	IB of LCL	c.810_812delGTC	c.1948–16T>G (exon 22 skipping)	None (residual protein)
10	9 / I	Lymphocyte	38.4%	58.4% (M)	n.d.	n.d.	RC of fibroblasts	c.1948–16T>G (exon 22 skipping)	c.2715+1G>A (aberrant splicing)	None (residual protein)
11	10 / I	Lymphocyte	55.4%	65.8% (M)	? (Wien)	? (Wien)	IB of LCL	c.3707G>A (aberrant splicing)	c.2835dupC	None (residual protein)
12	11 / I	Lymphocyte	40.2%	61.1% (M)	n.d.	n.d.	IB of LCL	c.274–57_–56insinvAlu Yb8nt36_319 +dup c.274–69_–57	c.3453_3456delCAAA	None (residual protein)
13	12 / I	Lymphocyte	27.6%	57.8%	0.11	1.08 (M) 2.9 (D)	IB of LCL	c.1948–16T>G (exon 22 skipping)	c.1948–16T>G (exon 22 skipping)	None (residual protein in T cells and LCL)
14	13 / I	Lymphocyte Fibroblast	20.9%	32.1% (M)	n.d.	n.d.	Mutation analysis (by sibling)	c.1948–6C>A (exon 22 skipping)	2775_2776CC>TT	2775_2776 CC (blood, LCL)
15	13 / II	Lymphocyte Fibroblast	25.0%	35.4% (M) 69.2% (M)	0	0.11 (M) 0.02 (D)	RC of fibroblasts	c.1948–6C>A (exon 22 skipping)	2775_2776CC>TT	Recombination
16	14 / I	Lymphocyte	n.d.	n.d.	0.04	1.78 (D)	IB of LCL	c.2444G>A	c.2444G>A	None (residual protein)
17	14 / II	Lymphocyte	n.d.	n.d.	0.12	3.1 (D)	Mutation analysis (by sibling)	c.2444G>A	c.2444G>A	None (no LCL)
18	15 / I	Lymphocyte	n.d.	n.d.	0.12	1.5 (D)	IB of LCL	c.696–2A>T (exon 10 skipping)	c.1321_1322delAG (aberrant splicing)	None (residual protein)
19	16 / I	Fetal blood	n.d.	n.d.	n.d.	3.7 (D)	RC of fetal fibroblasts	c.692T>Gpat	c.2444G>Amat	not done
20	17 / I	Lymphocyte	n.d.	n.d.	0.02	8.4 (D)	IB and RC of LCL	c.1948–6C>Amat, (exon 22 skipping)	2660delApat	None (residual protein)
21	18 / I	Lymphocyte	n.d.	n.d.	0.02	5.4 (D) 10.3 (D)	IB of LCL	c.2404C>T	c.2444G>A	None (residual protein)
22	19 / I	Lymphocyte	n.d.	n.d.	0.04	3.7 (D)	RC of fetal fibroblasts from 880/2 (early spontaneous abortion)	c.2444G>Apat	c.2715+1G>Amat (aberrant splicing)	None (residual protein)
23	20 / I	Lymphocyte	n.d.	n.d.	0.08	7.4 (D)	IB of LCL	c.757C>T	c.1367T>G	None (residual protein)
24	20 / II	Lymphocyte	n.d.	n.d.	0.20	8.9 (D)	IB of LCL	c.757C>T	c.1367T>G	None (residual protein)
25	21	Lymphocyte	n.d.	n.d.	Data missing	Data missing	Mutation analysis	c.1948–16T>G (exon 22 skipping)	c.1948–16T>G (exon 22 skipping)	1953G>T (W651C) (blood, LCL)
26	22 / I	Fibroblast	22.2% (fibroblast)	54% (fibroblast, 300 nM ≈ 100 ng/ml MMC)	0.04	0.16 (300 nM ≈ 100 ng/ml MMC)	Mutation analysis in fibroblasts (by sibling)	c.376A>G (aberrant splicing)	c.3803G>A	376A (blood,)
27	22 / II	Lymphocyte	n.d.	n.d.	0.12	>10 (M)	IB and IP of LCL	c.376A>G (aberrant splicing)	c.3803G>A	None (residual protein)
28	23 / I	Lymphocyte	n.d.	n.d.	0.10	6.0 (M)	IB, IP and RC of LCL	c.206–2A>T (exon 4 skipping)	g.22875_23333del459 (c.1414–71_c.1545+256del459)	None (residual protein)
29	23 / II	Lymphocyte	n.d.	n.d.	0.12	8.1 (M)	Mutation analysis (by sibling)	c.206–2A>T (exon 4 skipping)	g.22875_23333del459 (c.1414–71_c.1545+256del459)	None (residual protein)

MMC, M, mitomycin C; DEB, D, diepoxybutane; RC, retroviral complementation; IB, immunoblotting; IP, immunoprecipitation; LCL lymphoblast cell line; n.d., not determined

Supplementary Table S5. Clinical diagnostic data of the 29 cohort FA-D2 patients

Patient number	Kindred/ Sibling	Consanguinity Gender	Ethnicity Nationality	Age at diagnosis	Clinical presentation	Hematologic manifestations	Survival at lastfollow-up	Family history
1	1 / I	unkown f	Asian Indian	6 mo	IUGR, patent ductus arteriosus, pigmentation anomalies, microcephaly, low-set ears, hypoplastic thumb with duplicate nail (R), radial ray aplasia with cutaneous thumb (L), pelvic kidney (R), congenital hip dislocation (L), aplasia of the corpus callosum	BMF as of 2 y 4mo, transfusions from 3 y 2 mo, AML at 7.0 y	† 7 y 6 mo (AML, pneumonia)	No SABs; no known cancer
2	2 / I	absent f	Caucasian German	5 y 7 mo	GR, pigmentation anomalies, microcephaly, microphtalmia, low-set thumbs, duplicate kidney (R), dysplastic hips	BMF as of 5 y 7 mo, cortisol from 8 y, transfusions from 8 y 4 mo, androgen from 9 y 2 mo	† 11 y 4 mo (subarachnoidic hemorrhage)	1 SAB; MGM:Cervix ca, 40 y
3	3 / I	cousins of 1st° m	Caucasian Turkish	1 y 11 mo	IUGR, pigmentation anomalies, microcephaly, hypoplastic thumbs (L>R), syndactyly II/III toes, hypogenitalism, glomerulosclerosis	Stable partial mosaïcism, BMF as of 11 y, cortisol and androgen from 12 y, transfusions from 18 y 9 mo, BMT at 19 y 7 mo	† 20 y 7 mo (viral encephalitis following BMT)	No SABs, no known cancer
4	4 / I	cousins of 2nd° f	Caucasian Turkish	5 y 10 mo	IUGR, pigmentation anomalies, microcephaly, microphtalmia, hypoplastic thumb (R), hydocephalus internus, hypoplastic corpus callosum, mental retardation, hyperactivity attention deficit disorder	BMF as of 2 y 6 mo, transfusions from 2 y 6 mo, subdural hemorrhage 6 y, BMT at 7 y	8 y 3 mo	No SABs; no known cancer
5	4 / II	cousins of 2nd° m	Caucasian Turkish	4 y 5 mo	IUGR, microcephaly, microphtalmia, strabism, mental retardation, hyperactivity attention deficit disorder	BMF as of 3 y 3 mo, transfusions from 3 y 3 mo, oxymetholon from 5y 9 mo	6 y 11 mo	No SABs; no known cancer
6	5 / I	absent m	Caucasian German	2 y 6 mo	GR, microcephaly, microphtalmia, absent anthelix (R), radial ray hypoplasia, preaxial hexadactyly (R), duplicate pelvic kidney (R), maldescensus of the testes, micropenis, dysplastic hips, hypoplastic corpus callosum, misshaped brain ventricles, psychomotor retardation	BMF as of 2 y 9 mo, BMT at 3 y 3 mo.	4 y 4 mo	No SABs; PGM cancer 70 y, otherwise no cancer history
7	6 / I	absent f	Caucasian Italian	2 y	IUGR, pigmentation anomalies, microcephaly, microphtalmia, absent thumbs, short radii, absent anthelix (R), closed auditory canals	BMF as of 4.5 y	12 y	No SABs; no cancer history
8	7 / I	absent m	Caucasian German	3 y 9 mo	Pigmentation anomalies, microcephaly, 'flat' auricles-absent anthelix?, ptosis, short thumbs, hyperactivity attention deficit disorder	BMF as of 4 y	4 y 4 mo	No SABs; no cancer history
9	8 / I	absent f	Caucasian Tschech	2 y 11 mo	IUGR, microcephaly, brain atrophy, patent ductus arteriosus, esophagus atresy, tracheoesophageal fistula (IIIb), hypoplastic kidneys, polycystic ovary (L), triphangeal digitalized thumbs, pedes equinovari, rib anomaly (VACTERL-like association)	BMF as of 2 y 10 mo, transfusions from 2 y 11mo	† 5 y 10 mo (hemorrhage)	1 SAB (first trimester); no cancer in the family
10	9 / I	absent f	Caucasian Turkish	7 mo	IUGR, pigmentation anomalies, microcephaly, hydrocephalus internus, absent corpus callosum, microphtalmia, microstomy, low-set ears, hypoplastic thumbs, unilateral triphalangeal (R), pelivic kidney (L), hip luxation, psychomotor retardation	BMF as of 2 y	2 y 3 mo	1 SAB (first trimester); 1 pregnancy terminated because of hydrocephalus and renal agenesy; PGF bronchus ca
11	10 / I	absent f	Caucasian Austrian	10 y 10 mo	IUGR, pigmentation anomalies, microcephaly, hypoplastic thumbs, ectopic kidney (R)	BMF as of 10 y 10 mo, transfusions from 10 y 10 mo, MDS (RAEB-t) with del(7)(q32) at 10 y 10 mo, BMT at 11y 1 mo	11y 11 mo	No SABs; no cancer history
12	11 / I	absent m	Caucasian Danish	3 mo	IUGR, atresy of the duodenum, microcephaly, dilated lateral ventricles and stenosis of the aquaeduct (hydrocephalus), hypoplasia of the carpous callosum, microphtalmia, closed auditory channels, hypoplastic thumbs, micropenis	BMF as of 2 wks	5 mo	No SABs; PGM and MPGM breast ca., MGGF prostate ca.
13	12 / I	cousins of 1st° m	Caucasian Turkish	5 y 5 mo	IUGR, pigmentation anomalies, microcephaly, microphalmia, psychomotor retardation, Michelin tire baby syndrome	BMF as of 1 y 5 mo	5 y 8 mo	No SABs; no cancer history
14	13 / I	absent f	Caucasian German	34 y 2 mo	IUGR, microcephaly, mild radial ray hypoplasia	None	34 y	No SABs; no cancer history
15	13 / II	absent f	Caucasian German	21 y 11 mo	IUGR, microcephaly, radial ray hypoplasia, dysplasia of mandibula, anomalies of the teeth, dysplasia of hip (R), mental retardation	Transfusions from 17 y 6 mo, MDS(RARS-RAEB) at 17 y 6 mo	† 23 y 5 mo (pneumonia, invasive aspergillosis, hemorrhage)	No SABs; no cancer history
16	14 / I	cousins of 3° m	Caucasian Spanish	6 y	Patent ductus arteriosus, pigmentation anomalies, bifid thumb (R), hypogonadism	BMF as of 7 y (very mild hypoplasia of the myeloid series)	25 y	No SABs; MGF lung, PGFstomach ca.
17	14 / II	cousins of 3° m	Caucasian Spanish	8 mo	Pigmentation anomalies, microphtalmia, hypoplastic thumb (R), absent os metacarpale I (L), glandular hypospadia	Blood cell counts at low-range normal levels	20 y	No SABs; MGF lung, PGF stomach ca.
18	15 / I	absent f	Caucasian Spanish	5 y 3 mo	IUGR, pigmentation anomalies, microcephaly, microphtalmia, hypotelorism, annular pancreas	BMF as of 5 y 3 mo, androgen, G-SCF, EPO and transfusions from 5 y 3 mo, BMT at 10 y 11 mo	† 11 y 1 mo (graft failure / no take)	No SABs; MMGM colon ca.

Supplementary Table S5 (continued)

Patient number	Kindred/ Sibling	Consanguinity Gender	Ethnicity Nationality	Age at diagnosis	Clinical presentation	Hematologic manifestations	Survival at last follow-up	Family history
19	16 / I	absent m	Caucasian, maternal Irish and English, paternal Irish and Italian	22 wk of gestation	IUGR, absent thumb and radial aplasia (R), lateral cerebral ventricular dilation (hydrocephalus)	N/A	N/A, terminated with diagnosis of FA	3 first trimester SABs, 4th fetus with IUGR, radial aplasia, cystic hygromas, encephalocele, probably heart defects, terminated; MGM pancreas, MMGM breast, MGF melanoma & basal cell ca.
20	17 / I	absent m	Caucasian maternal German, paternal Dutch	4 y 5 mo	IUGR, pigmentation anomalies, microcephaly, microphtalmia	BMF as of 2 y, BMT at 5 y	9 y (4y post BMT)	1 SAB, M.3x basal cell, MMMGM melanoma, MMGF breast, PGM bowel ca.
21	18 / I	absent m	Caucasian Hispanic (Mexican)	7 mo	IUGR, café au lait spots, microcephaly, micro-phtalmia, hearing loss (auditory canals? sensory hearing impairment?), absent thumbs and radii, intestinal aresia, renal defects, genital anormalities (undescended testes), learning diabilities	None yet	10 y 3 mo	No SAB; one cancer;
22	19 / I	absent m	Caucasian maternal Irish, Dutch Yugoslavian French and Native American, paternal Irish and Sicilian	Newborn	Patent ductus arteriosus, pigmentation anomalies, low-set ears, malformed auricle (R), constriction bands of mid forearms (Michelin tire baby syndrome?), preaxial hexadactyly (R), hypoplastic thumb with ponce flottant (L)	BMF as of 1 y 4 mo	2 y 6 mo	5 miscarriages with one positive for FA; PGM breast, MPGM cervix and lung ca., MMGM brain tumor
23	20 / I	absent m	maternal African American / Caucasian, paternal African American	(1 mo)	IUGR; microcephaly; microphtalmia; hypoplastic thumb (L); hypoplastic metacarpal I (R); horseshoe kidney	none	1 y 4 mo	GM: 2 miscarriages. Cancer only in GreatGP generation
24	20 / II	absent f	maternal African American / Caucasian, paternal African American	4 y 6 mo	IUGR; café-au-lait spots; microcephaly; microphtalmia	BMF starting from 4 y 6 mo	5 y 9 mo	GM: 2 miscarriages Cancer only in GreatGP generation
25	21 / I	cousins of 1st° m	Caucasian Turkish	5 y 5 mo	IUGR, pigmentation anomalies, microcephaly, microphtalmia, pelvic kidney	BMF starting from 4 y; oxymethalone and prednisone from 5 y 5 mo	† 9 y (intracranial hemorrhage)	No SAB; no cancer history
26	22 / I	absent f	Caucasian Dutch	5 y	IUGR (asymmetrical); pigmentation anomalies; microcephaly; ventriculo-megaly (hydrocephalus) and multiple developmental anomalies of the brain, possibly holopros-encephaly; hypotelorism; microphtalmia; narrow auditory canals; hypoplastic os metacarpale I, renal aplasia (R); dysplasia of the hip (L); STH deficiency	BMF as of 5 y; transfusions from 6 y 9 mo + GCSF; BMT at 7 y 10 mo	9 y	No SAB; no cancer history
27	22 / II	absent m	Caucasian Dutch	3 y	IUGR; pigmentation anomalies; microcephaly; hypoplastic corpus callosum; hypertelorism; blepharophimosis; preaxial hexadactyly (L)	BMF as of 2 y 1 mo; transfusions from 5 y 8 mo; BMT at 7 y 7 mo	7 y 9 mo	No SAB; no cancer history
28	23 / I	absent m	Caucasian Dutch	8 y 6 mo	IUGR, pigmentation anomalies, microcephaly, Kartagener syndrome with situs inversus, mild mental retardation	BMF as of 8 y 5 mo; no transfusions; BMT at 9 y 5 mo	† 10 y 1 mo (gastro-intestinal hemorrhage due to necrotizing enterocolitis post BMT)	1 SAB; no cancer history
29	23 / II	absent m	Caucasian Dutch	5 y 8 mo	IUGR, pigmentation anomalies, microcephaly, microphtalmia	BMF as of 5 y 8 mo; BMT at 6 y 7 mo	7 y 10 mo	1 SAB; no cancer history

f, female; m, male; L, left; R, right; (IU)GR, (intrauterine) growth retardation; BMF, bone marrow failure; BMT, bone marrow transplantation; MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; SAB, spontaneous abortion

Supplementary Table S6A. *FANCD2* splice acceptor calculations

3´Splice site (acceptor)			
Exon	**Wild type/ variant**	**Sequence**	**Score (Maxent*)**
4	Consensus	ctcttctttttttctgcatagCTG	9.12
	IVS3-2A>T	ctcttctttttttctgcat**t**gCTG	0.76
10	Consensus	tctttttctaccattcacagTGA	7.39
	IVS9-2A>T	tctttttctaccattcac**t**gTGA	-0.97
13	Consensus	ttcctctctgctacttgtagTTC	6.19
	IVS12-1G>A	ttcctctctgctacttgta**t**TTC	-2.56
22	Consensus	tgtttgtttgcttcctgaagGAA	6.43
	IVS21-16T>G	tgtt**g**gtttgcttcctgaagGAA	5.58
	IVS21-6C>A	tgtttgtttgcttc**a**tgaagGAA	4.51
37a	Ex37 (consensus)	ACTTTTGTTGTTTTCTTCCGTGT	2.10
	3706C>A	ACTTTTGTTGTTTTCTTC**A**GTGT	10.14

* MaxEntScan::score3ss for human 3' splice sites
(http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html)

Supplementary Table S6B. *FANCD2* splice donor calculations

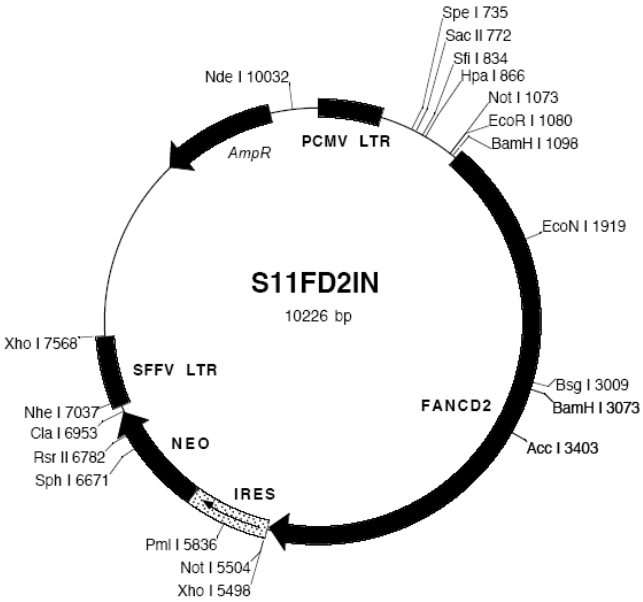
5´Splice site (donor)					
Exon	Wild type/ variant	Sequence	Score (splicefinder*)	Difference	Result
5	Consensus	CAGgtgtggag	LC4 12||3|2|		
	376A>G	C**G**Ggtgtggag	LC2 2|8||3|2	large	malfunction
9a	IVS (consensus)	acggtaactta	LC4 ||12|2||		
	IVS9-121C>G	ACGgtaa**g**tta	HC3 ||17||	large	gain of function
10	Consensus	AAGgtagaaaa	LC4 |12|||2|		
	782A>T	A**T**Ggtagaaaa	LC3 ||10|||2|	small	malfunction

* Splicefinder (http://www.uni-duesseldorf.de/rna/html/5__ss_mutation_assessment.php)

Supplementary figures

Supplementary Figure S1. Circular map of the vector S11FD2IN.

The retroviral expression vector S11FD2IN contains a bicistronic construct of the full-length *FANCD2* cDNA (FANCD2) and the neomycin resistance gene (NEO). Translation of the latter is ensured by an internal ribosomal entry site (IRES). Shown are also the LTRs, the restriction sites and their positions and the bacterial resistance (*AmpR*).



CHAPTER 5

Fanconi anemia complementation group FA-L: severe phenotype despite somatic reversion of the *FANCL* gene

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Human Mutation, to be submitted

Abstract

To date, twelve Fanconi anemia genes have been identified which interact in the recognition and repair of DNA damage. Eight of the FA proteins assemble into a nuclear complex which is necessary for monoubiquitination of FANCD2 during S phase of the cell cycle and in response to DNA damage. A member of the complex, the ring finger protein FANCL, provides the presumptive E3 ligase function. Here we describe the worldwide second patient assigned to subgroup FA-L via retroviral gene complementation. The patient presented with short stature, multiple congenital defects and bone marrow failure between ages 9 and 11 necessitating alternate donor hematological stem cell transplantation. Sequencing of fibroblast-derived *FANCL* cDNA and gDNA revealed compound heterozygosity for a 5-bp deletion (exon 7) and a missense substitution (exon 11). In contrast to skin fibroblasts, two independently established lymphoid cell lines lacked the typical hypersensitivity to MMC and showed proficient monoubiquitination of FANCD2 suggesting somatic reversion. Sequence analysis of gDNA from LCLs, peripheral blood mononuclear cells revealed a heterozygous base substitution at c.472-2A>T (IVS6-2A>T) that was not present in fibroblasts. Retroviral transduction of patient fibroblasts with the revertant cDNA sequence resulted in loss of MMC sensitivity, restoration of FANCD2 monoubiquitination and reappearance of FANCD2 nuclear foci, thus proving the restoration of FANCL function by the reverted sequence. The clinical course of the patient was marked by increasing cytopenia of all cell lineages except lymphocytes. This suggests that the reversion event occurred in a precursor cell of the lymphoid cell lineage rather than in a bone marrow stem cell, thus being unable to delay or prevent bone marrow failure.

