Molekulargenetische Ursachen und Folgen genetischer Instabilität am Beispiel des FA/BRCA Caretaker Pathways

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

Kornelia Neveling

- geboren in Rheinberg -

Würzburg 2007

Eingereicht am: .....

bei der Fakultät für Biologie

der Bayerischen Julius-Maximilians-Universität Würzburg

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. Martin J. Müller

1. Gutachter: Prof. Dr. Detlev Schindler

2. Gutachter: Prof. Dr. Thomas Dandekar

Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

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 dem Zulassungsantrag urkundlich vorgelegenen Graden keine anderen akademischen Grade erworben habe oder zu erwerben versucht habe.

Würzburg, den 20.12.2007

_____

(Kornelia Neveling)

Danksagung

Mein allererster und größter Dank geht an meine beiden „Chefs“ Prof. Dr. Detlev Schindler und Prof. Dr. Holger Höhn. Bei Prof. Schindler möchte ich mich bedanken, dass er mir damals diese Stelle angeboten und mich auf so nach Würzburg geholt hat. Die Entscheidung, nach Würzburg zu kommen, hat mich sowohl in beruflicher als auch in menschlicher Hinsicht sehr viel weiter gebracht. Ich möchte mich dafür bedanken, dass er es geschafft hat, das Interesse und die Begeisterung für die Humangenetik und insbesondere die Fanconi Anämie in mir zu wecken, dass er mich stets unterstützt hat und mir in allen Dingen immer hilfreich zur Seite stand. Bei Prof. Höhn bedanke ich mich für die große Unterstützung, auf die ich immer zählen durfte. Insbesondere danke ich für die vielen Ratschläge und Korrekturen beim Schreiben der Manuskripte, für ebensoviele Aufmunterungsversuche und Motivationshilfen, wenn es mal nicht so gut lief, für die angenehmen Freitagsseminare und die vielen kleinen und großen Hilfen, die er immer und ganz selbstverständlich geleistet hat.

Ein ebenfalls großer Dank geht an Prof. Dr. Thomas Dandekar, der so direkt und bereitwillig die Rolle des 2. Gutachters übernommen hat und mir bei Fragen immer hilfreich zur Seite stand.

Weiterhin möchte ich an dieser Stelle meinem ehemaligen Kollegen Reinhard Kalb danken. Wir haben viele Projekte gemeinsam bearbeitet, und dabei sowohl Begeisterungen als auch Enttäuschungen miteinander geteilt. Nicht nur durch die endlosen Diskussionen mit ihm, sondern allgemein, habe ich viel von ihm gelernt.

Ich danke auch allen anderen Mitgliedern unserer Arbeitgruppe für die schöne Zeit. Richard Friedl danke ich für die Einführung in die Durchflusszytrometrie, die Hilfe bei den vielen Computer- und Druckerproblemen und seine angenehme ruhige Art. Benni Gottwald danke ich für die vielen Hilfen in der Zellkultur und dass sie durch ihr Temperament „Leben“ ins Labor bringt. Ioannis Gavvovidis danke ich für die stete Hilfsbereitschaft und eine Einführung ins „Griechische“. Weiterhin danke ich Daniela Endt für das gute Zusammenarbeiten -auch unterwegs-, Bea Schuster für die tolle Weiterführung der Western Blots, und Lena Weissflog und Anne Krämer für die ihre nette und angenehme Art.

Ich danke außerdem Indrajit Nanda, Claus Steinlein und Prof. Dr. Michael Schmid für die gute Zusammenarbeit und ihre Hilfen bei der Zytogenetik.

An dieser Stelle möchte ich auch allen anderen Mitgliedern der Arbeitsgruppen Schmid, Felbor und Müller-Reible sowie Prof. Dr. Utz Fischer und dessen Arbeitsgruppe für die allgemeine Hilfsbereitschaft und das nette Arbeitsklima danken.

Zu großem Dank verpflichtet bin ich auch allen unseren nationalen und internationalen Kollaborationspartnern, insbesondere: Helmut Hanenberg, Wolfgang Schulz und Heiner Schaal (alle Düsseldorf), Heidemarie Neitzel (Berlin), Holger Tönnies (Kiel), Arleen Auerbach (New York), Jordi Surralles (Barcelona), Juan Bueren und Paula Rio (beide Madrid), Weidong Wang (Bethesda), Nazneen Rahman (Sutton, UK), Johan de Winter und Hans Joenje (beide Amsterdam), Tony Huang (New York), Margaret Knowles (Leeds).

Ein spezieller Dank geht an Michi, Julia, Sven und Gudrun, und alle meine Freunde zuhause. Sie haben dafür gesorgt, dass es neben dem Labor auch noch ein privates Leben gab. Der gemeinsame Sport, die Kino-Abende, das Babysitten oder einfach das nette Zusammensein waren mir immer sehr wichtig. Ich freue mich, in ihnen so gute Freunde zu haben.

Und ein ganz großer, besonderer Dank geht nicht zuletzt an meine Eltern und meinen Bruder, an die ich mich immer wenden kann und von denen ich immer die allergrößte Unterstützung, Hilfe und Rückhalt erhalten habe.

Table of Contents

Preface

The work presented in this thesis has been performed from May 2004 to December 2007 in the Division of somatic cell genetics of the Department of Human Genetics at the University of Wurzburg, Biocenter, under the direction of Professor Detlev Schindler.

My thesis deals with an inherited disease, Fanconi anemia, first described in 1927 by the Swiss pediatrician Guido Fanconi. This rare disorder exemplifies the close connection between genetic instability, physical defects, organ malfunctions and, most prominently, neoplasia. For a long time, Fanconi anemia was a disease only known to a small field of clinicians and basic researchers. This has changed during recent years as molecular defects in a newly defined family of caretaker genes have been recognized as cause of the disease. Presently, research on Fanconi anemia is conducted by scientists coming from very different fields, including areas such as DNA repair, cancer, stem cells, and development. Model organisms like drosophila, zebrafish, chicken and mouse are increasingly used to study the connection between defective caretaker genes and defective cellular function. The first FA gene was discovered in 1992, and since that time the pace of gene discovery has been astounding. During my work as a graduate student I have witnessed and participated in the discovery of three FA genes, bringing the total number of these genes to currently 13. Nevertheless, the exact details of the socalled “FA/BRCA” network and its precise role in the recognition and repair of DNA-interstrand crosslinks are not fully understood. We need to know more about how the FA proteins work, how they interact with other caretaker proteins, and how they manage to maintain genomic *stability* which is a prerequisite for the exceptional longevity of our species. Conversely, elucidation of the