Introduction

Fanconi anemia (FA) is a recessive inherited multisystem disease characterized by congenital abnormalities, progressive bone marrow failure, chromosome fragility and cancer susceptibility. To date, 13 complementation groups have been defined (FA-A, B, C, D1, D2, E, F, G, I, J, L, M and N) and, except for *FANCI* all of the corresponding FA genes have been identified [1-5]. The FA gene products cooperate in a caretaker network that maintains chromosomal stability based on effective resolution of DNA interstrand crosslinks and stalled replication forks [1]. In response to DNA damage and/or stalled replication forks eight of the FA proteins (*FANC-A, B, C, E, F, G, L* and *M*) assemble into a nuclear multi-protein complex (FA core complex) whose function is required for monoubiquitination of the central protein FANCD2 at Lys561 [5, 6]. Monoubiquitinated FANCD2 is targeted to nuclear foci containing proteins such as BRCA1, BRCA1 interacting protein 1 (BRIP1/FANCJ), BRCA2/FANCD1 and RAD51 which participate in DNA double strand repair via homologous recombination [7-11].

The process of ubiquitination is tightly controlled by an enzymatic cascade involving at least three types of enzymes: an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and a substrate specific ubiquitin ligase (E3) [12]. The presumptive E3 ligase activity for monoubiquitination of FANCD2 is provided by FANCL which appears to function as the catalytic subunit of the FA core complex, and recently UBE2T was found to be the corresponding E2 [13]. FANCL contains three WD40 repeats known as sites of protein-protein interaction and a C terminal PHD (plant homeodomain) zinc finger motif [14]. Meetei et al. [14] demonstrated that FANCL is able to ubiquitinate itself *in vitro* and that mutations in *FANCL* result in defective FANCD2 monoubiquitination. Interestingly, both *FANCL* and *FANCD2* belong to the small group of evolutionarily conserved FA genes for which relatively few patients have been described. One would therefore expect that mutational inactivation of *FANCL*, entails severe phenotypic consequences.

To date, only a single patient displaying a *FANCL* defect has been reported, whose lymphoblasts (EUFA868) has been the basis for the identification of the FA-L subtype [14]. In our laboratories, we have determined the complementation group of more than 400 FA patients via retroviral gene

transfer, DNA sequencing [15, 16] and DHPLC screening. Among the patients studied we found only a single patient belonging to complementation group FA-L. We here present a brief case report of this patient who to the best of our knowledge is the second only FA-L patient worldwide, and the first FA-L patient on whom clinical information is available. This patient is of additional interest since she developed revertant mosaicism involving a novel type of compensatory mutation.

Materials and Methods

Diagnosis and cell culture

Peripheral blood and skin biopsy samples were referred to the participating laboratories for diagnostic testing with suspicious of FA. Diagnostics, genetic subtyping and mutation analysis were performed with informed consent according to the Declaration of Helsinki. The study was approved by the institutional review boards of the participating centers.

The clinical suspicion of FA was initially confirmed by the detection of cellular hypersensitivity to DNA crosslinking agents. Chromosome breakage rates were assessed in phytohemagglutinin (PHA)-stimulated 72 h whole blood cultures either by mitomycin C (MMC), diepoxybutane (DEB) or nitrogen mustard (NM) sensitivity assays. MMC was added at the time of culture initiation at final concentrations of 50 and 100 ng/ml, DEB was added 24 h after culture initiation at a final concentration of 100 ng/ml for the last 48 h and NM was likewise added 24 h after culture initiation at a concentration of 50 ng/ml for 48 h. The number of break events was scored according to the type of individual aberration. In the Wuerzburg laboratory, mononuclear blood cells isolated via Ficoll-Paque PLUS (Amersham Biosciences, Munich, Germany) were cultured for 72 h and subjected to cell cycle analysis using an analytical, dual-laser equipped flow cytometer (LSR; Becton Dickinson, Erembodegem, Belgium). Cell cycle distributions reflecting DNA content were quantitated using the MPLUS AV software package (Phoenix Flow Systems, Inc., San Diego, CA, USA). Cell cycle testing for FA-typical G2 phase arrest in response to MMC has previously been shown to arrive at the same diagnostic conclusions as standard chromosome breakage analysis [17, 18]. Lymphocytes and EBV transformed LCL were maintained in RPMI medium (Gibco, Karlsruhe, Germany) with 16% fetal bovine serum. Fibroblast cultures were established using standard cell culture procedures and MEM medium supplemented with glutamine (Invitrogen, Karlsruhe, Germany) and 16% fetal bovine serum. All cell cultures were kept in high humidity incubators equipped with CO_2 and O_2 sensors in an atmosphere of 5% (v/v) CO_2 and 5% (v/v) O_2 by replacing ambient air with nitrogen. For cell cycle analysis cells of logarithmical growing cultures (fibroblasts, lymphoblasts) were harvested 48 after treatment with 0 and 10-20 ng/ml mitomycin C. Prior to flow cytometry living cells were stained using the DNA binding dye Hoechst 33342 (Molecular Probes, Eugene, OR, USA) (4µg/ml, 1h, 37°C) or alternatively, nuclei were stained with DAPI as described previously (Schindler and Hoehn, 1999)

Assignment to complementation group FA-L

In the context of this study, we analysed 43 DNAs of lymphoblastoid cell lines (LCL) originating from well-characterized FA patients previously excluded from complementation groups FA-A, B, C, D1, D2, E, F and G. For complementation analysis we used retroviral complemention, immunoblot and/or DNA sequence analysis. *FANCL* screening was by heteroduplex analysis and direct DNA sequencing in 13 fragments. DHPLC profiles were run both spiked with control DNA and unspiked in order to detect homo- and heterozygous alterations. We found 1 synonymous missense variant, S327S, and 8 intronic variants (Tab. S1), but no change that could be classified as *bona fide* mutation.

In another screen of 12 previously unassigned FA lymphoblasts and 8 unassigned FA-fibroblasts, all deficient in FANCD2 monoubiquitination, we used retroviral vectors for complementation analysis, expressing wt *FANCL* from bicistronic constructs either with an IRES-*GFP* or with a selectable IRES- neomycin resistance gene cassette [15, 16]. Among the 8 fibroblast lines tested, one was complemented by the *FANCL* vector as assessed by flow cytometric cell cycle analysis following MMC treatment (10-15 ng/ml, 48 h) (see below).

Immunofluorescence

Cells were fixed with 3.7% (w/v) in PBS diluted paraformaldehyde for 15 minutes followed by permeabilization with 0.5% Triton X-100 in PBS for 5 min. After blocking for 30 minutes (10% FBS, 0.1% NP-40 in PBS) FANCD2 was detected using mouse monoclonal FANCD2 antibodies (Fl17, Santa Cruz Biotechnology, Heidelberg, Germany) in a dilution of 1:200. Cells were subsequently washed three times in TBS. As secondary antibodies we used Alexa 594 coupled anti-mouse antibodies (Molecular Probes, Leiden, The Netherlands) diluted in TBS (1:1000). After incubation (45 min, RT) cells were washed three times and covered with DAPI containing Vectashield mounting media (Vector Laboratories).

Immunoblotting

FANCD2 immunoblotting was performed as described by Garcia-Higuera et al. [6] with minor modifications. Around 16 hours prior lysis MMC (50 ng/ml) was added to the culture medium. Cells were lysed with 1x lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM ß-Glycerophosphate, 0,1 mM Na_3VO_4, 0.3% Nondipet P-40, 0,2% Triton X proteinase inhibitors (Roche Applied Science). Whole cell extracts were separated on 7% Tris-acetate gels (Invitrogen, Karlsruhe, Germany) and transferred overnight at 4° C onto PVDF membranes (HybondP, Amersham Bioscience, Munich, Germany). Immunoblots were blocked in 5% non-fat dry milk (Roth) in PBS/0.05% Tween 20 (PBS-T). As primary antibody, we used mouse monoclonal FANCD2 antibody Fl17 (Santa Cruz Biotechnology, Heidelberg, Germany) at a dilution of 1:800. The secondary horseradish peroxidase-conjugated antibody (Amersham Bioscience, Munich, Germany) was diluted 1:4,000. Chemiluminescence detection was done by standard ECL reagent (also provided by Amersham Bioscience).

Genetic work up

For mutation analysis total RNA was extracted using the Micro to Midi total RNA isolation kit (Invitrogen, Karlsruhe, Germany) and reverse transcribed by Superscript II (Invitrogen, Karlsruhe, Germany). *FANCL* ORF was amplified using Phusion polymerase (Finnzyme, Espoo, Finnland), purified with GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience, Munich, Germany) and sequenced. Identified mutations were confirmed at the gDNA level. Genomic DNA was prepared using a modified salting-out technique [19].

For exon scanning sequencing we amplified exons and adjacent intron regions. Sequencing of PCR products was performed using ABI-PRISM big-dye terminator chemistry and the ABI 310 instrument (Applied Biosystems). All primer sequences will be provided by request. For allele specific analysis PCR products (cDNA and gDNA) were cloned into the vector pCR2.1 via TOPO TA cloning (Invitrogen, Karlsruhe, Germany).

Splice assay

The potential disturbance of splicing by the base substitution c.891C>G was examined by cloning the genomic region into the vector SVcrev. For amplification following primer were used: sense, 5´-GAA**GAATTC**GTAATAATGTTTCCATAAATATAG; antisense 5´-GAA**CTCGAG**CTCATATAAGCATATTTGATGG. Both PCR product and target vector were cut by EcoR1 and XhoI, followed by ligation (14 °C, ON). The inserted sequence was controlled by

DNA sequencing. Transfection was done as previously described [20].

Sequences

Nucleotide sequence NM_018062 (NCBI) was used as human *FANCL* cDNA reference. The genomic reference sequence was ENSG00000115392 (Ensembl). For sequence alignment ClustalX was used.

Results

Case report

The patient was born prematurely in the 35th week of pregnancy as the second child of non-consanguineous parents of German ancestry. The clinical diagnosis of FA was considered at age 3 following the onset of mild thrombopenia, neutropenia and megaloblastic anemia. Confirmation at that time was by DEB testing and cell cycle analysis of peripheral blood mononuclear cells [17, 21]. Physical examination after birth and prior to transplantation revealed short stature, hypopigmentation and malformations of the skeleton, eyes and inner organs. In addition to severe growth retardation (3rd percentile) the patient exhibited microcephaly, malposition of thumbs, rudimentary preaxial polydactyly, cutanous syndactyly II and III, congenital hip dysplasia and Klippel-Feil deformity. Microphtalmia was present together with belpharophimosis and Sicca syndrome. Malformations of inner organs included duodenal atresia with malrotation II and renal hypotrophy combined with vesicorenal reflux causing stage IV chronic renal failure. Statomotoric development was delayed.
At age 6 the patient was started on androgen treatment (Primobolan S) which was continued until bone marrow transplantation at age 11. The combined treatment with androgens and erythrocyte transfusions (at Hb levels below 8 g/dl) stabilized her blood cell counts for nearly 3 years. At age 9 gradual decrease of thrombocytes was noted which was followed by a decrease of neutrophil counts 2 years later, necessitating haematological stem cell transplantation (HSCT). HSCT was successfully performed using a protocol based on fluorarabine and an unrelated HLA matched alternate stem cell donor. Two years after transplant the 13 year old patient developed a squamous cell carcinoma of the tongue that was detected during a routine control examination. The carcinoma was successfully removed and there is no evidence for recurrence at the time of writing.

Assignement to complementation group FA-L and mutation analysis

Patient derived primary fibroblast cultures were screened for complementation after retroviral transfer of *FANCA, -C, -D2, -E, -F, -G* and *–L* cDNAs. As shown in figure 1, in contrast to transduction with *FANCF* cDNA, transfer of *FANCL* cDNA into patient cells resulted in the restoration of FANCD2 monoubiquitination (Fig. 1A). Likewise, transduction with *FANCL* cDNA led to functional correction of the MMC-sensitive cellular phenotype as evidenced by decreased G2 phase cell cycle fractions (Fig. 1B). On the basis of these complementation studies our patient was assigned to FA complementation group FA-L.

Subsequent mutation analysis employed *FANCL* cDNA derived from primary fibroblast cultures followed by confirmation at the gDNA level. The patient was found to be heterozygous for a 5 base pair deletion in exon 7 (c.483delATCAC) and a base substitution in exon 11 (c.891C>G, I297M) (see Fig. 1C). The deletion causes a frameshift and premature termination at amino acid position 186 (E161DfsX186), whereas heterozygous skipping of exons 10 and 11 was noted in connection with the exon 11 mutation. To examine whether the exon 11 mutation could influence mRNA splicing we searched for exonic splice enhancer (ESE) motifs using the ESE finder programme (rulai.cshl.edu/tools/ESE/). The base substitution obviously leads to loss of a potential SC35 binding site accompanied by strengthening of a putative SRp40 motif. However, using mini gene based splicing

assays no differences of splicing could be found (Fig. S1). Cloning of patient cDNA showed a certain degree of exon 10 and 11 skipping in both alleles, implying alternative splicing. Retroviral transfer of cDNA containing the c.891C>G change failed to complement patient fibroblasts, thus indicating the true pathogenetic nature of the mutation.

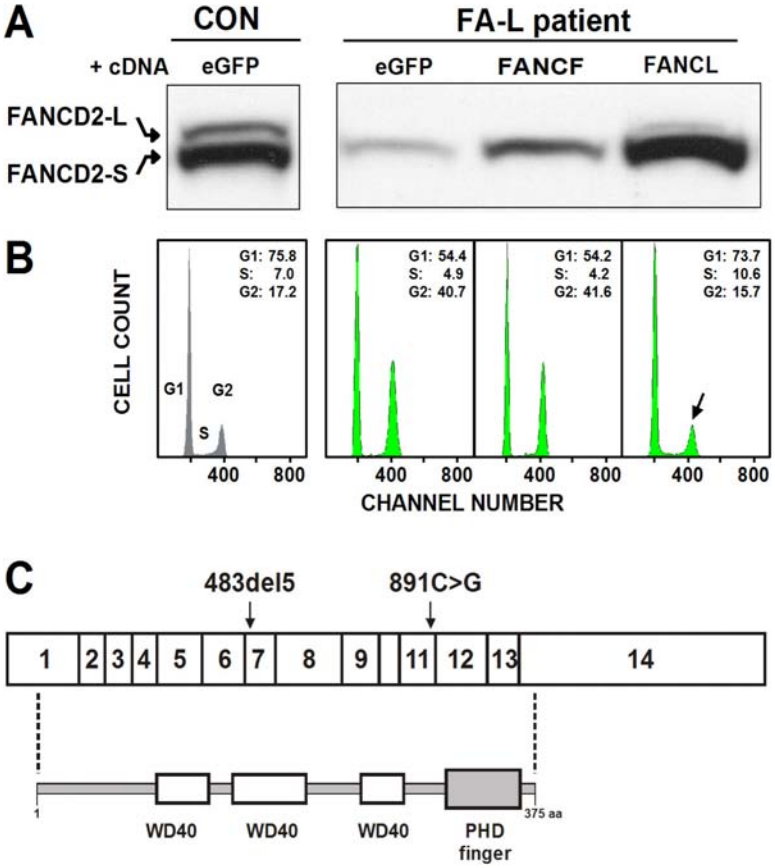

Fig. 1. Assignment to complementation group FA-L and mutations in *FANCL* A) Retroviral transduction of *FANCL* cDNA into patient fibroblasts restores FANCD2 monoubiquitination, reflected by the appearance of both native (FANCD2-S) and monoubiquitinated FANCD2 (FANCD2-L) after MMC treatment. In contrast, transduction of vectors containing *eGFP* (enhances green fluorescent protein) or *FANCF* yield only a single FANCD2-band, indicating lack of monoubiquitination B) MMC sensitivity of primary fibroblasts results in prominent G2 phase peaks (*eGFP* and *FANCF*) but is reversed to control levels (CON) after transduction with *FANCL*-cDNA. C) cDNA cartoon of *FANCL* illustrating the location of the identified mutations.

Somatic reversion

Cell cycle analysis of peripheral blood mononuclear cells obtained at age 3 had revealed sensitivity towards MMC. In contrast, prior to bone marrow transplantation at age 10 peripheral blood mononuclear cells proved MMC resistant reflecting the development of a complete mosaic status of peripheral blood cells by this age. Two independent lymphoid cell lines (LCL) established at patient ages 3 and 8 proved MMC-resistent as evidenced by cell cycle analysis (Fig. 2B). In these LCLs, both the native and the monoubiquitinated FANCD2 isoforms were detected via immunoblotting reflecting functional reversion of the B-cell derived cell lines (Fig. 2A). A *de novo* 10 base pair deletion in *cis* of the 5 bp deletion was detected as molecular cause of the reversion to MMC resistance, obviously leading to restoration of the ORF and thereby restoration of function of the mutated *FANCL* allele (Fig. 2C). The genomic cause of the somatic deletion proofed to be a heterozygous base substitution c.472-2A>T (IVS6-2A>T) present in lymphoblasts, bone marrow and peripheral blood mononuclear cells (at patient age 10) but not in patient´s fibroblasts (see Fig. 2D). This base substitution causes destruction of the natural splice acceptor site of exon 7 by affecting the conserved AG dinucleotides and, in turn, utilization of the following downstream AG for splicing (Fig. 2E). The alternatively used 3´ splice site is located 10 bases downstream of the regular exon boundary, and one base pair preceding the germline deletion (c.483delATCAC). The predicted protein contains a tyrosine instead of threonine at position 158 followed by a deletion of 5 amino acids (T158Ydel5) (Fig. 2F). This alteration affects the second WD40 repeat which is located between aa 147-202. In order to examine the function of the altered protein we cloned the

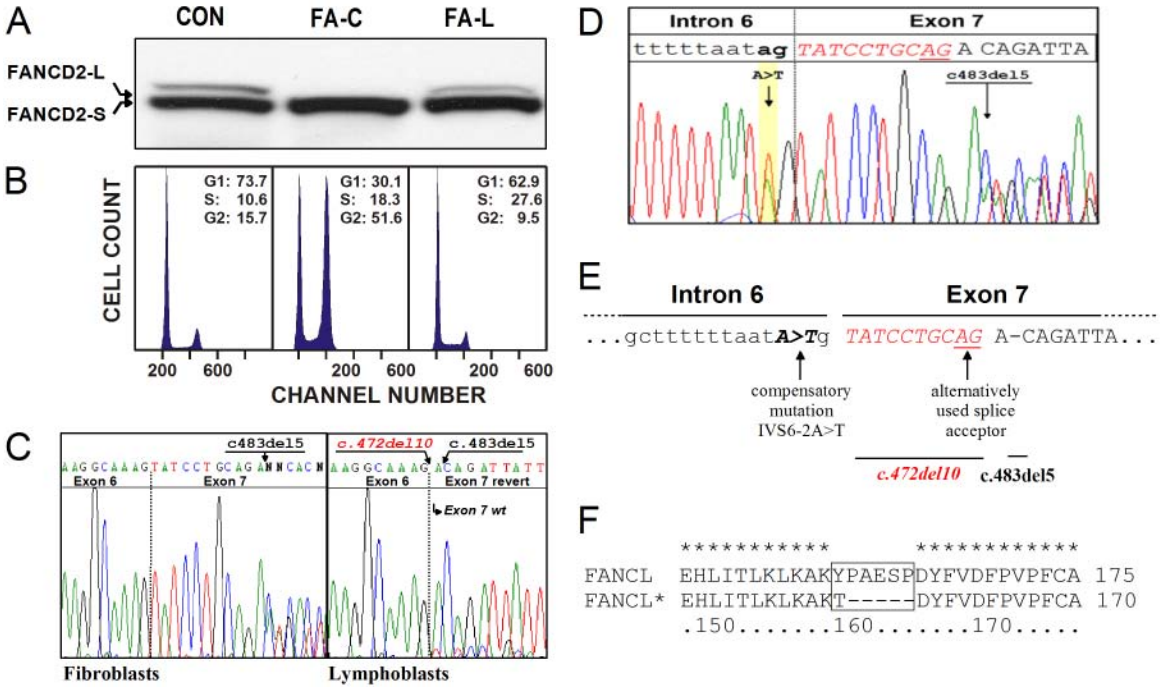

Fig. 2. Molecular characterization of somatic reversion in patient lymphoid cells (LCL). A) In contrast to a *FANCC* deficient cell line (FA-C) patient LCLs (FA-L) are proficient in FANCD2 monoubiquitination. (B) (left to right) control cells, FA-C and FA-L LCLs subjected to flowcytometric analysis. In contrast to FA-C cells, FA-L LCLs show lack of mitomycin C sensitivity as reflected by the low G2 phase arrest after MMC exposure. C) Compared to patient fibroblasts patient derived LCLs show two deletions at cDNA level: a germline (c.483del5) and a somatic deletion (r.472del10). D) gDNA derived from peripheral blood mononuclear cells and LCLs shows coexistence of the germline mutation in exon 7 and a base substition in *cis*, IVS6-2A>T (c.472-2A>T), abrogating the normal splice acceptor. E) At the mRNA level, the IVS6-2A>T change results in the usage of a cryptic site located 10 bp downstream and loss of the initial 10 nt (red) of exon 7. (F) The lack of altogether 15 nt at mRNA level (FANCL*) restores the reading frame including exchange of 1 aa (T158Y) and the deletion of 5 aa.

revertant cDNA into a retroviral vector (Fig. 3A) and transduced patient derived fibroblasts with this construct. As shown in figure 3B, the introduced cDNA abolished the hypersensitivity of patient fibroblasts towards MMC, proving complementation. The comparison between transduced wildtype and revertant *FANCL* cDNAs revealed no differences regarding complementation efficiency as measured by our cell cycle assay. In addition, DNA damage induced FANCD2 foci formation was restored (Fig. 3C) and no significant differences in number of foci per nuclei or number of foci containing nuclei was observed after retroviral transduction of either wt-*FANCL* or reverted *FANCL* cDNAs (Fig. 3D).

Discussion

Genotype-phenotype correlations

To date, only a single patient with a *FANCL* defect has been reported in the literature. This patient, whose LCL served as basis for the identification of the *FANCL* gene [14] is since deceased and unavailable for study. Here we present the clinical and molecular data of the only surviving FA-L patient currently known. This patient clearly belongs to the more severely affected FA phenotypes with short stature, congenital malformations, microcephaly, microphthalmia, childhood onset of bone marrow failure and juvenile onset of neoplasia. At the cellular level, patient derived fibroblasts and T-lymphocytes (age 3) proved highly sensitive towards MMC which is typical for all FA complementation groups. One of the underlying mutations causes a frameshift, and, presumably, protein truncation. The other allele shows loss of function caused by a missense mutation. The

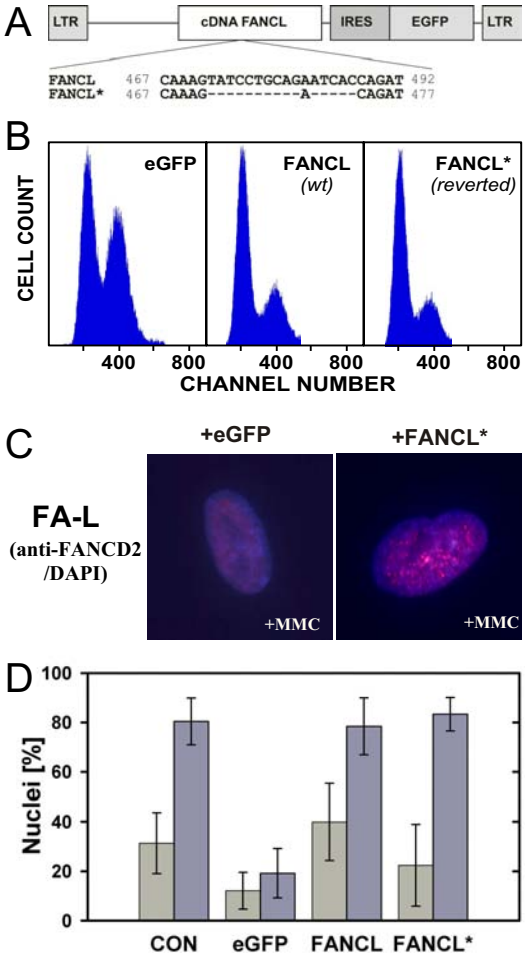

Fig. 3. Proof of the truly compensatory nature of the somatic reversion event A) Cartoon of a retroviral *FANCL* vector construct containing the reverted allele present in patient LCLs (r.472del10 and r.483del5), denoted FANCL* (or S11FL*IEG). B) Flow cytograms after transfection of patient fibroblasts with (from left to right) eGFP only, wildtype *FANCL*-cDNA and *FANCL**-cDNA (containing the reverted sequence). Both wildtype and reverted cDNAs reduce the G2-phase accumulation of MMC-treated patient fibroblasts, indicating MMC-resistance conveyed by both wildtype and reverted cDNA. This experiment classifies IVS6-2A>T and the resulting 10-nt skipping as a true compensatory mutation. C) Patient fibroblasts failed to show nuclear FANCD2 (red) foci after exposure to MMC (left), while transduction with *FANCL** cDNA restored MMC-induced FANCD2 assembly into nuclear foci (right). Nuclei were counterstained with DAPI (blue). (D) Quantitative evaluation (mean and SD of 200 nuclei each) of nuclear focus formation without (light bars) and with (dark bars) MMC treatments.

first FA-L patient mentioned above was described with skipping of exon 11 based on a homozygous or hemizygous insertion of 177 bp in the polypyrimidine tract of the splice acceptor site leading to little or no protein in whole cell extracts of lymphoid cell line [14]. The available data imply that neither of the two known FA-L patients carries defined biallelic null mutations. Leaky mutations due to aberrant splicing are known to predominate in the target of FANCL, FANCD2 (chapter 4), and only very few mutations have been found in *FANCM*, another evolutionarily conserved FA gene [22, 23]. Patients carrying mutations, let alone biallelic null mutations, in any of these conserved genes occur much less frequently than patients with mutations in the evolutionarily recent genes *FANCA, FANCC* or *FANCG*. This underlines the functional importance of the highly conserved FA genes. FANCL provides the presumed E3 ligase activity for FANCD2 monoubiquitination, and both FANCL and FANCD2 seem to play important roles in ontogenesis. The murine FANCL/PHF9 homologue was identified as the gene underlying the mouse phenotype of gcd (germ cell-deficient) denoted *POG* (proliferation of germ cells) [24]. *POG* was shown to be essential for proper primordial germ cell proliferation in the embryonic stage, but is not needed for spermatogonial proliferation after birth [25].

Somatic reversion in patient T- and B-lymphoid cells

At age 11, examination of primary T-lymphocytes and of B-cell derived LCLs (established at ages 3 and 8) revealed loss of MMC sensitivity that had not been detected in primary T-lymphocytes obtained at age 3. Therefore, the shift to MMC-resistance of peripheral blood mononuclear cells (mostly primary T-lymphocytes) must have taken place between ages 3 and 10. As other cell lineages such as thrombocytes, erythrocytes and neutrophils continued to decline during this time necessitating bone marrow transplantation, the reversion event must have taken place in a lymphoid precursor cell rather than in a hematopoietic stem cell. So far, revertant mosaicism has been described for FA patients belonging to complementation groups A, C and D2 [26-30]. Mechanisms of reversion include intragenic crossover, gene conversion, back mutation, and compensating mutations in *cis* [26, 30, 31]. Only two patients, both belonging to complementation group FA-A,

showed functional correction of a frameshift mutation by a somatic alteration in *cis* similar to what we observed in our patient [30]. One of the reported *FANCA* frameshift mutations, a microdeletion (c.1615delG), was compensated by two additional single base-pair deletions located downstream of the germline mutation. The other example, a microinsertion (c.3556insG), was compensated by a second site insertion (c.3580insCGCTG). In both cases, the open reading frame of *FANCA* was restored enabling the expression of a functional protein.

A novel type of compensatory mutation in cis

Our patient provides an example for another so far unique type of compensatory mutation upstream of a 5 base pair deletion (483delATCAC, exon 7) in the *FANCL* gene. The mutation of the natural splice acceptor of exon 7 results in usage of an alternative splice site, located 10 base pairs downstream, leading to restoration of the open reading frame. The deduced amino acid sequence is predicted to contain a tyrosine instead of a threonine at position 158 affecting the first third of second WD40 in FANCL. Loss of the WD40 repeats results in defective FANCD2 monoubiquitination and FA core complex assembly [32]. However, WD-repeats are minimally conserved domains of approximately 40–60 amino acids containing both variable and characteristic domains, such as a tryptophan-aspartic acid (WD) dipeptide at the C terminus. The putative common function of these repeats is the assembly of multiprotein complexes by serving as a rigid platform or as a scaffold for protein-protein interactions [33, 34]. In our patient, the compensatory deletion evidently did not alter protein function, indicating that no structurally important domain was affected. Functionality of the reverted gene product was proven by transduction of *FANCL* cDNA containing the compensatory mutation into patient-derived primary fibroblasts. This transduction resulted in loss of MMC sensitivity and restoration of FANCD2 monoubiquitination. In addition, the MMC induced recruitment of FANCD2 into nuclear foci was restored to normal levels reflecting the truly compensatory nature of the somatic reversion event.

In summary, we report the second only patient ever assigned to complementation group FA-L, and the first patient for whom clinical data are available. The patient is severely affected, showing growth retardation and a multitude of congenital defects that are typically found in FA patients. Thrombocytopenia and macrocytic anemia were noted at age 6, and her failing bone marrow required a life-saving hematopoietic stem cell transplantation at age 11. Like the other previously described FA-L cell line, our patient carried compound heterozygous mutations one of which is a missense mutation which may account for viability. Over the course of 6 to 7 years our patient`s native and cultivated T- and B-cells converted to MMC resistance caused by a novel type of compensatory mutation. The reversion event must have taken place in a precursor cell of the lymphoid cell lineages, since the other blood cell types showed continuous decline. Hematological improvement has been observed in a number of mosaic FA-patients (e.g. Lo ten Foe et al, 1997; Gross et al, 2002; Soulier et al, 2005), but such global improvement of bone marrow function requires that the somatic reversion event takes place in a hematopoietic stem cell (Mankad et al, 2006). Only two years after HSCT, the patient developed a squamous cell carcinoma of the tongue, a frequent post-transplant complication in FA patients [35]. Fortunately, this tumor was detected at an early stage and could be successfully removed.

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Supplementary material

A) Supplementary Tables

Table S1. Polymorphisms/variants found during genomic screening of *FANCL* in 36 FA patients.

Position		Frequency
Exonic	c.982T>C (p.S327S)	15/36
Intronic	c.217-11T>C *(IVS3-11T>C)*	6/36
	c.273+20insT *(IVS4+20insT)*	2/36
	c.374+35delT *(IVS5+35delT)*	3/36
	c.541-27A>G (*IVS8-27A>G)*	3/36
	c.691+59delTAA (*IVS8+59delTAA*	11/36
	c.775+22C>T (*IVS9+22C>T)*	12/36
	c.775+31A>G *(IVS9+31A>G)*	9/36
	c.1021-65G>A *(IVS13-65G>A)*	11/36

B) Supplementary Figures

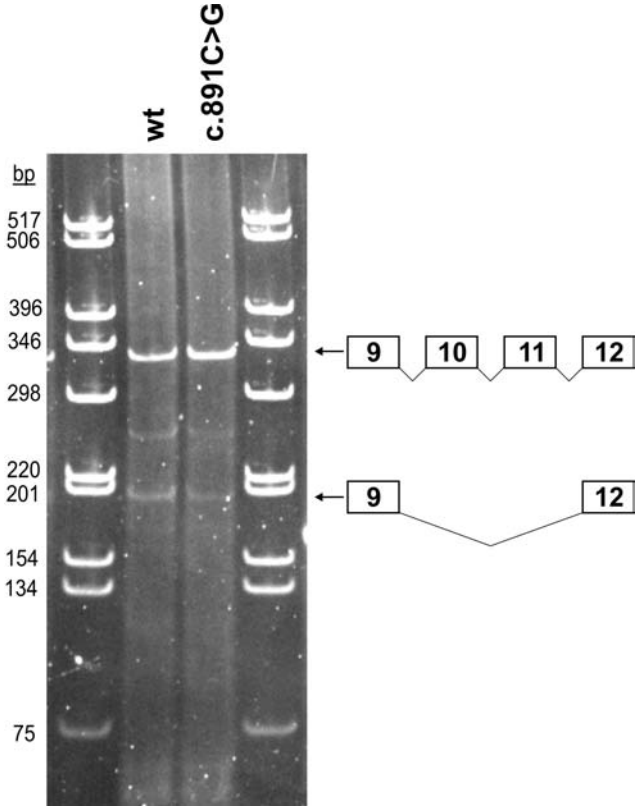

Figure S1. Minigene assay probing *FANCL* splicing involving c.891C>G. The entire genomic region of *FANCL* exons 9 to 12 was cloned into the vector SVcrev and transfected into human cells. Isolated mRNA was reverse transcribed, amplified with vector specific primers and separated on agarose. Both patient and control DNA show identical splice products.

CHAPTER 6

The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia

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Abstract

Seven Fanconi anemia-associated proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG and FANCL) form a nuclear Fanconi anemia core complex that activates the monoubiquitination of FANCD2, targeting FANCD2 to BRCA1-containing nuclear foci. Cells from individuals with Fanconi anemia of complementation groups D1 and J (FA-D1 and FA-J) have normal FANCD2 ubiquitination. Using genetic mapping, mutation identification and western-blot data, we identify the defective protein in FA-J cells as BRIP1 (also called BACH1), a DNA helicase that is a binding partner of the breast cancer tumor suppressor BRCA1.

Fanconi anemia is a genetic disorder associated with developmental abnormalities, bone marrow failure, predisposition to cancer and genomic instability [1]. Fanconi anemia has extensive genetic heterogeneity; eleven complementation groups have been defined [2] and nine associated genes have been identified (*FANCA*, *FANCB*, *FANCC*, *BRCA2* (also called *FANCD1*), *FANCD2*, *FANCE*, *FANCF*, *FANCG* and *FANCL*) [3-5]. FANCD2 monoubiquitination is intact in two complementation groups (FA-D1 and FA-J). Whereas biallelic mutations in *BRCA2* have been found in individuals with FA-D1 (Ref [6]), the gene mutated in FA-J has not yet been identified. The goal of this study was to identify new downstream gene(s) in the Fanconi anemia pathway. Cell lines and genomic DNA samples were derived from individuals with Fanconi anemia and their family members registered in the International Fanconi Anemia Registry (IFAR) after obtaining informed written consent. The studies were approved by the Institutional Review Board of The Rockefeller University.

We selected five individuals from four families in the IFAR [1] for a genome-wide scan using 50K SNP arrays on the basis of the following criteria: (i) unassigned complementation group using retroviral complementation studies or linkage; (ii) normal expression of the two isoforms of FANCD2 (FANCD2-S and FANCD2-L) [3]; and (iii) suspected homozygosity by descent originating from consanguinity or a founder effect. A previous genome-wide scan of 460 microsatellite markers in DNA from consanguineous families in the IFAR [7] identified the position of *FANCA*, the gene most commonly mutated in Fanconi anemia, but did not detect additional Fanconi anemia–associated genes in the individuals studied, including individual IFAR346/1, whom we also studied here. We also carried out genome scans of three Inuit individuals (IFAR983/1, IFAR983/2 and IFAR1001/1) using microsatellite markers (O.L., unpublished data) but identified no region of shared homozygosity. We suspected that the region of homozygosity was smaller than the 10-cM average distance between the microsatellite markers used in these studies, and so we screened DNA from the five individuals using 50K SNP arrays (Supplementary Methods). We identified a statistically significant region of homozygosity of 6 Mb on chromosome 17q23, flanked by SNPs rs10515150 and rs2665850 (positions 53,267,127 and 59,307,844, respectively; Fig. 1a). This region contains 48 SNPs for which Inuit siblings IFAR983/1 and IFAR983/2 share the same haplotype; the haplotype of the other Inuit individual IFAR1001/1 differs by only one SNP (rs1037364), leaving a 4.5-Mb region of shared haplotype among the Inuit individuals (position 54,792,947–59,307,844). Each of the Hispanic individuals IFAR943/1 and IFAR346/1 has a unique haplotype. The extent of the homozygous region was different in each individual (Fig. 1a). Microsatellite marker data from the previous scan [7] for individual IFAR346/1 showed that two markers (*D17S808* and *D17S794*) lie in the candidate region (positions 58,025,847 and 58,170,985, respectively). This individual was homozygous with respect to marker *D17S808* but heterozygous with respect to marker *D17S794*, reducing the shared homozygous region among the five individuals to 5 Mb (positions 53,267,127–58,170,985; Fig. 1a). The initial 6-Mb interval contains 68 known genes and hypothetical proteins, from which we chose two candidate genes on the basis of their biological functions: *RAD51C* [8] and *BRIP1* (also called *BACH1*) [9]. We found no mutations in the coding regions or the adjacent intron portions

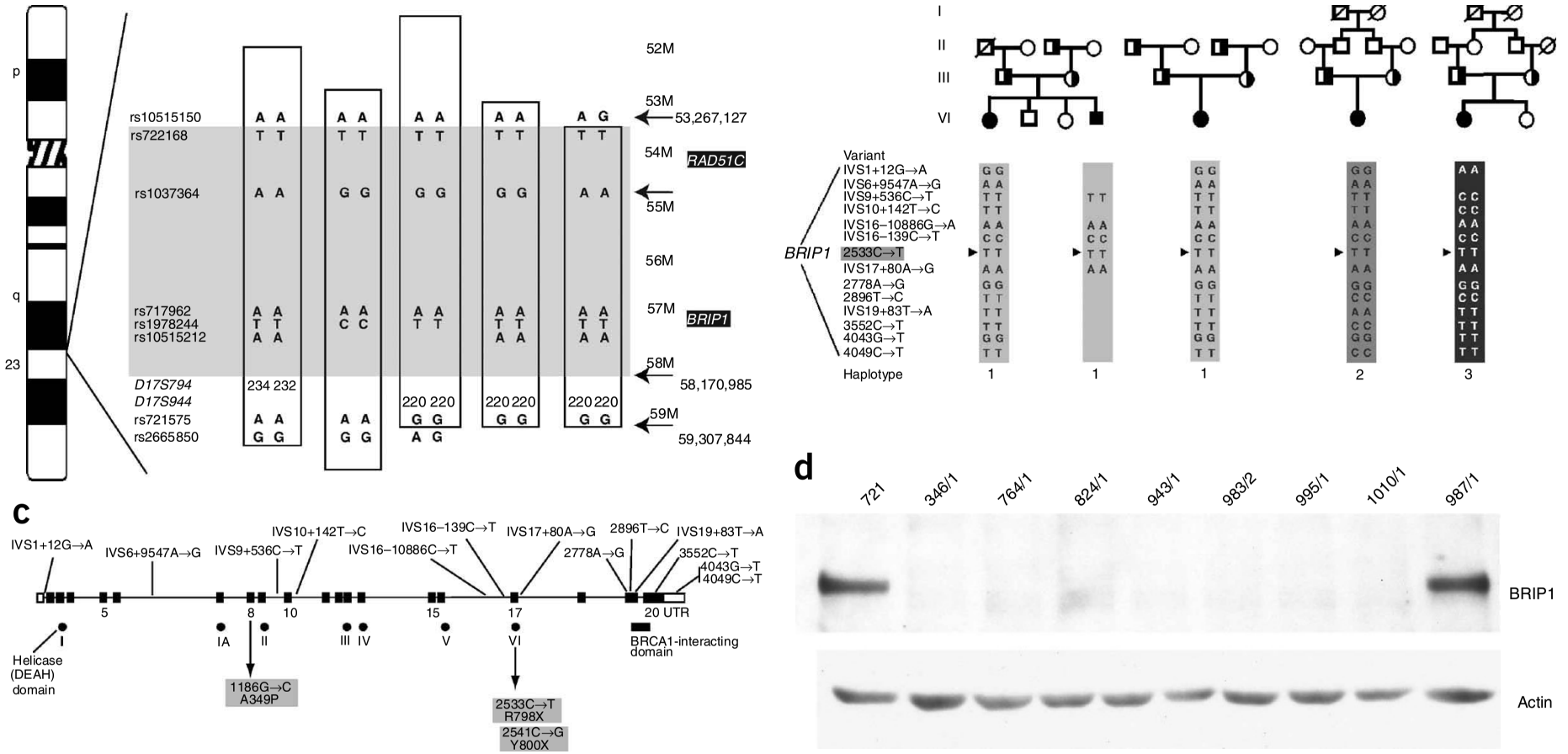

Fig.1. BRIP1 is defective in Fanconi anemia. (**a**) The 5-Mb region of shared homozygosity among the five individuals is highlighted. The map position and the candidate genes (*RAD51C* and *BRIP1*) are indicated on the right. The open boxes indicate the full region of homozygosity for each individual. (**b**) Pedigree structure of the four families in this study. All individuals are homozygous with respect to the 2533C>T mutation; carriers of this mutation are indicated by half-filled symbols. Haplotype analysis shows that the Inuit individuals share the same haplotype (Hap1, light gray) and that the Hispanic individuals each carry an independent haplotype (Hap2 and Hap3, dark gray and black, respectively). (**c**) Schematic representation of the *BRIP1* gene. Exons are indicated by black boxes; untranslated regions (UTR), by open boxes. The seven protein helicase domains (black circles) and the BRCA1-interacting region (black rectangle) are shown below the diagram. SNPs are indicated above the diagram, in their respective locations. The R798X, Y800X and A349P mutations are highlighted. Long introns were reduced in size. (**d**) Whole-cell extracts prepared from exponentially growing lymphoblastoid cell lines from a wild-type control (721) and from an individual with Fanconi anemia not of the FA-J complementation group (IFAR987/1) were immunoblotted with BRIP1 antiserum and compared with cells extracts from individuals with *BRIP1* mutations. Actin served as a loading control.

of *RAD51C*. Immunoblotting with an antiserum to RAD51C detected a normal protein in all individuals (data not shown). Sequencing of the coding regions and the adjacent intron portions of *BRIP1* identified a 2533C>T nonsense mutation in exon 17, resulting-in a premature stop codon (R798X; Supplementary Fig. 1). All five individuals were homozygous with respect to this mutation. Screening of family members for this mutation confirmed the autosomal recessive inheritance (Fig. 1b). Sequence analysis for 13 SNPs in *BRIP1*, including the 3 intronic variants observed on the SNP arrays (IVS6+9547A>G, IVS9+536C>T and IVS16-10886G>A), showed variability for eight variants among the five individuals (Fig. 1b,c).

Haplotype analysis indicated that the Inuit individuals (IFAR983/1, IFAR983/2 and IFAR1001/1) shared the same intragenic haplotype (Hap1), indicative of a founder effect in this small isolated population. Intragenic haplotypes for individuals IFAR346/1 (Hap2) and IFAR943/1 (Hap3) were unique. All variants except IVS10+142T>C and 4043G>T were previously described [10]. The appearance of the same mutation on at least three different haplotypes from diverse populations is notable, especially for a rare disorder such as Fanconi anemia.

We screened an additional 18 individuals from the IFAR with Fanconi anemia of an unassigned complementation group for mutations in *BRIP1*. Three additional individuals (Table 1) were homozygous with respect to the 2533C>T (R798X) mutation, and three were heterozygous, including individual IFAR764/1 of European ancestry who was previously assigned to the complementation group FA-J [2]. The second mutation in two of the heterozygous individuals was identified by genomic DNA sequencing and was confirmed in family members. Individual IFAR995/1 carries a second nonsense mutation (2541C>G; Y800X) in exon 17, and individual IFAR781/1 carries a missense mutation (1186G>C; A349P) in exon 8 (Table 1). The second mutation in individual 764/1 is 80037A T in intron 11. The base substitution generates a new GT splice donor site that activates a cryptic AG splice acceptor at positions 79071-79072. cDNA from this individual shows an insertion of 963 bp between exon 11 and 12 sequence (1628_1629ins963bp).

Table 1 Characteristics of individuals with Fanconi anemia and mutations in *BRIP1*

Individual	Maternal allele	Paternal allele	DEB-treated breaks/cell (%)[a]	Baseline breaks/cell	Ethnicity	Phenotypic and hematologic abnormalities
346/1	2533C→T, R798X	2533C→T, R798X	4.6 (80)	0.1	Hispanic	Growth retardation; café-au-lait spots; bilateral hypoplastic thumbs; microphthalmia; BMF at 6 y
764/1	2533C→T, R798X	ND	2.0 (32)	0.1	Eur-Amer	Severe growth retardation; café-au-lait spots; microphthalmia; urogenital abnormalities; hearing loss; developmental delay; no BMF at 6.5 y
781/1	1186G→C, A349P	2533C→T, R798X	0.35[b]	0.08	Eur-Amer	IUGR; radial and ulna aplasia; bilateral club feet; cleft palate; abnormal facies; severe GI, urogenital, cardiovascular, respiratory and CNS abnormalities; stillborn fetus, gestational age 22 wk
824/1	2533C→T, R798X	2533C→T, R798X	5.7 (95)	0.24	Eur-Amer	Severe growth retardation; café-au-lait spots; microphthalmia; radial ray, urogenital and heart abnormalities; conductive hearing loss; no BMF at 5 y
894/1	2533C→T, R798X	2533C→T, R798X	8.80 (100)	0.28	Irish traveler	Severe growth retardation; café-au-lait spots; microphthalmia; hydrocephalus; radial ray defect; BMF at 2 y
943/1	2533C→T, R798X	2533C→T, R798X	9.6 (95)	0.16	Hispanic	Growth retardation; café-au-lait spots; thumb and kidney abnormalities; developmental delay; BMF at 3.5 y; CMMol at 13.5 y; died at 14 y
983/1	2533C→T, R798X	2533C→T, R798X	13.3 (100)	0.5	Inuit	IUGR; radial ray and genital abnormalities; died at 6 wk
983/2	2533C→T, R798X	2533C→T, R798X	23.3 (100)	0.48	Inuit	Severe growth retardation; café-au-lait spots; abnormal thumbs; developmental delay; BMF at 5.5 y
995/1	2533C→T, R798X	2541C→G, Y800X	10.1 (100)	0.2	Mixed[c]	IUGR; microphthalmia; radial ray, vertebral, urogenital, heart, CNS and ear abnormalities; died at 4 d
1001/1	2533C→T, R798X	2533C→T, R798X	14.3 (100)	3.7	Inuit	Growth retardation; café-au-lait spots; hypoplastic thumbs; kidney and anal abnormalities; other skeletal (Klippel-Feil and Sprengel) malformations; hearing loss; BMF at 5.5 y
1010/1	2533C→T, R798X	2533C→T, R798X	18.1 (100)	1.5	Eur-Amer	Growth retardation; café-au-lait spots; radial ray and cardiac abnormalities; hearing loss; developmental delay; BMF at 5.5 y

[a]Percentage of aberrant cells is indicated in parentheses. [b]Fetal fibroblasts treated with 0.01 μg ml^{-1} DEB. [c]Paternal ancestry is African American and European American; maternal ancestry is European American and Hispanic. BMF, bone marrow failure; CMMol, chronic myelomonocytic leukemia; CNS, central nervous system; Eur-Amer, Americans of European descent; GI, gastrointestinal system; IUGR, intrauterine growth retardation; ND, not determined.

Because we identified mutations in *BRIP1*, we analyzed the BRIP1 protein by immunoblotting (Supplementary Methods). Two different BRIP1 antisera recognized a single species of ~150 kDa in wild-type cells. This protein was not evident in lymphoblastoid cell lines established from individuals who were homozygous (IFAR346/1, IFAR824/1, IFAR943/1, IFAR983/2 and IFAR1010/1) or heterozygous (IFAR764/1 and IFAR995/1) with respect to 2533C>T (R798X; Fig. 1d). We interpret these data to indicate that these individuals are BRIP1-deficient, but the presence of a truncated protein cannot be excluded by these analyses, as the antibodies both bind in the C terminus. Such a protein would not retain BRCA1 binding capacity, as the BRCA1-BRIP1 interaction domain lies in residues 888-1,063 (ref. 9), a region lost from the mutant gene product (Fig. 1c).

Previous analyses point to a functional interaction between monoubiquitinated FANCD2 and BRCA1 at presumptive sites of DNA damage [3]. As FANCD2 monoubiquitination is intact in the BRIP1-deficient cells, our findings suggest that the Fanconi pathway converges on BRCA1 and the BRIP1 helicase as mediators of chromosome maintenance downstream of FANCD2. Further, given the implication of both BRCA1 and BRIP1 in recombinational DNA repair [11-13], we suggest that DNA double-strand breaks are the lesions that underlie the pathology of Fanconi anemia.

Acknowledgments

We thank the affected individuals and their families for providing tissue samples for these studies; the many physicians who referred their patients to the IFAR and helped in sending the tissue samples for their contributions; A. Dawson, B. Chodirker and G. Graham for providing samples from the Inuit families, which were instrumental to the success of the mapping experiments; S. Cantor for the monopool BRIP1 antibody; Myriad Genetic Laboratories for sequencing *BRCA2* in IFAR samples; F. Lach, S. Arama, B. Zhang and Y. Flit for technical assistance; and J. Morales, C. Zhao and J. Lowe for advice. This work was supported in part by grants from the US National Institutes of Health (to A.D.A. and to J.P.), by the Joel and Joan Smilow Initiative (J.P.); by Kinderkrebsklinik Duesseldorf e. V. (H.H.); by CIEMAT and the Marcelino Botin Foundation (P.R.); by the Schroeder-Kurth Fund and the Deutsche Fanconi-Anamie-Hilfe (K.N., R.K. and D.S.); and by the Fanconi Anemia Research Fund (H.H.).

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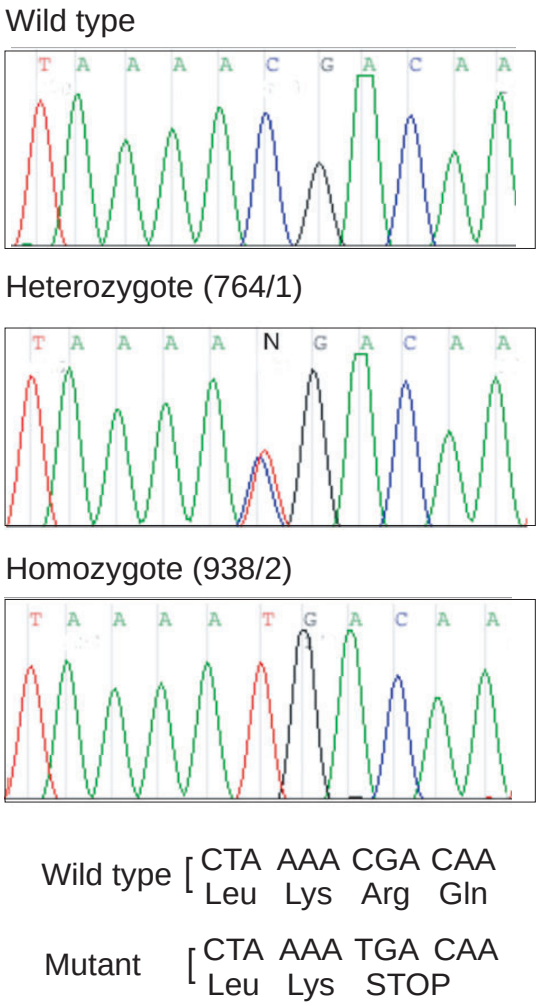
Supplementary Material

1) Supplementary Figure 1 (right).
Chromatograms displaying the wild type and mutation c.2533C>T in exon 17 of BRIP1, and illustration of the creation of a premature stop codon R798X. The protein sequence is shown below the genomic sequence. Patients IFAR764/1 (FA-J) and IFAR983/2 are heterozygous and homozygous, respectively, for the c.2533C>T mutation.



2) Supplementary Methods

Subjects and cell lines
Cell lines and genomic DNA samples were derived by standard methods from FA patients and family members registered in the IFAR after obtaining informed written consent. The studies were approved by the Institutional Review Board of The Rockefeller University. The diagnosis of FA was confirmed in patients with phenotypic manifestations of FA by study of baseline and DEB-induced chromosomal breakage in phytohemagglutinin (PHA)-stimulated cultured peripheral blood lymphocytes as described [1] (see **Table 1**). Complementation studies with retroviral vectors containing normal *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, *FANCG* and *FANCL* cDNAs were as described [2]. *BRCA2* was sequenced by Myriad Genetic Laboratories, Salt Lake City, UT. Immunoblotting for detection of monoubiquitinated FANCD2 was performed as described [3].

50K SNP array
A genome wide screen of DNA from five FA patients that had normal FANCD2 monoubiquitination was performed at The Rockefeller University Genotyping Resource Center, using the GeneChip® Human Mapping 50K Array 240 Xba (Affymetrix Inc**.** Santa Clara, CA) according to the manufacturer's guidelines. GeneChip® DNA Analysis Software (GDAS) provided the score and SNPs were annotated using data from the NetAffxTM Analysis Center (Affymetrix, Inc. Santa Clara, CA). SNPs with ambiguous positions were excluded [2].

Statistical Analysis.
To localize a genomic region harboring a potential FA gene, we searched for runs of consecutive SNPs for which all five individuals are homozygous (a form of linkage analysis). These runs were ranked by their lengths. To determine whether the longest runs can be considered statistically significant, a graphical analysis was employed where the run length (y-axis) was plotted against rank (x-axis).

PCR primers and conditions

Rad51C primers flanking exons 1-9 were designed using the Primer3 software (Whitehead Institute; http://frodo.wi.mit.edu/cgibin/ primer3/primer3_www.cgi). PCR conditions were as described [4]. *BRIP1* primers flanking exons 1-20 are described elsewhere [5], (http://lpg.nci.nih.gov/struewing/pubs/analysis/). Exon 19 was divided into 19a and 19b and exon 20 was divided into 20a, 20b and 20c. All 25 fragments were amplified using a single Touchdown PCR program that was a modification of the one described elsewhere (http://lpg.nci.nih.gov/struewing/pubs/analysis/). *BRIP1* cDNA primer sequences are available upon request.

Sequencing

PCR products were cleaned using ExoSAP-IT (USB, Cleveland, OH) according to manufacturer's protocol. Sequencing was performed by Genewiz Inc. (North Brunswick, NJ) with Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction kits (Applied Biosystems, Forester City, CA) on a 3730xl DNA analyzer. Sequence data were generated by means of Sequencing Analysis v.5.1 (Applied Biosystems, Forest City, CA) and analyzed with Sequencher v.4.5 (Gene Codes Corporation, Ann Arbor, MI).

Immunoblotting

Immunoblotting was carried out as previously described [6]. Cells were lysed in RIPA buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Sodium Deoxycholate, 0.1% SDS). Primary antibodies used were mouse BACH1 monopool (a gift from Sharon Cantor), mouse BACH1 1:500 (Novus Biologicals, NB-100-189), and mouse monoclonal actin 1:2500 (Sigma). Secondary antibodies used were ImmunoPure Protein A/G- or mouse IgG- HRP conjugated (Pierce). Detection was by ECL (Amersham Biosciences).

References for Supplementary Methods

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CHAPTER 7

Biallelic mutations in *PALB2*, which encodes a BRCA2 interacting protein, cause Fanconi anemia subtype FA-N and predispose to childhood cancer

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Abstract

Fanconi anemia is a rare, recessive, chromosomal instability disorder characterized by growth retardation, congenital malformations, progressive bone marrow failure, cancer predisposition and cellular hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC) [1,2]. Biallelic BRCA2 mutations cause Fanconi anemia subtype FA-D1 and predispose to childhood malignancies [3,4]. PALB2 was recently identified as a nuclear partner of BRCA2 and knockdown of PALB2 sensitizes cells to MMC [5]. We identified biallelic truncating PALB2 mutations in seven individuals affected with Fanconi anemia and solid tumors in early childhood. Transduction of cells from one of these individuals with wild-type PALB2 restored PALB2 expression and reversed MMC-induced G2 phase arrest. These genetic, protein and complementation data demonstrate that biallelic PALB2 mutations cause a new subtype of Fanconi anemia, FA-N, and, similar to biallelic BRCA2 mutations, confer a high risk of childhood cancer.

Fanconi anemia is a genetically heterogeneous condition with 12 complementation groups currently recognised, 11 of which have been attributed to distinct genes, *FANCA* (FA-A), *FANCB* (FA-B), *FANCC* (FA-C), *BRCA2* (FA-D1), *FANCD2* (FA-D2), *FANCE* (FA-E), *FANCF* (FA-F), *FANCG* (FA-G), *BRIP1* (FA-J), *FANCL* (FA-L) and *FANCM* (FA-M). These genes encode interacting proteins that participate in the recognition and repair of DNA interstrand cross-links [6,7].

BRCA2 is a DNA repair protein with a key role in the repair of double-strand DNA breaks by homologous recombination [8]. *BRCA2* was originally identified through positional cloning of a familial breast cancer predisposition gene and monoallelic (heterozygous) mutations are associated with high risks of breast and ovarian cancer [9]. Subsequently, biallelic *BRCA2* mutations were found to cause a rare subtype of Fanconi anemia, FA-D1 [3].

The phenotype of biallelic *BRCA2* mutations differs from other Fanconi anemia subtypes, most strikingly in the risk and spectrum of associated cancers. Collectively, all Fanconi anemia subtypes are associated with greatly increased risks of cancer, primarily acute myelogenous leukaemia and adult squamous cell carcinomas, particularly in the oral cavity and the pharyngo-esophageal and female genital regions [1]. Childhood solid tumors occur very rarely in Fanconi anemia and, to date, have been almost exclusively associated with FA-D1. The risk of childhood embryonal tumors, particularly Wilms tumor and medulloblastoma, is very high in biallelic *BRCA2* mutation carriers [3,4,10-12].

Although many cases of Fanconi anemia with childhood solid tumors are attributable to *BRCA2*, we identified individuals with this phenotype in whom *BRCA2* mutations could be excluded. This raised the possibility that deficiency of other proteins might give rise to this combination of features and we considered proteins functionally related to BRCA2 the most credible candidates.

PALB2 (for 'partner and localizer of BRCA2') was recently identified as a nuclear partner of BRCA2 [5]. PALB2 colocalises with BRCA2, promoting its localisation and stability in key nuclear structures, which in turn facilitates BRCA2 functions in DNA repair. Furthermore, knockdown of PALB2 sensitizes cells to MMC treatment which results in interstrand cross-links and double-strand breaks[5]. Sensitivity to MMC is a hallmark of Fanconi anemia and these data therefore recommended *PALB2* as a candidate Fanconi anemia gene.

We sequenced the 13 exons and intron-exon boundaries of *PALB2* in 82 individuals with Fanconi anemia not due to known genes. We identified pathogenic mutations in seven families (Figure 1a, Table 1). In four affected individuals (GESH, IFAR-847, LNEY, IFAR-849) we identified biallelic mutations that result in premature protein truncation. Seven of the mutations were small insertions or deletions that result in translational frameshifts, or base substitutions that generate premature stop codons. RNA analysis in LNEY demonstrated that the second mutation in this individual,

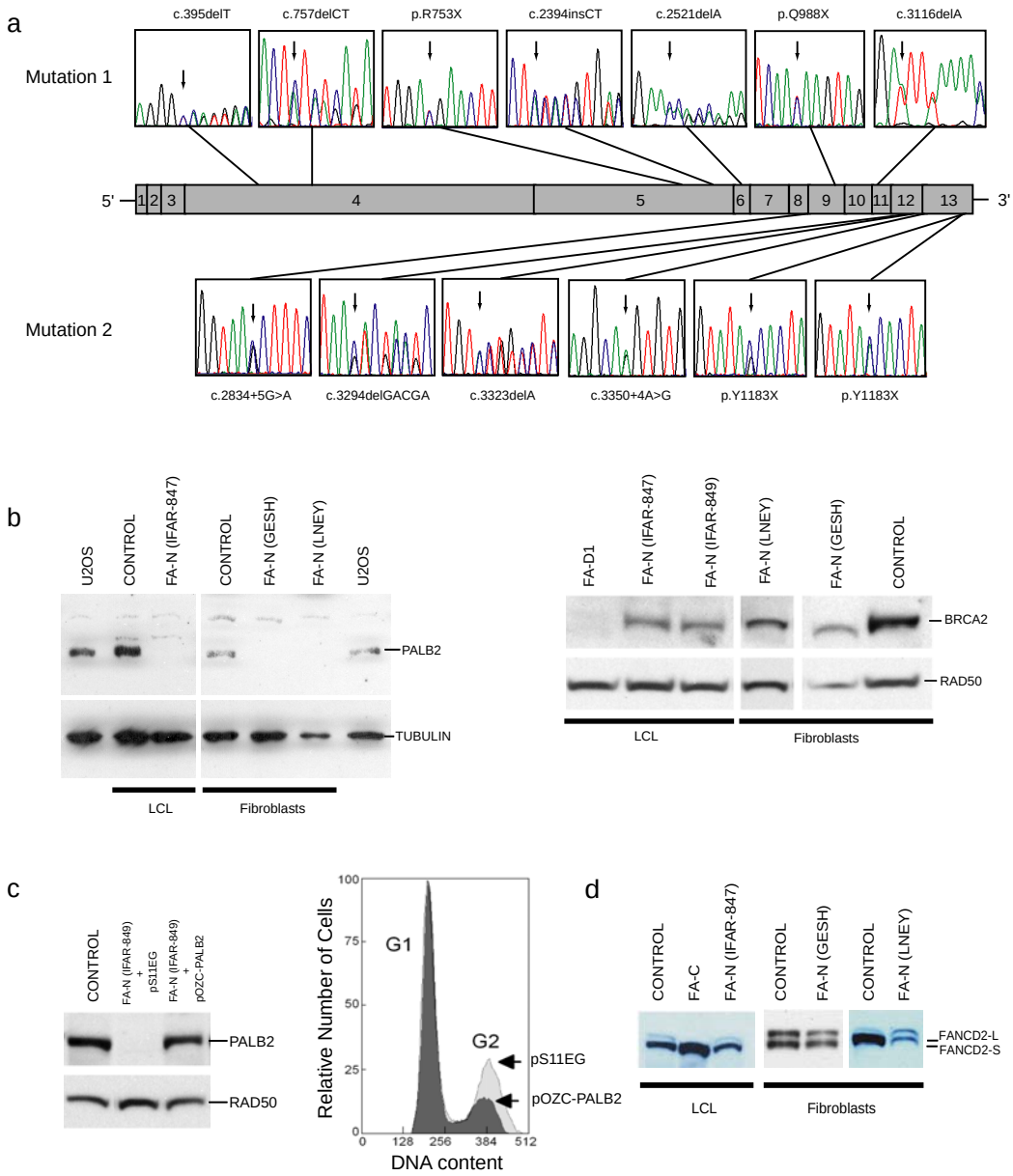

Figure 1. Evidence that PALB2 deficiency causes FA-N. **a**) Mutation analysis. Schematic representation of *PALB2* showing the position of mutations identified in FA-N cases. Further details of the mutations and the individuals that they were identified in are given in Table 1. The c.3549C>G, p.Y1183X mutation was identified in two separate families. **b**) PALB2 and BRCA2 immunoblotting. PALB2 protein is absent in cells from FA-N cases IFAR-847 (LCL), GESH (fibroblast) and LNEY (fibroblast) and is present in control cells (left hand panel). BRCA2 is present in FA-N and control cells and is absent in FA-D1 cells (right hand panel). Tubulin or RAD50 were used as loading control. **c**) Complementation of cellular FA phenotype. Transduction of cells from IFAR-849 with the vector pOZC-PALB2 restores PALB2 expression similar to that in a normal control, whereas the mock vector pS11EG (expressing GFP) does not. The transduction leads to reduction in MMC-induced G2 phase arrest in cells transduced with pOZC-PALB2 (black) compared to that in the mock-transduced line pS11EG (grey) grown for the same time at identical MMC concentration. This effect has been reported in complemented FA cell lines from other subtypes [23] **d**) FANCD2 monoubiquitination. There is normal FANCD2 monoubiquitination in control cells and cells from FA-N cases IFAR-847, LNEY and GESH, whereas FA-C cells show absent FANCD2 monoubiquitination. This indicates that PALB2 acts downstream of FANCD2 in the FA-BRCA pathway.

c.3350+4A>G, results in aberrant splicing and generates two abnormal products, r.3202_3350del149, in which exon 12 is skipped, and r.3350insGCAG, which utilises a cryptic splice donor site. Both changes introduce translational frameshifts. Analysis of parental DNA demonstrated that all the mutations had been inherited from different parents, consistent with autosomal recessive inheritance. No sample was available from the affected individuals LOAO, ICR-40 and ICR-60, but

their parents all carried *PALB2* mutations. We also sequenced *PALB2* in 352 control chromosomes (176 normal individuals). No truncating or splice junction variants were identified providing further evidence that such mutations are pathogenic in the individuals with Fanconi anemia.

One mutation, c.3549C>G, p.Y1183X, was seen in two separate families. A different mutation at the same nucleotide, c.3549C>A, also results in p.Y1183X and was identified in a third family. c.3549C is in the last exon of *PALB2* and there are only three amino acids after codon 1183 before the protein terminates. Truncating mutations close to the end of a protein are generally expected to escape nonsense-mediated RNA decay [13]. However, there was no detectable PALB2 protein in lymphoblastoid cells from IFAR-847 and IFAR-849, who both carry p.Y1183X, indicating that the mutation results in a null allele and confirming its pathogenicity (Figure 1b).

Western blot analysis using antibodies to PALB2 on lymphoblastoid cells or fibroblasts from four individuals with biallelic *PALB2* mutations showed absence of PALB2 protein in each case (Figure 1b). Transduction of cells from IFAR-849 with an expression construct carrying wild type PALB2 restored PALB2 expression and reversed MMC-induced G2 phase arrest (Figure 1C). The combined genetic, protein and complementation data provide strong evidence that *PALB2* mutations underlie a new Fanconi anemia complementation group that we have designated subtype FA-N.

Eight of the known Fanconi anemia proteins, (A, B, C, E, F, G, L and M), form a nuclear core complex that mediates monoubiquitination of FANCD2. Activated FANCD2 is translocated to DNA repair foci where it colocalizes with various proteins involved in the DNA damage response, including BRCA2 [6,7]. Given the close functional relationship between BRCA2 and PALB2, one would predict that PALB2 acts downstream of FANCD2 in the FA-BRCA pathway. We confirmed this in different cell types from IFAR-847, GESH and LNEY which show normal monoubiquitination of FANCD2 (Figure 1d).

The phenotype of FA-N is, in many ways, typical of Fanconi anemia and includes growth retardation and variable congenital malformations (Table 1) [14]. The cellular phenotype of PALB2 deficiency is similar to BRCA2 deficiency and more severe than other Fanconi anemia subtypes, with elevated spontaneous chromosome breakage rates and markedly reduced lymphocyte survival and increased chromosome breakage on exposure to MMC (Figure 2a, 2b). There was also no

Table 1. PALB2 mutations and clinical features of FA-N cases.

		Mutation 1		Mutation 2		Clinical Features	
ID	**Origin**	**Nucleotide change**	**Effect**	**Nucleotide change**	**Effect**	**Cancer (age at diagnosis)**	**Other features**
LOAO [a]	Albanian[b] / Morrocan [c]	c.395delT	p.V132fs	2834+5G>A[d]	Presumed splice defect	medulloblastoma (3.5yr)	Growth retardation, radial ray hypoplasia, absent right kidney.
GESH	White (German)	c.757_758delCT	p.L253fs	c.3294_3298 delGACGA	p.K1098fs	Wilms tumor (0.9 yr), AML (0.9 yr), medulloblastoma (1 yr)	Severe growth retardation, hypoplastic thumbs, left pelvic kidney, anal atresia, microcephaly, congenital cataract, microphthalmia.
IFAR-847	Hispanic[b] / White (N Am)[c]	c.2257C>T	p.R753X	c.3549C>A	p.Y1183X	Wilms tumor (1yr)	Severe IUGR, postnatal growth retardation, microcephaly, microphthalmia, skin hyperpigmentation.
LNEY	White (German)	c.2394_2395insCT	p.T799fs	c.3350+4A>G[e]	r.ex12del/p.G1068fs r.3350insGCAG/p.F1118fs	medulloblastoma (4 yr)	Growth retardation, microcephaly, microphthalmia.
ICR-40[a,f]	White (N Am)[b] / Black (N Am)[c]	c.2521delA	p.T841fs	c.3323delA	p.Y1108fs	Wilms tumor (1.5 yr), medulloblastoma (1.5 yr)	Growth retardation, microcephaly, skin hyper- and hypopigmentation, horseshoe kidney, gonadal dysgenesis.
ICR-60[a]	White (UK)	c.2962C>T	p.Q988X	c.3549C>G	p.Y1183X	medulloblastoma (2.3 yr)	Growth retardation, microcephaly, hypoplastic thumb.
IFAR-849	White (N Am)	c.3116delA	p.N1039fs	c.3549C>G	p.Y1183X	neuroblastoma (0.7 yr), AML (2 yr)	Growth retardation, microcephaly, VSD, ASD, thumb and radial anomalies, skin hyperpigmention.

[a]No samples were available from the affected individuals and the mutations were therefore identified in parental samples. [b]Origin relates to mutation 1. [c]Origin relates to mutation 2. [d]This mutation is thought to effect splicing as it occurs at a known donor splice site. [e]This mutation resulted in either skipping of exon 12 or insertion of the intronic nucleotides GCAG after nucleotide 3350. [f]This individuals clinical details have previously been published [22]. AML, acute myelogenous leukaemia; IUGR, intrauterine growth retardation; ASD, atrial septal defect, VSD, ventricular septal defect; N Am, North American ancestry.

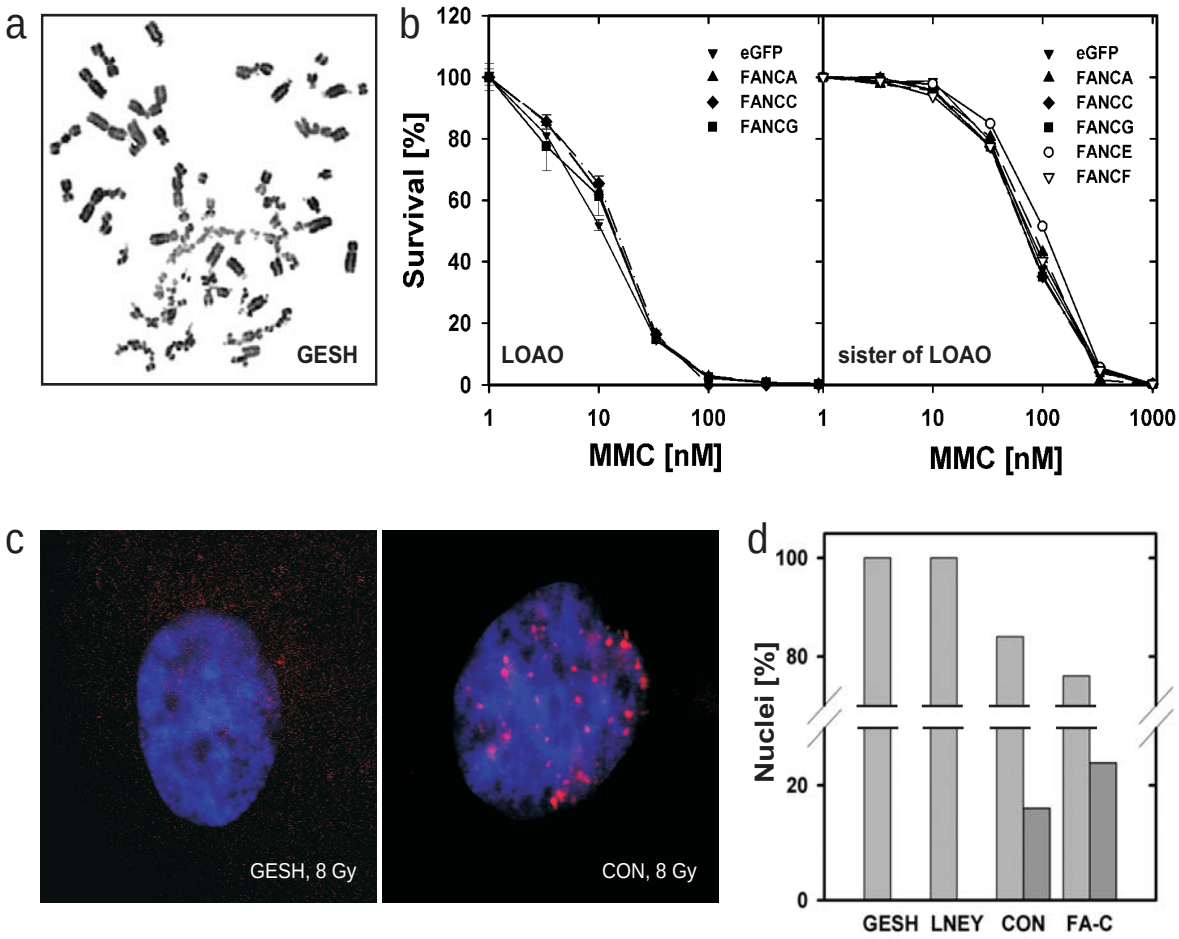

Figure 2. Cellular phenotype of PALB2 deficiency. **a**) Severe chromosomal damage in a fibroblast metaphase from GESH after treatment with 50 ng/ml MMC. **b**) Dose-response curves demonstrate diminished survival of lymphocytes from patient LOAO as opposed to his heterozygous sibling. The cells were transduced with eGFP or Fanconi anemia cDNAs as indicated. **c**) No cells with >5 RAD51 nuclear foci were detectable in fibroblasts from GESH after induction of DNA damage, whereas a normal control clearly showed nuclei (blue) with >5 RAD51 signals (red). **d**) The proportion of nuclei with >5 RAD51 foci was ~0 in fibroblasts from GESH and LNEY compared to 16% in control cells and 24% in FA-C cells.

formation of nuclear RAD51 foci in PALB2-deficient fibroblasts after ionising irradiation. Again, this is similar to cells with biallelic *BRCA2* mutations and differs from other Fanconi anemia subtypes (Figure 2c, 2d) [15].

FA-N is associated with unusually early mortality due to childhood cancer. All seven FA-N cases developed cancer in early childhood including three Wilms tumors, five medulloblastomas, two cases of AML and one neuroblastoma (Table 1). One individual developed three malignancies within the first year of life and three individuals had two cancers. Cancer treatment was unsuccessful in six patients, all of whom died before four years of age. LNEY is currently alive at 4.5 years but is in the early stages of treatment for medulloblastoma. The cancer spectrum of biallelic *PALB2* mutations is very similar to that associated with biallelic *BRCA2* mutations, which also confer high risks of embryonal tumors [4]. The reasons for the association between childhood solid tumors and deficiency of BRCA2 or PALB2 are unclear, but seem likely to be related to functions not shared by other FA proteins.

Monoallelic (heterozygous) *BRCA2* mutations are associated with high risks of breast and ovarian cancer and lesser risks of other cancers such as prostate and pancreatic cancer [9]. Given the intimate functional links between PALB2 and BRCA2 and the similar phenotypes associated with biallelic mutations in the genes that encode them, it is plausible that monoallelic *PALB2* mutations

confer susceptibility to adult cancer. Of interest in this regard are the cancer histories in the seven FA-N families we have identified. The mother of ICR-40 had early-onset bilateral breast cancer and has a strong family history of breast cancer affecting her sister, mother and other more distant relatives. *BRCA1* and *BRCA2* mutations have been excluded in this family. No other first-degree relatives of FA-N cases are known to have developed cancer, although most are still less than 50 years of age. However, the maternal grandmother of ICR-60 and the maternal great grandmother of GESH both developed breast cancer at 52 years and 20 years respectively. Mutational analyses of *PALB2* in individuals with adult-onset cancer, particularly familial breast cancer, will clarify the role of monoallelic *PALB2* mutations in cancer susceptibility.

Mutations in *BRCA2* and *PALB2* together account for almost all our individuals with both Fanconi anemia and childhood solid tumors. However, there are rare Fanconi anemia cases, not due to known genes, whose cells show normal monoubiquitination of FANCD2. Thus it is possible that other genes encoding proteins that physically and/or functionally interact with BRCA2 cause currently unrecognised subtypes of Fanconi anemia.

METHODS

Samples and cell lines

Genomic DNA was obtained from 82 individuals with Fanconi anemia and / or their parents with informed consent. These individuals came from several different clinicians and repositories. All had a clinical diagnosis of Fanconi anemia. This diagnosis was confirmed by study of baseline and DEB- or mitomycin C-induced chromosomal breakage or cell cycle arrest in PHA-stimulated cultured peripheral blood lymphocytes as previously described [16,17], except in cases that died before suitable samples could be obtained. In some cases, diagnosis of Fanconi anemia was based on fibroblast analysis. None of the cases included in this study were known to be due to an existing Fanconi anemia gene. However, the extent to which known genes were formally excluded varied, depending on the available samples. Methods used for exclusion were sequencing, retroviral complementation group analysis [18], and western blot analysis for FANCD1 [19], FANCD2 [20] and FANCJ [21]. *BRCA2* mutations had been excluded in all cases by sequencing of all exons and intron/exon boundaries in genomic DNA from either the case and/or parents. The 176 samples from normal individuals were from the 1958 Birth Cohort Collection (see http://www.cls.ioe.ac.uk/Cohort/Ncds/mainncds.htm). This research was approved by the London Multicentre Research Ethics Committee (05/MRE02/17).

PALB2 sequencing

Primers were designed to amplify the 13 exons and intron-exon boundaries of *PALB2* (Supplementary Table 1 online). We used a touchdown 68-50°C protocol to amplify all products which were sequenced using the BigDyeTerminator Cycle sequencing kit and a 3730 automated sequencer (ABI Perkin Elmer). Sequencing traces were analysed using Mutation Surveyor software (www.softgenetics.com) and by visual inspection.

Immunoblotting

PALB2 immunoblots were performed with samples containing 50 μg total protein each on 7% NuPage Tris-Acetate polyacrylamide gels (Invitrogen). Membranes were probed with polyclonal rabbit anti-PALB2 antiserum raised against the first 120 amino acids of PALB2 at a concentration of 1:1000 [5]. Secondary antibody was ECL donkey anti-rabbit IgG horseradish peroxidase-linked whole antibody (GE Healthcare) at 1:2000. Detection was by the chemiluminescence technique using the ECL system (Amersham). BRCA2 immunoblots were performed with samples containing 80 μg

total protein each on 3-8% NuPage Tris-Acetate polyacrylamide gels (Invitrogen). Membranes were probed with polyclonal rabbit anti-BRCA2 antiserum (Ab-2, Calbiochem), raised against amino acids 3245-3418 of BRCA2, at a concentration of 1:200. Secondary antibody (1:5000) and detection were the same as for the PALB2 immunoblots. Immunoblotting for detection of monoubiquitinated FANCD2 was performed using previously described methods [20].

Transduction and complementation

For complementation studies, the γ-retroviral vectors pOZC-PALB2 [5] and pS11EG (expressing GFP) were packaged in PG 13 cells and used for transduction of patient and control cell lines. Gene transfer was monitored by CD25 (pOZC-PALB2) or GFP (pS11EG) expression. Transduced cells were grown for 48 h in the presence of MMC at concentrations of 12 (fibroblasts) or 15 ng/ml (LCL). The cells were vitally stained with Hoechst 33342 fluorescent dye (Molecular Probes) at 16 μg/ml. DNA histograms were recorded by flow cytometry.

Lymphocyte survival assay

Lymphocyte survival was determined using CD3/CD28/IL2-stimulated lymphocytes. Transduction was with FANC cDNAs (as indicated in Figure 2B) and GFP cDNA as a control, separately cloned into a S11-type γ-retroviral vector as described [18]. The cells were exposed to various concentrations of MMC for 5 days. Live/dead cell ratios were determined by propidium iodide exclusion/uptake on flow cytometry.

Immunofluoresence

To examine RAD51 foci formation, nuclear foci were induced by ionizing irradiation with 8 Gy of fibroblast cultures grown on glass slides. Cells were fixed 8 h later using 4% paraformaldehyde in PBS (pH 6.8) and permeabilized with 0.1% Triton X-100. Primary antibody was mouse monoclonal anti-RAD51 (GeneTex) at a dilution of 1:100. Secondary antibody was Alexa594-conjugated goat anti-mouse IgG (Molecular Probes) at 1:200. DAPI was used as DNA counterstain. Nuclear foci were counted on a fluorescence microscope.

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CHAPTER 8

Human RAD50 deficiency causes impairment of ATM- and NMR-related functions

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to be submitted

Abstract

The NBS1/MRE11/RAD50 (NMR) complex plays a central role in the cellular response to DNA double strand breaks. Mutational inactivation of NBS1 causes the Nijmegen breakage syndrome (NBS) while inactivation of MRE11 causes an ataxia telangiectasia-like disorder (AT-LD), both radiosensitive conditions of autosomal recessive inheritance. Here we present the first patient with biallelic mutations in *RAD50*, the third member of the NMR complex. With the exception of immunodeficiency, the patient´s phenotype resembles NBS. She is compound heterozygous for the null allele R1093X and the hypomorphic allele X1313Y, the latter giving rise to residual protein. Her mononuclear blood cells show marked sensitivity towards ionizing irradiation (IR) including chromosomal instability and accumulation in the G2 phase of the cell cycle. In RAD50 deficient cells, MRE11 and NBS1 fail to relocalize into nuclear foci following IR. In addition, these cells show attenuated activation of the ATM kinase and of its downstream targets Chk2 and p53, while IR induced phosphorylation of NBS1 at Ser343 is abrogated. Our data show, that RAD50 modulates, like NBS1 and MRE11, the ATM-mediated DNA damage response and the G1/S cell cycle checkpoint, and is important for nuclear localization of MRE11, and NBS1 focus formation.

Eukaryotic cells have developed effective mechanisms to deal with DNA damage in order to maintain genome integrity [1, 2]. One important player in the cellular response to DNA double strand breaks is the NBS1/MRE11/RAD50 (NMR) complex, which is involved in several functions including signalling of DNA damage, DNA repair and restart of collapsed replication forks [3]. Null mutations in either of the three genes of the NMR complex are not viable in vertebrates [4-6]. However, hypomorphic mutations in *NBS1* and *MRE11* have been shown to cause the radiosensitivity syndromes NBS and AT-LD [7].

Previously, a then 4-year-old girl of non-consanguineous German ancestry was reported as having a NBS-like condition [8]. Her diagnosis was based on typical congenital (see supplemental table 1) and haematological laboratory findings including spontaneous chromosomal instability and cellular hypersensitivity to ionizing radiation. At age 15, she presented mild to moderate delay of psychomotor development, mild spasticity and modestly impaired senso-motor coordination resembling progressive ataxia. Physical examination revealed height (130 cm), weight (29 kg), and head circumference (43 cm) well below the 3rd percentile. Of note, the girl had never experienced recurrent or severe infections, and biannual laboratory studies showed normal serum levels of immunoglobulin (Ig) classes G, A, M, D, and the IgG subclasses. Lymphocyte counts, subset distributions, and lymphocyte response to mitogen were within normal limits. There was no evidence of lymphoid malignancy such as occurs in individuals with NBS. Patient-derived lymphoblasts and primary fibroblasts showed reduced levels of NBS1, MRE11 and complete loss of RAD50 as revealed by immunoblot (Fig. 1A). However, traces of residual RAD50 protein were detected after extended exposure (Fig.1B) including a wildtype-sized RAD50 protein band and an additional band corresponding to a slower migrating isoform with an increased molecular weight of approximately 5-10 kDa. Sequence analysis of the entire coding region of *RAD50* at mRNA (lymphoblasts) and genomic (lymphocytes and fibroblasts) level revealed compound heterozygous mutations affecting highly conserved amino acid residues (Fig.1C-F). The maternal mutation (c.3277C>T, R1093X) is located in exon 21 and causes premature termination (Fig. 1C). The paternal mutation c.3939A>T (located in exon 25) affects the *RAD50* stop codon by changing the ochre triplet into a tyrosine codon (X1313Y) (Fig. 2D). Translation of the paternal allele (X1313Y) is predicted to result in a somewhat larger polypeptide compared to wildtype sequence. It is extended by 66 additional

carboxyterminal amino acids encoded by the 3´-UTR sequence. Translation termination of the resulting ORF takes place 198 bases downstream of the mutated stop codon at position 4135-4137 (Fig. 2H). The prediction of a larger polypeptide encoded by the aberrant paternal allele is consistent with both the larger protein band observed on immunoblots (Fig.1B) and the larger protein band in a cDNA based protein truncation test (Fig.1G). Notably, cDNA sequencing indicated a quasi-hemizygous expression of the paternal allele, implying nonsense mediated mRNA decay for the maternal allele. Screening of 150 chromosomes from a random population sample failed to reveal similar changes present in our patient.

In human wildtype cells the RAD50, MRE11 and NBS1 proteins are known to colocalize in large nuclear foci following irradiation (ref. [9]). As shown in figure 2, NBS1, MRE11 and RAD50 are

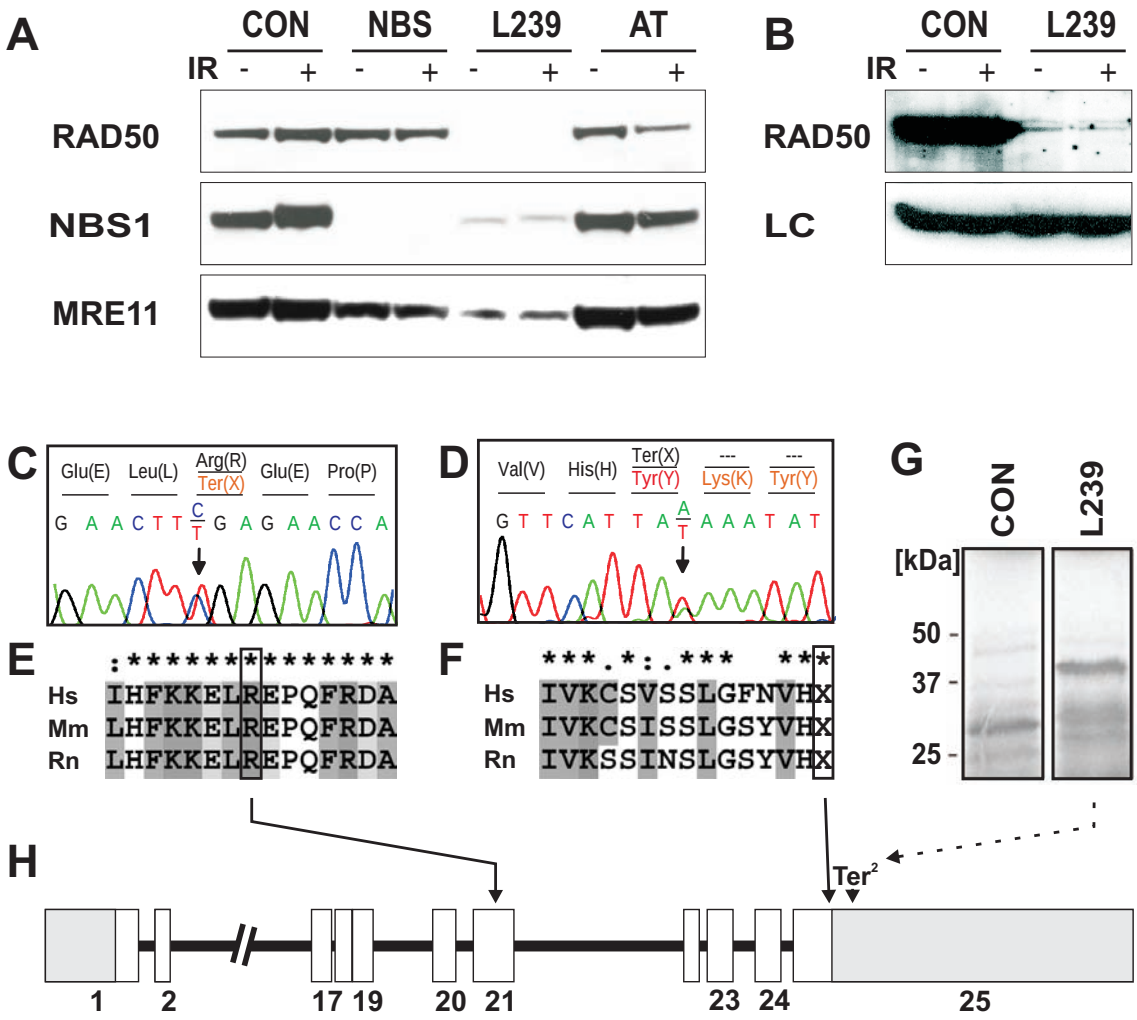

Fig. 1: RAD50 deficiency caused by hypomorphic mutations. Immunoblot analysis of NBS1, MRE11, RAD50 in whole cell extracts of either untreated (-) and 6 Gy irradiated lymphoblasts (+) revealed lack of RAD50 protein in patient derived cells (L239) and reduced amounts of NBS1 and MRE11 compared to normal control (CON) and AT lymphoblasts (A). However, residual levels of RAD50, a normal sized and larger variant, could be detected after overexposure (B). Mutation analysis revealed to heteroallelic base substitutions, 3277C>T (R1093X) (C) and 3939A>T (X1313Y) (D), causing changes of amino acid residues conserved within vertebrates (Hs: *Homo sapiens*, Mm: *Mus musculus*, Rn: *Rattus norvegicus*, ClustalX alignment) (E, F). The allele carrying the mutation 3939A>T occur in a quasi hemizygous state at mRNA level resulting in protein extension, which is reflected by the band shift in the protein truncation test (G). Loss of the natural termination signal results in an extension of the open reading frame for 196 (66 aa) until the next stop codon follows indicated in the illustrated *RAD50* gene by Ter[2] (H).

diffusely distributed throughout the nucleus in control fibroblasts, and irradiation (IR) causes relocation into distinct nuclear foci. In RAD50 deficient fibroblasts NBS1 was also located predominantly in the nucleus, however lacking the ability to form IR induced foci (Fig. 2A). In contrast, in RAD50 deficient fibroblasts MRE11 was found predominantly in the cytoplasm independent of IR. Transduction of *RAD50* cDNA into TERT-immortalized patient derived fibroblasts restored both nuclear relocation of MRE11 and focus formation of RAD50 and MRE11 in response to IR (Fig. 2B). Our data indicate that intact RAD50 is essential for nuclear localisation of MRE11 and DNA damage induced recruitment of NBS1 and MRE11 into nuclear foci.

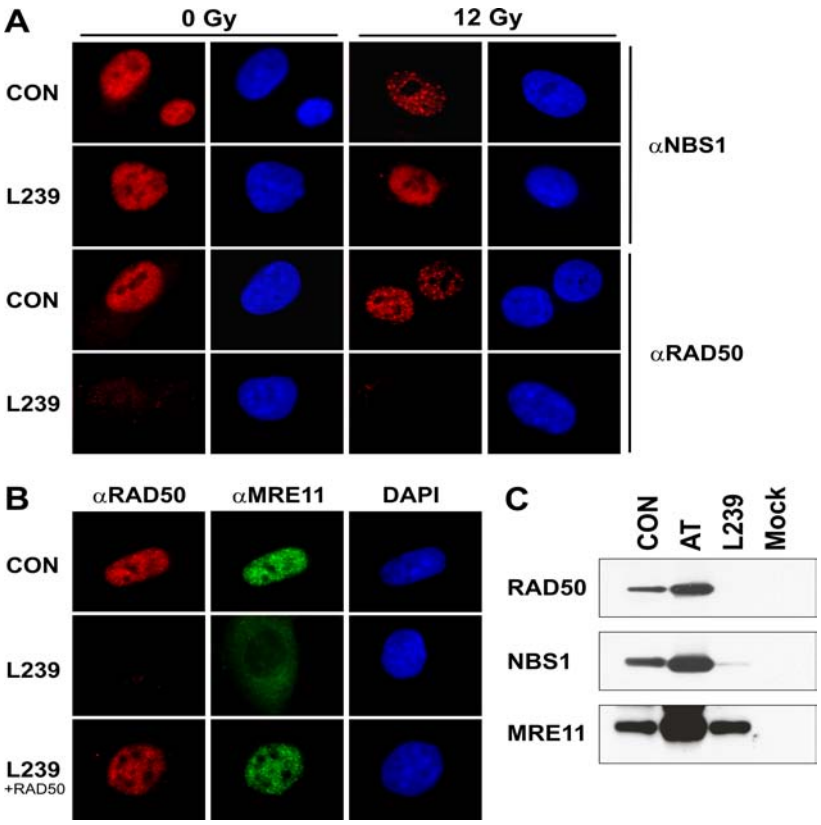

Fig. 2: Irradiation induced relocation of NBS1, MRE11 and RAD50. Immunofluorescence of primary fibroblasts with mutant *RAD50* (L239) and normal controls assessed for their ability to form irradiation induced nuclear foci (A). NBS1 is located predominantly in the nucleus, however the patient derived cells show no radiation induced relocation of NBS1 into nuclear foci. RAD50 protein was not detected in patient fibroblasts (A,B). As shown in TERT immortalized fibroblasts RAD50 deficient cells show a cytoplasmatic localization of MRE11. Nuclear localization and focus formation of MRE11 and RAD50 is restored after transduction of RAD50 cDNA (B). Both complex partners NBS1 and RAD50 could be co-immunoprecipitated with anti-MRE11 antibodies in normal lymphoblast protein extracts. In comparison, RAD50 deficient lymphoblasts show a strongly reduced level of NBS1-MRE11 interaction (C).

As shown in figure 2C NBS1 and RAD50 protein could be coimmunoprecipitated in lymphoblasts using antibodies against MRE11 (and vice versa, data not shown). In patient cells the interaction of the complex partners MRE11 and NBS1 is strongly reduced compared to controls suggesting a stabilizing function of RAD50 for the MRE11-NBS1 interaction.

A common biological marker for ATM activation is its autophophorylation at residue 1981 [10] and IR-induced activation of ATM appears to be impaired in NBS1 cells [11]. We observed that in RAD50 deficient fibroblasts the DNA damage-induced ATM autophosphorylation was partly reduced after 6 Gy irradiation (Fig. 3C). In addition, the radiation-induced ATM-dependent phosphorylation of p53 via Chk2 at serine 15 was reduced by 50% relative to wildtype control cells (Fig. 3B), and there was no phosphorylation of NBS1 at Ser343 (Fig. 3A). Importantly, ATM autophosphorylation, phosphorylation of p53Ser15 and NBS1Ser343 were restored after transfection of full length RAD50 cDNA into RAD50 deficient lymphoblasts (Fig. 3C). Thus RAD50 at least partially influence ATM kinase activity and ATM-dependent signalling after IR. Our conclusion is further supported by the finding of reduced p21/WAF1 transcription levels in response to IR in our RAD50 deficient patient cells (data not shown).

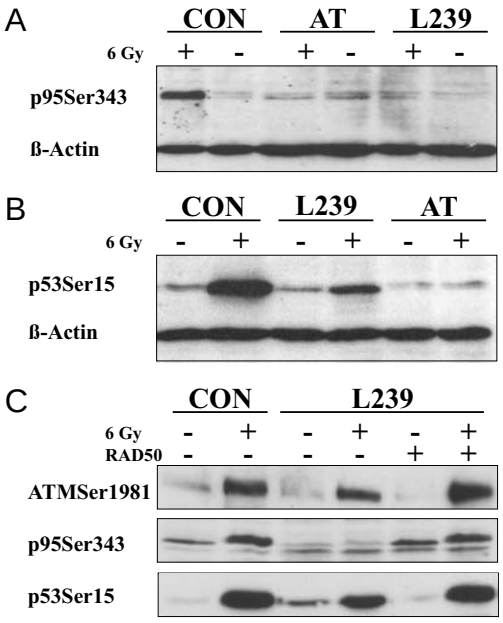

Fig.3. Role of RAD50 in ATM-dependent IR induced signalling. In both ATM (AT) and RAD50 deficient (L239) lymphoblasts is no IR induced phosphorylation of NBS1 at serine 343 detectable, which is present in irradiated controls (A). In addition the phosphorylation of p53 at Ser15 in response to IR is reduced in AT and RAD50 deficient cells (B). Reduction of ATM autophosphorylation at Ser1981, phosphorylation of NBS1 (Ser343) and p53 (Ser15) is restored after transient transfection of RAD50 cDNA (C)

A common cellular feature of NBS, AT and AT-like disorders is a significant increase in chromosomal instability. We analysed patient's lymphocytes for the occurrence of aberrant chromosomal structures. We found spontaneous chromosomal instability (1996, 0.54 breaks/metaphase; 2001, 0.88 breaks/metaphase) as well as radiation-induced chromosomal breaks (supplemental table 2). Chromosome painting assays using probes of chromosomes 1, 2 and 4 uncovered spontaneous translocations (23/ 1000 metaphases) (Fig. 4A), comparable to what has been shown for cells from AT and NBS patients [12]. In contrast, the frequency of translocations of chromosomes 7 and 14 was lower than reported for AT or NBS (GTG banding: 4% (1996); 0% 2001; chromosome painting: 1.5% (2001)). Compared to AT or NBS cells, the RAD50 deficient cells showed an unexpected frequency of chromatid exchanges (chte) (blood 7.6 chte/100 metaphases; LCL 2.7 chte/100 metaphases) (Figure 4B). All chte-figures consisted of non-homologous chromosomes involved in quadriradial-type interchanges.

Radiosensitivity of AT or NBS cells is reflected by the accumulation in the G2 phase of the cell cycle. Patient-derived lymphocytes, lymphoblasts and fibroblasts were found to accumulate in the G2 phase of the cell cycle after IR in a dose dependent manner (Suppl. Fig. 1), indicating accumulation of unrepaired DNA lesions - or checkpoint defects. Using BrdU quenching combined with DNA staining we found that more RAD50 deficient lymphoblasts leave the first G0 phase 48 h after irradiation at 2.5, 4 and 8 Gy compared to wildtype cells (Fig. 4C), suggesting that the G1/S phase checkpoint may be ´leaky´ in RAD50 deficient cells like it was shown for NBS1 cells [13]. After low irradiation doses, a certain proportion of control cells accumulate in the first S phase, while RAD50 deficient cultures failed to accumulate. This may reflect radioresistant DNA synthesis (RDS) comparable to what is known for MRE11 and NBS1 [7].

Little is known about the developmental functions of RAD50 since null mutations in *RAD50* cause embryonic stem cell lethality in mice [4]. Here we show that *RAD50* deficiency is associated with a phenotype resembling NBS rather than AT-LD, indicating distinct roles for MRE11 and RAD50/NBS1 in neuronal development. This is of special interest since MRE11 binds to both NBS1 and RAD50 [14, 15]. Importantly, the phenotype of the RAD50 deficient patient also differs from classical NBS by the absence of any immunological defects and the presence of quadriradial exchange figures, which are typical for Bloom-syndrome and Fanconi anaemia patients but not for NBS [16]. This suggests RAD50-specific functions that are independent of NBS1 and MRE11.

MRE11 and RAD50, but not NBS1, have been shown to be involved in the activation of ATM at early time points after low doses of IR [17]. In agreement with this, we have shown that RAD50 deficiency causes reduction of ATM autophosphorylation and reduction of ATM-dependent responses. Our findings strongly support the role of MRE11/RAD50 as a sensor of DNA damage [18, 19]. However, the role of RAD50 may be direct or indirect, since RAD50 deficiency also

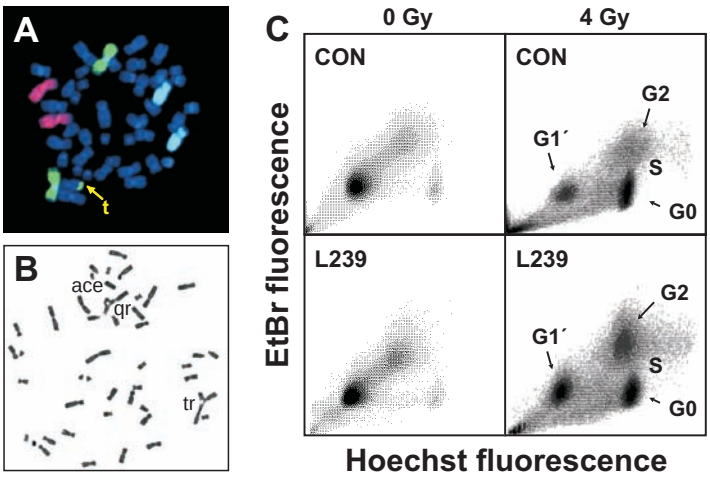

Fig. 4. Chromosomal aberration and cell cycle checkpoints. Chromosome painting of chromosome 1 (green), 2 (red) and 4 (light blue) revealed spontaneous translocation (t) of a chromosome 1 fragment to a non-homologous chromosome in patient derived lymphocytes. Cytogenetic analysis of Giemsa stained metaphases also showed pontaneous chromatid exchanges (chte). **qr** = asymmetrical quadriradial interchange with complete exchanges and acentric fragment (**ace**); **tr** = symmetrical triradial interchange with incomplete exchanges (B). After irradiation with the indicated doses BrdU was added to the culture medium of an assynchronic growing culture. After 48 h the nucleus was double stained with Hoechst 33258 and etidium bromide (EtBr). BrdU incorporation quenches Hoechst fluorescence, which enable separation of each cell cycle. The growth of RAD50 deficient and controls were comparable in untreated cells. Irradiation (4 Gy) results predominately in G0 and S phase cell cycle arrest of the control cells. In contrast, RAD50 deficient cells show a difference in the cell cycle distribution: slightly reduced G0, low S phase and high G2 phase fractions (C).

affects the nuclear localization of MRE11 and its interaction with NBS1. In addition, mutational inactivation of RAD50 causes a reduction of the expression levels of the remaining components of the complex, a phenomenon also observed in cells where NBS1 or MRE11 are mutated [7, 9].

In conclusion, our data strengthen the clinical and molecular relationship among the three members of the NMR complex but also highlight some functional differences between the MRE11, NBS1 and RAD50 proteins. The appearance of immunodeficiency with *NBS1* but not with *RAD50* mutations may reflect an NMR independent role of RAD50 in lymphoid-cell specific recombination events [20]. However, the occurrence of chromatid exchange figures implies the participation of RAD50 in recombinational types of DNA repair other than non-homologous end-joining, typical for gene rearrangements of immune cells [21]. Our findings and the availability of a patient-derived RAD50 deficient cell line provide the basis for the delineation of the biological significance of the individual proteins that make up the NMR complex.

Methods

Cell cultures and treatments.

Whole blood was stimulated by 2.5% phytohemagglutinin. Lymphoblast cell lines were established by EBV transformation of Ficoll-isolated blood lymphocytes. Primary fibroblast cells were grown from skin explants. The RAD50-deficient fibroblasts, but not those of the parents, displayed a prematurely senescent morphology (akin to WRN cells) and poor growth even at early passages. Suspension or adherent cell cultures were maintained in RPMI 1640 or D-MEM (Invitrogen, Karlsruhe, Germany), respectively, supplemented with 10-20% heat inactivated FBS (Sigma, Taufkirchen, Germany), 1% L-glutamine and antibiotics in an humidified atmosphere with 5% CO_2 at 37 °C. Ionizing radiation employed either a ^{60}Co- or an X-ray source (dose rate, 1.5 Gy/min), both controlled by dosimetry.

For cell cycle analysis fibroblasts and lymphoblasts were harvested 48 h after treatment and nuclei were stained with DAPI. For bivariant flow cytometry BrdU was added to the culture medium, directly after irradiation. The harvested cells were double stained with Hoechst 33258 (Sigma-Aldrich, Taufkirchen, Germany) and ethidium bromide. Flow histograms were recorded on

an analytical dual-laser-equipped flow cytometer (LSR1, BD Biosciences, Heidelberg, Germany). Cell cycle distributions were quantified using the M-Plus AV software package (Phoenix Flow Systems, San Diego, CA, USA) as previously described [22].

Cytogenetic analysis.
T-lymphocytes and LCLs were examined for spontaneous and radiation-induced chromosomal instability. Exponentially growing cells were irradiated in the G2-phase of the cell cycle, using an X-ray therapeutic radiation source (dose rate, 1.5 Gy/min). Colcemid (0.06 µg/ml) was added to the cultures 2 h after irradiation and the cells were harvested 2 h later. Metaphase plates were prepared and stained using standard cytogenetic techniques. Chromosome aberrations were scored according to ISCN nomenclature. Cytogenetic analysis was performed using standard techniques. Metaphases were GTG-banded using pancratin and giemsa. Chromosome painting standard was performed as described previously [23], using hybridization probes for chromosome 7 and 14 or 1, 2 and 4.

Genomic work up
Total RNA and genomic DNA were isolated from peripheral white blood cells, lymphoblasts and fibroblasts. RNA was reverse-transcribed using random hexamers (Amersham Bioscience, Munich, Germany) to prime first-strand cDNA reactions. The entire coding region of *RAD50* was amplified by PCR and sequenced in ten overlapping fragments using primers derived from the published cDNA sequence. For the amplification of genomic DNA we constructed primers based on exon-flanking intronic sequences derived from two published P1 clones which allowed us to assemble the exon-intron structure of the *RAD50* gene. Specifically, our genomic PCR conditions were as follows:

5'-AACAGCACCCAGCACCTAGC-3' and 5'-CCTACACCTGTGGAGCCCTA-3'	(exon 1, annealing 59°C)
5'-GGTAAACTTCTGTGGTTCTC-3' and 5'-GGTTACTACTGGGTGCTAAA-3'	(exon 2, annealing 53°C)
5'-CCAACACTGGTGCTTATTAA-3' and 5'-CAGTACTTTCTGCCCAATTT-3'	(exon 3, annealing 51°C)
5'-AACGGGATAGGTGAAGGGCC-3' and 5'- GGTTGCTGCTGGATGAGAGG-3'	(exon 4, annealing 59°C)
5'-GTGACAGCATAATATCCCAC-3' and 5'-TTTAGCCAGTCCACGATGTA-3'	(exon 5, annealing 53°C)
5′-CAGCCATGTAAGCTATAGTGAG-3′ and 5′-ATGTGGATGGCAAAATGGATTC-3′	(exon 6, annealing 61°C)
5′-GATCATATTTTCTTATGTTTGTAC-3′ and 5′-CATCATAGTAGGAAAAACGACC-3′	(exon 7, annealing 56°C)
5′-CTCGTGAATCTGCAGCTATCTC-3′ and 5′-CAATTCAAGTAGAGAATATGATGC-3′	(exon 8, annealing 56°C)
5′-TTCTTCAGTGTACATATATTCC-3′ and 5′-GAACCAAAGAGTCAGAAGATCAAG-3′	(exon 9, annealing 56°C)
5′-CCTTAGAGCATATATAGTGCC-3′ and 5′-GGCTATTTTAAGTACCAGACAG-3′	(exon 10, annealing 61°C)
5′-GATATAATGTGGAGATATAGAC-3′ and 5′-CAGGTAAGCATGAAATAAGAG-3′	(exon 11, annealing 56°C)
5′-GGTCATACCAAACTCTTGTC-3′ and 5′-CAAAGGTGTCAAAGTATCCTG-3′	(exon 12, annealing 61°C)
5′-ACAACCGTATTCAGAATACTG-3′ and 5′-CTGCTACATGTACAGTGAAGG-3′	(exon 13, annealing 61°C)
5′-GAACACAATGTCACTTCTGTGG-3′ and 5′-CCTGTACCTGAATACTAGCTAC-3′	(exon 14, annealing 61°C)
5′-AAGATTTTGAATAATGCAGTAAG-3′ and 5′-CATGTGCTCGCAATGTCAAAGTC-3′	(exon 15, annealing 56°C)
5′-GCATTTGTGGATTCCATAGACC-3′ and 5′-CCTGGGTGACAGAACGAGACTG-3′	(exon 16, annealing 56°C)
5′-GAGCCTGGCACATAGAAAGTG-3′ and 5′-GACGTGGTGCTATGAACATAAG-3′	(exon 17, annealing 61°C)
5′-CCTGTTATGTGCCCTTAAGTAC-3′ and 5′-GCATTTCTATTCAATGGATCTTC-3′	(exons 18-19, annealing 61°C)
5′-GTCACCAGTTGCCTGTTACAG-3′ and 5′-CTTCACATTCCAGTAATAAAGAC-3′	(exon 20, annealing 56°C)
5′-TCTATGACTTTTCCACTTCAGG-3′ and 5′-ACTGCAATAAGAAAATCCCCAG-3′	(exon 21, annealing 58°C)
5′- CCAAGCAGCAAAGTTTTGCTGCTG-3′ and 5′- CATGATGAGAGGTCATAAGGGG -3′	(exon 22, annealing 56°C)
5′-GCTACAGAGCATAGGTTCCTC-3′ and 5′-CTCTTTCAGTTACTTGGGTGAG-3′	(exon 23, annealing 56°C)
5′-CCCTGCTGAAAAGATCATGTC-3′ and 5′-GTGAGATACTTACTCAACCAG-3′	(exon 24, annealing 56°C)
5′-GCACAAGTTCATGTGTCTGAC-3′ and 5′-ATACACTTTCTGAGGACCTAC-3′	(exon 25, annealing 60°C)

Purified PCR products were sequenced on ABI 310 instruments (PerkinElmer Applied Biosystems). All reagents supplied by the manufacturer. For further genotyping we also used restriction enzyme digest of exon 21 and exon 25 PCR products, respectively, followed by electrophoresis of the fragments on 2 % agarose gels. The c.3277C>T substution destroyed a *Taq*I site of wildtype exon 21 (185 and 146 bp fragments after PCR digest) resulting in a single, uncleaved PCR product of 331 bp length. The c.3939A>T substitution created a new recognition site for *Psi*I that resulted in digestion fragments of 285 and 52 bp length in mutant alleles. Restriction enzymes were purchased from New England BioLabs.

Protein analysis

Subcultures of lymphoblast or fibroblast lines were left untreated or exposed to 6 Gy of ionizing radiation and harvested after 30 min. Extracts were prepared by resuspending of the cells in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.1 mM ortho-vanadate, 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 1 µg/ml aprotinin, 0.2% Triton X-100, and 0.3% nonidet P-40) followed by incubation on ice for 30 min. Supernatants were collected after centrifugation at 13,000*g* for 10 min. To determine the RAD50, NBS1, and MRE11 protein levels and the extend of radiation-induced NBS1 phosphorylation, whole cell extracts were separated on 7.5% SDS-PAGE gels at 20 µg total protein/lane. The gels were blotted onto Hybond-C nitrocellulose membranes (Amersham Pharmacia, Munich, Germany) using carbonate buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, pH 9.9) and 20% methanol at 35 V for 3-4 hours. Alternativley, 7% Tris-Acetate Gels (Invitrogen, Karlsruhe, Germany) and manufacturer supplied buffers were used. After blocking with 5% skim milk and 0,05% Tween 20 (Sigma, Taufkirchen, Germany) in PBS, the membranes were probed with mouse monoclonal anti-RAD50 antibody (RAD50-13B3, diluted 1:5000, GeneTex, MA, USA), rabbit polyclonal anti-NBS1 antiserum (anti-hp95, diluted 1:5000, Novus Biologicals, Littleton, CO, USA) or mouse monoclonal anti-MRE11 antibody (MRE11-12D7, diluted 1:400, GeneTex, MA, USA). To detect radiation-induced p53 phosphorylation, we used 10% SDS-PAGE gels and probed immunoblots with mouse monoclonal anti-phospho p53 (serine-15 specific) antibody (diluted 1:1000, New England Biolabs, MA, USA). For comparison with a protein of uniform abundance, blots were reprobed with monoclonal anti-β-actin antibody (dilution 1:5000, Sigma Aldrich, Taufkirchen, Germany). We used horseradish peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit IgG as secondary antibodies (dilution 1:5000, Sigma Aldrich, Taufkirchen, Germany), and enhanced chemiluminescence (ECL, Pierce, Amersham Bioscience, Munich, Germany) for visualization and autoradiography. Signals were quantitated densitometrically by the AlphaDigiDoc 1000 software (Biozym, Hess. Oldendorf, Germany).

For immunoprecipitation primary antibody (rabbit anti-NBS1 (Novus Biologicals, Littleton, CO, USA) or rabbit anti MRE11 (Novus Biologicals, Littleton, CO, USA) was added to 220 µg (Controls) or 440 µg (L239) protein solution (200 µl), rotated at 4 °C for 1 h followed by immunoprecipitation overnight with protein G-Sepharose (Amersham Biosciences, Munich, Germany). Immunoprecipitates were washed extensively and separated by denaturating gel electrophoresis. For immunodetection mouse anti-NBS1, MRE11 or RAD50 (all provided by GeneTex, San Antonio, Tx, USA)

For the protein truncation test we used the TNT®Quick Coupled Transcription/Translation System (Promega, Southampton, UK) and ^{35}S-Methionin. The template was generated by amplifications of a cDNA region spanning nucleotides 3175 to 4188. For *in vitro* transcription and translation a T7 promotor and a Kozak consensus sequence was linked to the forward primer. The synthesized peptides were separated on a SDS page and detected by autoradiography.

Immunofluorescence

Normal control and RAD50-deficient fibroblasts were seeded onto coverslips in 12-well-plates (Greiner), grown for 24 h and exposed to 12 Gy of ionizing radiation. The cells were incubated for an additional 14 h with fresh growth media, followed by fixation and permeabilization in 4% paraformaldehyde and 0.1% Triton X-100 for 15 min. After washing with PBS, the coverslips were blocked in PBS with 5% goat serum (Jackson ImmunoResearch) at RT for 1 h. The cell monolayers were covered with rabbit polyclonal anti-NBS1/p95 antiserum (Novus Biologicals, Littleton, CO, USA) at a 1:100 dilution, rabbit polyclonal anti-MRE11 antiserum (Novus Biologicals, Littleton, CO, USA) at a 1:200 dilution, or mouse monoclonal anti-RAD50 antibody (GeneTex) at a 1:200 dilution, and incubated at RT °C for 1 h. After washing with PBS, the cells were incubated with Alexa Fluor 594 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse conjugate (Molecular

Probes), respectively, at a 1:200 dilution, for 1 h at RT. In the case of double staining Alexa Fluor 488 goat anti-rabbit IgG was used. Nuclei were counterstained with DAPI at 1 ng/ml for 2 min. After 3 final washing steps, the coverslips were mounted in Vectashield mounting medium (Vector). They were examined by fluorescence microscopy on an Axiophot microscope (Zeiss). Images of the same nuclei were captured using Texas red, FITC and DAPI filters and an Applied Imaging System with EasyFish version 1.2 software.

Complementation. The *RAD50* cDNA was a gently gift of Dr. H. Kim. The coding region was amplified by PCR and cloned into the Vektor pREP (Invitrogen, Karlsruhe, Germany) using BamH and Not1 cutting sites. Lymphoblasts were transfected using FuGene reagent (Roche applied science). Successful transfection was controlled by immunoblotting.

The RAD50 ORF was subsequently subcloned into pCLNXS (Imgenex) using EcoR1/BamH1 restriction sites. After packing into retroviral particles hTERT-immortalized RAD50(-/-) fibroblasts were transduced (efficiency: ~30%). Expression of RAD50 was monitored via immunofluorescence and WB.

GenBank accession numbers. *HsRAD50* cDNA, NM_005732; *HsRAD50* genomic sequences (P1 clones), AC004041 and AC004042; *MmRAD50* cDNA, U66887; *RnRAD50* cDNA, NM_022246, RAD50 protein sequences: AAB07119.1 (human), CAI24691.1 (mouse) and AF218576_1 (rat).

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Supplementary Tables

Suppl. Tab. 1. Clinical and laboratory findings of the presented RAD50 deficient patient

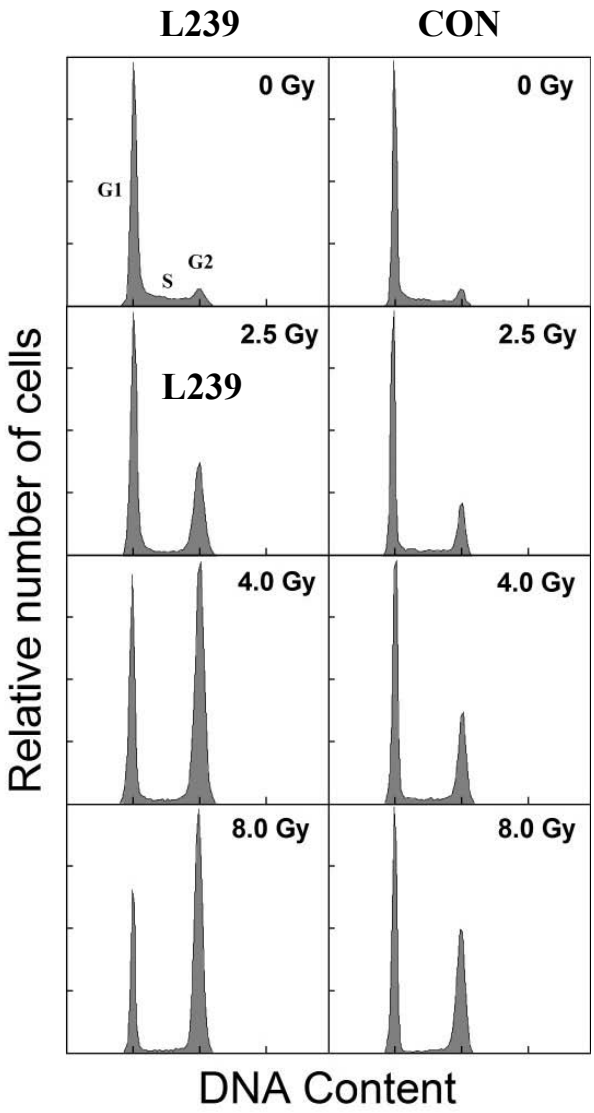
CLINICAL FINDINGS
Congenital abnormalities ● NBS like facies ● Microcephaly (<3rd percentile) ● Pre- and postnatal growth retardation ● Skin hyper- and hypopigmentation, ∅ 15 mm ● Hyperopia
Progressive case ● Early childhood progressive ataxia ● Mild spasticity
Immunological status ● No recurrent and severe infections ● Normal Ig status (IgG,A,M,D and IgG subclasses) ● Normal lymphocyte counts ● Normal alpha Fetoprotein levels

Suppl. Tab. 2. Spontaneous and radiation-induced chromosomal aberrations in RAD50-deficient, NBS, and AT cells analysed from giemsa-stained metaphases.

	0 Gy		**0.5 Gy**		**1.0 Gy**	
	%[a]	b/m[b]	%[a]	b/m[b]	%[a]	b/m[b]
Patient blood	24	0.54	76	2.78	90	3.98
Patient LCL	10	0.14	62	1.12	91	2.15
NBS[c] blood *(n = 1/ 1/ 1)*	12	0.16	60	1.16	74	2.16
NBS[c] LCL *(n = 24/ 11/ 11)*	9± 7	0.14±0.15	55±11	1.16±0.32	70±12	2.38±0.88
AT LCL *(n=8/ 6/ 6)*	11±4	0.18 ±0.10	69±14	1.52±0.29	82±17	2.78±1.06
Controls blood *(n = 96/ 50/ 50)*	1±2	0.02 ±0.02	15±8	0.19±0.11	22±8	0.27±0.10
Controls LCL *(n = 38/ 18/ 18)*	2±3	0.03±0.04	14±8	0.19±0.15	23±9	0.35±0.18

[a]Percentages of aberrant metaphases; [b]breaks/metaphase. [c]all tested NBS cells had truncating mutations in the *NBS1* gene.

Supplementary Figures



Suppl. Fig. 1. IR dose dependent cell cycle distribution of control (CON) and RAD50 deficient (L239) lymphoblasts. Cell cycle analyses of patient derived cells revealed a prominent arrest in the G2 phase 48 h after irradiation indicating a high sensitivity against irraditiation.

General discussion

Efficient DNA repair is necessary to maintain genomic integrity despite the occurrence of DNA damage caused by endogenous and exogenous agents. The cellular DNA damage response is tightly controlled and includes a network of protein-protein interactions that influence central mechanisms including DNA repair, replication, cell cycle progression and programmed cell death [1, 2]. Failure of these processes results in chromosomal instability, mutagen sensitivity and, in certain cases, in cancer susceptibility [3]. Biallelic germline mutation in some of these caretaker genes are known to cause human diseases, like Fanconi anemia (FA), Nijmegen breakage syndrome (NBS), Ataxia telangiectasia (AT), Bloom syndrome (BLM) or Cockayne Syndrome (CS). These rare diseases serve as “experiments of nature” in our quest to understand the molecular, cellular and phenotypic consequences of defective genome maintenance.

At the molecular level the proteins involved in chromosomal instability syndromes show varied patterns of molecular crosstalk [1]. For example, some of the proteins of the FA core complex were immunoisolated together with BLM [4], a highly conserved helicase-type caretaker gene. Likewise, the central protein of the FA pathway, FANCD2, was shown to colocalize with NBS1 [5] and is phosphorylated by ATM [6] and ATR [7]. Despite these close functional correlations, the distinctive cellular phenotypes of each of the caretaker gene syndromes imply distinctive functions of the FA proteins that are not shared with other caretaker proteins like ATM or BLM. In some cases the presence of overlapping clinical features between FA and NBS requires analysis at the molecular level in order to arrive at the correct diagnosis [8, 9]. Since NBS and AT belong to the group of radiosensitivity syndromes it has long been controversial whether FA cells are sensitive towards ionizing radiation [10-12]. In chapter 3 I present experimental data showing that FA fibroblasts display no systematic differences regarding cellular survival following X-ray or UV compared to non-FA controls. This apparent lack of major radiosensitivity holds for all tested complementation groups and was confirmed by others [13]. A very recent study reported that under physiological conditions FA fibroblasts show no increased radiosensitivity. Under conditions of extreme hypoxia (<0.1 % v/v oxygen), however, lower cell survival than normal was observed [14]. Whether DNA crosslink repair functions under such severely hypoxic conditions remains unclear. It is very clear, however, that FA cells are sensitive to ambient concentrations of oxygen (i.e. 21% v/v), and modest reduction of oxygen tension in tissue culture incubators from 21 to 5% (v/v) results in the restoration of near normal growth of these cells [15].

A central step in the FA pathway is the monoubiquitination of the evolutionarily conserved FANCD2 protein [16, 17]. The functional importance of FANCD2 is indicated by the existence of orthologs in drosophila [18, 19], caenorhabditis [20, 21], zebrafish [22] and mice [23]. Disruption of *Fancd2* in zebrafish and mice is associated with severe developmental defects [22, 23]. However, up to now little was known about the kinds of mutations that are compatible with survival of FA-D2 patients, and there was no information on possible genotype-phenotype correlations. In a comprehensive study to which seven research groups from six countries contributed patient cell lines, we assigned 32 FA patients to complementation group FA-D2 (chapter 4). Most of the patients show a severe phenotype including growth retardation, a high prevalence of microcephaly, microphthalmia and

radial ray defects. In addition, most patients developed bone marrow failure early in their lives. The subsequent genetic analysis was carried out as part of this thesis in the Wurzburg laboratory. It revealed an unexpected high frequency of mutations that affect correct splicing which may explain the presence of residual levels of FANCD2 protein in all patient-derived cell lines. The existence of residual protein has previously been described for 5 other FANCD2 deficient cell lines [16, 17, 24], suggesting that the viability of FA-D2 patients depends on the presence of leaky or hypomorphic mutations. Thus, my study of the mutational patterns in FA patients belonging to complementation group FA-D2 provides additional proof for the essential role of the *FANCD2* gene, both during development and in the DNA damage response.

Somatic reversion

Like in Bloom syndrome, revertant mosaicism in somatic cells is frequently observed in Fanconi anemia [25, 26]. Somatic reversion can be caused by intragenic recombination, gene conversion, back mutation, and compensatory second site mutations. Development of somatic mosaics has been reported for complementation group FA-A [27, 28], C [27], D2 [24] (without genetic data) and as part of this thesis work for complementation groups FA-L and FA-D2. As described in chapter 5 the second only FA-L patient that has become known worldwide displayed a novel kind of somatic reversion. A 5 bp deletion was compensated by an upstream in *cis* deletion caused by mutation of the canonical AG dinucleotide of the splice acceptor, resulting in usage of an alternative splice site. Another interesting case of revertant mosaicsm based on splicing was found in a patient belonging to complementation group FA-D2 (chapter 4). Patient derived primary fibroblasts were homozygous for the mutation c.1947-16T>G, which causes skipping of exon 22. However, the lymphoblasts proved MMC resistant and showed only heterozygous skipping of exon 22. Genomic analysis revealed an additional heterozygous base substitution within exon 22, which obviously is responsible for correct exon recognition. Nevertheless, a worsening clinical course in both patients required bone marrow transplantation, indicating that the reversion events took place in a hematopoietic precursor cells rather than in a genuine bone marrow stem cell

Identification of new genes

Historically, the identification of the first FA genes (such as *FANCC* and *FANCA*) provided few hints as to their molecular function. Exept for *FANCG*, which is identical to the known DNA-repair gene *XRCC9*, the FA genes share neither homology to other proteins nor to known functional domains. This situation changed when Howlett and coworkers [29] found that the breast cancer associated protein 2 (BRCA2) is defective in FA patients belonging to complementation group FA-D1. This discovery strongly suggests the involvement of at least some of the FA proteins in DNA repair via homologous recombination. In addition, some of the subsequently discovered genes were found to contain protein domains with defined biochemical functions. For example, FANCL or PHF9/Pog has a RING domain with ubiquitin E3 ligase activity [30] – and the latest two FA genes, *FANCJ* (identical with *BRIP1/BACH1*) is a DNA helicase [31, 32] and *FANCM* (homolog to Hef) belongs to the group of translocases [33]. Even though the function of most of the FA genes is not fully understood, the current view is that of a complex molecular crosstalk between FA proteins and other proteins involved in initiation, regulation and execution of DNA repair [1, 2, 34].

The concept of FA proteins being part of a multiprotein network received strong support by the identification of FANCJ (chapter 6). Via positional cloning, two groups independently discovered that the BRCA1 interacting protein 1 (*BRIP1/BACH1*) is identical to the gene which is defective in Fanconi anemia patients belonging to rare complementation group J [31, 32] (see also chapter 8). This implies a participation of BRCA1 in the FA pathway. In parallel *BRIP1* was identified as a FA candidate gene by molecular analysis of DT40 chicken cell lines [35]. However, the phenotype

of chicken brip1 mutant cells in response to DNA damage differs from that of chicken brca1 mutant cells suggesting distinctive functions for BRCA1 and BRIP1 [35]. In addition, Bridge and coworker [35] showed that the phenotype could be reversed by expression of BRIP1 lacking the BRCA interaction site. Further analysis of FANCJ will therefore provide further insight into DNA repair via the FA/BRCA pathway.

Another most recently discovered FA gene is *PALB2* (chapter 7), which was originally identified as a nuclear binding partner of BRCA2/FANCD1 [36]. PALB2 colocalizes with BRCA1 and BRCA2 within nuclear foci after hydroxyurea treatment. Knock down of PALB2 results in reduced BRCA2 protein levels, lack of DNA damage-induced BRCA2 focus formation, and increase of MMC sensitivity. In a candidate gene approach PALB2 was identified as mutated in some of the unassigned FA patients. All of these PALP2-mutated patients show a rather severe clinical phenotype and early childhood onset of malignancies. Their phenotype is reminiscent of that seen in children with biallelic mutations in BRCA2, and BRCA2 is a key player in the RAD51 dependent DNA repair via homologous recombination.

The discovery of BRIP1 and PALP2 as bona fide Fanconi anemia genes that interact with the known cancer genes BRCA1 and BRCA2 connects the FA pathway even closer to the cancer field. These discoveries also underline the importance of recombinational types of DNA repair processes in the maintenance of genomic stability and, therefore, in the prevention of cancer.

*The NBS1/MRE11/*RAD50 *complex*

As a central member of the non homologous end joining (NHEJ) pathway, the NBS1/MRE11/ RAD50 (NMR) complex plays an important role in the repair of DNA double strand breaks. It was shown that the complex modulates the ATM mediated DNA damage response via attenuation of ATM activation [37-39] and, possibly, of ATR [40, 41]. Therefore, a function of the NMR complex as a primary DNA damage sensor was suggested [42]. In a feedback loop NBS1 is phosphorylated by ATM, a modification which is required for activation of an intra S Phase checkpoint [43, 44]. The discovery of the worldwide first patient with biallelic mutations in the *RAD50* gene as the third member of the NMR complex (see chapter 8) contributes greatly to our understanding of these putative interactions. Like MRE11 and NBS1 deficient cells [45], RAD50 deficient cells were found to display checkpoint defects, reduced activation of ATM, and reduced activation of its downstream targets Chk2 and p53. However, the clinical and molecular data clearly suggests some differences between defects in NBS1, MRE11 or RAD50 proteins. Biallelic mutations in MRE11 cause an AT-like syndrome [46], NBS1 deficiency causes the Nijmegen breakage syndrome (NBS) [47, 48], and (as part of my thesis) it was shown that RAD50 deficiency causes a NBS-like syndrome without immunodeficiency. These differences among the respective clinical phenotypes imply some independent functions of these genes during ontogenesis, a notion which is supported by the fact that RAD50 null mutations are lethal in mice. Mice carrying the hypomorphic *Rad50S* allele exhibit precipitous apoptotic attrition of hematopoietic cells [49]. In contrast, our RAD50 patient showed no reduction of cell counts and no evidence of immunodeficiency. Therefore, the comparison of these partially overlapping but distinctive phenotypes provides the possibility to better understand the developmental and cellular functions of the individual NMR complex members, and to sort out the respective complex-dependent and complex-independent functions. Again, as pointed out in the case of the Fanconi anemia genes, our RAD50 deficient patient cells represent a most instructive “experiment of nature” that offers new insights into the functions of the individual members of the NMR complex which plays a central role in the DNA damage response of human cells.

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Summary

Human caretaker genes play a central role in the DNA damage response. Their defects cause a number of rare diseases which show genetic instability and increased propensity to malignant cell growth. The first of these diseases to be described in this thesis is Fanconi anemia (FA), a rare chromosome instability disorder with recessive inheritance characterized by progressive bone marrow failure, variable congenital malformations, and cancer predisposition. There are at least 13 FA complementation groups (FA-A , B, C, D1, D2, E, F, G, I, J, L, M and N), each representing mutations in a distinct gene. To date, except *FANCI* all the corresponding genes have been identified, denoted as *FANC-A, B, C, D1/BRCA2, D2, E, F, G, J/BRIP1/BACH1, L/PHF9, M/Hef* and *N/PALB2*. Further information is provided in **chapters 1 and 2.**

FA cells are characterized by high sensitivity to DNA crosslinking agents and to elevated oxygen tension, but it is controversial whether they are also radiosensitive. Systematic testing (**chapter 3**) of primary skin fibroblast cultures from all currently known FA complementation groups revealed no increased sensitivity towards ionizing radiation (IR) and ultra-violet light (UV) when growing cells at physiological (5% v/v) oxygen levels. Despite considerable interstrain variations FA cells showed no systematic differences to cell cultures derived from healthy controls, whereas positive controls (Ataxia telangiectasia and Cockayne syndrome) proved highly sensitive to IR or UV.

Lack of radiosensitivity was also shown for the FANCD2 gene, a central gene in the FA/BRCA pathway whose mutational inactivation was studied in a large patient cohort. FA patients excluded previously from complementation groups FA-A, -C, E, F, G or L were screened for mutations in *FANCD2*. Even though mutation analysis of *FANCD2* is complicated by the presence of pseudogene regions, biallelic *FANCD2* mutations were identified in a series of 32 patients (**chapter 4**). The predominant types of mutations result in aberrant splicing causing exon skipping, exonisation of intronic sequence, activation of cryptic and creation of new 3´ splice sites. Many alleles were recurrent and could be associated with ethnicity. Interestingly, residual FANCD2 protein was observed in all available patient cell lines, and functionality was indicated by the presence of the monoubiquitinated FANCD2 isoform. This suggests that viability of FA-D2 patients depends on the presence of hypomorphic or leaky mutations.

In **chapter 5** the worldwide second FA patient belonging to complementation group FA-L is reported. Genetic analysis of patient derived fibroblasts revealed heterozygosity for a 5-bp deletion (exon 7) and a missense substitution (exon 11). In contrast to the tested fibroblasts two independent lymphoid cell lines proved resistant to the DNA crosslinking agent mitomycin C and showed proficient FANCD2 monoubiquitination. The functional reversion due to a compensating mutation in the splice acceptor site results in aberrant splicing and the restoration of the open reading frame. However, the revertant mosaicsm was restricted to the lymphatic cell lines such that there was no clinical improvement involving the other hematopoietic cell lineages, and bone marrow transplantation was required to treat the patients` bone marrow failure.

A direct link of Fanconi anemia to other DNA repair processes was provided by the identification of the BRCA1 interacting protein 1, *BRIP1/BACH1*, as a genuine FA gene (**chapter 6**). Genetic mapping of consanguineous Inuit families resulted in the identification of truncating mutations in *BRIP1*. In contrast to most of the other FA patients FANCD2 monoubiquitination was intact, linking these patients to complementation group FA-J. Biallelic mutations in *BRIP1* were found in eight

additional patients, one of whom was assigned previously to FA-J by somatic cell fusion. Therefore it could be shown that the postulated *FANCJ* gene is identical with *BRIP1*. This finding emphasizes the close connection between the BRCA- and the FA-family of genes, both involved in the DNA damage response.

Biallelic mutations in *BRCA2/FANCD1* cause a severe form of Fanconi anemia with childhood malignancies. Recently, a BRCA2 interacting protein was identified as a "partner and localizer of BRCA2" (PALB2) which confers cellular MMC resistance. A candidate gene approach revealed biallelic mutations in seven FA patients that developed solid tumors in early childhood (**chapter 7**). Patient cells show no or little PALB2 protein, lack of MMC induced RAD51 foci formation, and high chromosomal instability. Transduction of PALB2 cDNA complemented the MMC sensitive phenotype. Therefore, biallelic mutations in PALB2 cause a new subtype of FA, denoted as FA-N, which is connected with a high and early cancer risk.

With respect to one of the most prominent but least understood caretaker gene syndromes, Fanconi anemia, this thesis has expanded our knowledge as follows: 1. refutation of major cellular radiosensitivity of FA cell lines regardless of complementation group, 2. detection of hypomorphic mutations and residual protein levels as a prerequisite for viability of the *FANCD2* gene, 3. description of the worldwide second patient belonging to complementation group FA-L whose lymphocytes exhibit a novel type of somatic reversion, 4. participation in the discovery and functional characterization of two novel FA genes (*FANCJ* and *FANCN*).

The last chapter of my thesis deals with a DNA repair pathway that is activated following exposure to ionizing radation. One of the central proteins responding to radiation-induced DNA damage is the product of the *ATM* gene which signals to a myriad of other proteins in response to DNA double strand breaks, including the NMR complex. This complex formed by the NBS1/MRE11/RAD50 proteins is thought to act as a specific sensor of DNA double-strand breaks. Mutations of MRE11 and NBS1 are associated with the radiation sensitivity syndromes Ataxia-telangiectasia-like disorder (AT-LD) and Nijmegen breakage syndrome (NBS), respectively. **Chapter 8** presents the first ever identified patient with RAD50 deficiency due to biallelic germline mutations in the RAD50 gene. An 18-year-old German girl who has a variant form of NBS without immunodeficiency was found to be compound heterozygous for a nonsense mutation and the loss of the natural termination signal in the *RAD50* gene. RAD50 protein expression was reduced to less than one tenth of normal in her fibroblasts and lymphoblastoid cells. At the nuclear level, RAD50 deficiency was associated with a high frequency of spontaneous chromatid exchanges and with the failure to form MRE11 and NBS1 nuclear foci in response to irradiation. ATM autophosphorylation, phosphorylation of p53 at serine 15 and the transcriptional induction of p21/WAF1 mRNA were reduced, and there was no evidence for Ser343 phosphorylation of NBS1 in RAD50 deficient cells following irradiation. These defects could be complemented by expression of wildtype RAD50 cDNA.
Our data shows that RAD50 modulates, like NBS1 and MRE11, the ATM-mediated DNA damage response and the G1/S cell cycle checkpoint. In addition, RAD50 appears to be required for nuclear localization of MRE11, and for NBS1 focus formation, underlining its importance for the proper function of the NMR complex. Owing to the studies performed within the framework of this thesis, RAD50 deficiency can now be added to the growing list of human caretaker gene syndromes with pronounced radiosensitivity that is distinctive at both the cellular and the clinical level from deficiencies involving the other members of the NMR complex.

Zusammenfassung

Die sogenannten “Caretaker”-Gene spielen eine zentrale Rolle in der zellulären Antwort auf DNA Schäden. Mutationen in diesen Genen führen zur Ausprägung von einigen seltenen Erbkrankheiten, die sich durch genetische Instabilität und in vielen Fällen durch erhöhtes Krebsrisiko auszeichnen. Eine dieser Erkrankungen, die im Rahmen dieser Arbeit untersucht wurde, ist Fanconi Anämie (FA). Dabei handelt es sich um eine sehr seltene chromosomale Instabilitätserkrankung, die einem rezessiven Erbgang folgt. Der klinische Phänotyp von FA ist gekennzeichnet durch ein variables Spektrum von kongenitalen Fehlbildungen, progressivem Knochenmarksversagen und einer Prädisposition für Neoplasien. Durch somatische Zellfusionstudien und in einigen Fällen durch direkte genetische Zuordnung konnten bisher 13 Komplementationsgruppen definiert werden (FA-A , B, C, D1, D2, E, F, G, I, J, L, M and N), von denen jede einen Defekt in einem bestimmten Gen widerspiegelt. Inzwischen sind, mit Ausnahme von *FANCI*, alle korrespondierenden FA Gene identifiziert. Ein Überblick über den aktuellen Stand ist in den **Kapiteln 1 und 2** dieser Arbeit zu finden.

Zellen von FA-Patienten zeichnen sich durch eine hohe Sensitivität gegenüber DNA quervernetzenden Substanzen und einer hohen Empfindlichkeit gegenüber einer erhöhten Sauerstoffkonzentration aus. Zur Frage der Radiosensitivität von FA-Zellen gab es bisher nur widersprüchliche Daten. Mit Hilfe einer systematischen Analyse von primären Fibroblasten aller (zum Zeitpunkt der Untersuchung) bekannten Komplementationsgruppen konnte gezeigt werden, dass unter physiologischen Sauerstoffkonzentrationen keine erhöhte Sensitivität gegenüber ionisierender Strahlung (IR) und ultraviolettem Licht (UV) besteht (**Kapitel 3**). Trotz einigen Zellinien-spezifischen Schwankungen waren die Unterschiede zwischen Patientenzellen und gesunden Kontrollen marginal, verglichen mit der eindeutig erhöhten Sensitivität von Zellen positiver Kontrollen (Ataxia telangiactasia (IR-Kontrolle) bzw. Cockayne Syndrom (UV-Kontrolle)).

Die in Kapitel 3 nachgewiesene fehlende Radiosensitivität von FA-Zellen ist im Kontext des FANCD2-Gens von besonderem Interesse, da diesem Gen eine Schlüsselposition im FA/BRCA Doppelstrangbruch-Reparaturweg zukommt. Das Screening von FA-Patienten, bei denen bereits eine Zuordnung zu den Komplementationsgruppen FA-A, C, E, F, G und L ausgeschlossen war, führte zur Identifikation einer Reihe von FA-D2 Patienten. In einer großen Kohortenstudie wurden 29 FA-D2 Patienten mit klinischen Informationen sowie 3 zusätzliche FA-D2 Patienten hinsichtlich ihrer Mutationen in *FANCD2* untersucht (**Kapitel 4**). Für alle 32 Patienten konnten biallelische Mutationen nachgewiesen werden, auch wenn das Vorhandensein von pseudogenen Regionen die Mutationsanalyse erschwerte. Wie in keinem der anderen FA-Gene führt die Mehrzahl der Mutationen in *FANCD2* zu verändertem Spleißenverhalten, wie zum Beispiel zur Exklusion eines Exons, zur Exonisation intronischer Bereiche, der Aktivierung von kryptischen oder der Erzeugung von neuen 3´ Spleißstellen. Viele Allele wurden mehrfach gefunden und konnten einem ethnischen Hintergrund zugeordnet werden. In allen verfügbaren lymphoblastoiden Patienten-Zelllinien wurde residuales FANCD2-Protein detektiert, wobei das Vorhandensein der monoubiquitinylierten Isoform auf ein funktionelles Protein hindeutet. Zusammen mit der Tatsache, dass in keinem der Patienten eindeutige biallelische Nullmutationen nachgewiesen werden konnten, ist somit offenbar das Vorhandensein von hypomorphen Mutationen und entsprechendem Restprotein für die Lebensfähigkeit von FA-D2 Patienten essentiell.

In **Kapitel 5** wird der weltweit zweite FA Patient vorgestellt, welcher der Komplementationsgruppe FA-L zugeordnet werden konnte. Die genetische Analyse von primären Fibroblasten dieses Patienten führte zum Nachweis einer 5 Basen umfassenden Deletion in Exon 7 sowie zu einer *Missense*-Mutation in Exon 11. Im Gegensatz zu den untersuchten Fibroblasten zeigten zwei unabhängig etablierte lymphoblastoide Zellinien keine erhöhte Sensitivität gegenüber Mitomycin C, was auf einer somatische Reversion beruhen kann. Diese Vermutung wurde durch den Nachweis einer induzierbaren Monoubiquitinylierung von FANCD2 in Lymphoblasten bestätigt. Die funktionelle Reversion basiert auf einer zur Deletion *in cis* befindlichen kompensatorischen Mutation im Spleiß-Akzeptor von Exon 7, die zu einem veränderten Spleißprodukt und der Wiederherstellung des Leserahmens führt. Die somatische Reversion beschränkt sich jedoch auf die lymphatischen Zellreihen und hatte daher nur einen partiellen Einfluss auf den Krankheitsverlauf. Ein allgemeines Knochenmarksversagens machte eine Transplantation unumgänglich. Die Konditionierung wurde jedoch auf das vorhandene hämatopoietische Mosaik abgestimmt, um eine Graft vs. Host Reaktion zu vermeiden.

Die Identifizierung des BRCA1 interagierenden Proteins 1 (BRIP1/BACH1) als ein *bona fide* FA-Gen (**Kapitel 6**) verdeutlicht die direkte Verknüpfung des FA/BRCA DNA Reparaturweges mit anderen DNA Reparaturwegen. Trunkierende Mutationen in *BRIP1* wurden in zwei blutsverwandten Eskimofamilien mittels genetischer Positionsanalysen gefunden. Im Gegensatz zu den meisten anderen FA Patienten war die Monoubiquitinylierung von FANCD2 trotz MMC Empfindlichkeit intakt, was für eine Zuordnung zur Komplementationsgruppe FA-J sprach. Biallelische Mutationen in *BRIP1* wurden in 8 weiteren Patienten gefunden, von denen einer ursprünglich durch somatische Zellfusion zu FA-J zugeordnet wurde. Dadurch konnte gezeigt werden, dass das postulierte *FANCJ* Gen mit *BRIP1* identisch ist. Als eines der wenigen FA Gene besitzt BRIP1 bekannte funktionelle Domänen (DNA Helikase), die somit biochemisch für die Entschlüsselung des FA/BRCA Weges genutzt werden können.

Biallelische Muatationen in *FANCD1*, welches identisch mit dem bekannten Brustkrebsgen BRCA2 ist, führen zu einer besonders schweren Form von Fanconi Anämie, die bereits im frühen Kindesalter mit einem sehr hohen Krebsrisko verbunden ist. Kurz vor Abschluss dieser Arbeit gelang die Charakterisierung eines mit BRCA2 interagierendes Proteins, welches als „partner und localizer of BRCA2“ (kurz PALB2) bezeichnet wurde und offenbar zelluläre Resistenz gegenüber Mitomycin C (MMC) bedingt. Ein genetischer Screen von bisher nicht-klassifizierten FA-Patienten führte zur Identifizierung von sieben Patienten mit biallelischen Mutationen in *PALB2* (**Kapitel 7**). Alle Patienten entwickelten bereits im frühen Kindesalter bösartige Tumore. Der zelluläre Phänotyp von PALB2- defizienten Zellen weist eine Störung der MMC induzierten RAD51 Foci-Bildung auf. Darüberhinaus zeigen PALB2-defiziente Zellen eine spontane chromosomale Instabilität, die durch eine MMC-Gabe stark erhöht werden kann. Die Transduktion von *PALB2* cDNA führt zur Reduktion der MMC induzierten G2 Phasen Arretierung und zur Reversion des MMC-sensitiven Phänotyps. Biallelische Mutationen in *PALB2* verursachen somit eine Form von Fanconi Anämie, die mit hohem Krebsrisiko verbunden ist. PALB2 wird nach der gängigen FA-Nomenklatur als *FANCN* bezeichnet und liegt der neu definierten Komplementationsgruppe FA-N zugrunde.

Die vorliegende Arbeit hat in mehreren Punkten zur Verbesserung des derzeitigen Wissenstandes über die komplexe genetisch bedingte Erkrankung Fanconi Anämie beigetragen 1. trotz molekularer Wechselwirkung von FA-Proteinen mit Komponenten aus anderen DNA-Reparatursystemen zeigen Zellen von FA Patienten unabhängig von der Komplentationsgruppe weder Strahlen- noch UV-Sensitivität; 2. die Existenz von hypomorphen Mutationen in *FANCD2* und das Auftreten von Restprotein korreliert offenbar mit der Lebensfähigkeit von biallelischen Mutationen im *FANCD2* Gen. 3. unter den bisher nicht klassifizierten Patienten wurde der weltweit zweite Patient der Komplementationsgruppe FA-L gefunden, in dessen Blutzellen ein neuartiger Mechanismus von somatischer Reversion nachgewiesen werden konnte 4. die Beteiligung an der Identifizierung

und funktionellen Charakterisierung von zwei neuen FA-Genen (*FANCJ* und *FANCN*), welche die FA-abhängigen DNA-Reparaturwege direkt mit den Brustkrebsgenen *BRCA1* und *BRCA2* in Verbindung bringen.

Das letzte Kapitel der Arbeit befasst sich mit einem DNA-Reparaturweg, der insbesondere nach Strahlenexposition aktiviert wird. Eines der zentralen Proteine, das an der Erkennung von strahleninduzierten DNA Schäden beteiligt ist, ist die Kinase ATM. Eine Reihe von Proteinen wird von ATM phosphoryliert, womit eine DNA Schadensantwort bei Doppelstrangbrüchen ausgelöst wird. Der NMR-Proteinkomplex, bestehend aus NBS1, MRE11 und RAD50, ist an der Aktivierung der ATM Kinase beteiligt und wird daher als ein Sensor für DNA Doppelstrangbrüche angesehen. Biallelische Mutationen in MRE11 und NBS1 führen zu den Radiosensitivitätssyndromen „Ataxia telangiectasia-like disease“ (AT-LD) bzw. „Nijmegen breakage syndrome“ (NBS). In **Kapitel 8** wird der erste Patient mit RAD50 Defizienz vorgestellt. Bei einer 18 jährigen Patientin mit einem NBS- ähnlichem klinischem Phänotyp, jedoch ohne Immundefizienz, wurden eine prämature Stopp-Mutation und der Verlust des natürlichen Translationsterminations-Signals gefunden. Die Expression von RAD50 ist in Fibroblasten und Lymphozyten der Patientin auf weniger als 10% reduziert. Auf zellulärer Ebene ist eine hohe Chromosomenbruchrate, eine dosisabhängige Arretierung in der G2 Phase des Zellzyklus, Defekte in den G1 und S Phase Kontrollpunkten, sowie das Fehlen der IR-induzierten Relokalisation von NBS1 und MRE11 zu beobachten. Korrekte Relokalisation und normale Phosphorylierungsmuster wurden experimentell durch transiente Transfektion von RAD50 cDNA wiederhergestellt, was die Bedeutung von RAD50 für diese Prozesse belegt.

Die vorgestellten Daten zeigen, dass RAD50, ebenso wie NBS1 und MRE11, die ATM abhängige DNA Schadensantwort und die G1/S Zellzykluskontrolle moduliert. RAD50 scheint für die nukleäre Lokalisation von MRE11, sowie für die induzierte Relokalisation von MRE11 und NBS1 in nukleäre Foci notwendig zu sein. Als Ergebnis dieser Untersuchungen kann die RAD50-Defizienz zu der wachsenden Liste von Caretaker-Syndromen als eine neue Radiosensitivität-Erkrankung hinzugefügt werden. Das Krankheitsbild unterscheidet sich trotz enger molekularer Interaktion zwischen RAD50, NBS1 und MRE11 im NMR-Komplex von den Defekten in diesen Genen.

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Curriculum Vitae

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EDUCATION

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07/1996 – 04/1997	Military service
05/1997 – 08/2002	Student of Biology, University of Wuerzburg, Germany Major: Microbiology, Minor: Human Genetics and Physiology; graduation 05/2002 Diploma-thesis: *Genetische und funktionelle Untersuchungen zum Nijmegen Breakage Syndrome (NBS) und zu verwandten Störungen ("NBS-like-disorders")*
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SCIENTIFIC MEETINGS

10/2002	14th Annual Scientific Fanconi Anemia Research Fund Symposium, Philadelphia, USA
10/2003	14th Annual Meeting of the German Society of Human Genetics, Marburg, Germany
11/2003	15th Annual Scientific Fanconi Anemia Research Fund Symposium, Houston, USA
07/2004	European Human Genetics Conference and 15th Annual Meeting of the German Society of Human Genetics, Munich, Germany
09/2004	8th DNA Repair Workshop 2004, Ulm, Germany
10/2004	16th Annual Scientific Fanconi Anemia Research Fund Symposium, Boston, USA

03/2005	16th Annual Meeting of the German Society of Human Genetics, Halle, Germany
04/2005	ICGEB-ESF Workshop "The Pathology of pre-mRNA Splicing: Diagnostic and Mechanistic Aspects", Trieste, Italy
05/2005	European Human Genetics Conference, Prag, Czech Republic
09/2005	17th Annual Scientific Fanconi Anemia Research Fund Symposium, Geneve, Switzerland
11/2005	9th Joint Meeting; Signal Transduction: Receptors, Mediators and Genes, Weimar, Germany
03/2006	17th Annual Meeting of the German Society of Human Genetics, Heidelberg, Germany
10/2006	18th Annual Scientific Fanconi Anemia Research Fund Symposium, North Bethesda, USA

Special courses

11/2002	DNA-sequencing
03/2004	Flow cytometry
03/2006	Real-time user seminar

AWARDS

09/2005	Gene discovery award, Fanconi Anemia Research Fund, Geneva, Switzerland
10/2005	Ehrenauszeichnung, Deutsche Fanconi Anemie Hilfe e.V, Gersfeld, Germany

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B) PRESENTATIONS

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ERKLÄRUNG

Erklärung gemäß §4 Absatz 3 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universität Würzburg vom 15. März 1999

1. Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation selbstständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

2. Ich erkläre weiterhin, dass die vorliegende Dissertation weder in gleicher, noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

3. Ich erkläre des Weiteren, dass ich außer den mit den Zulassungsantrag urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, den 09. Oktober 2006

Reinhard Kalb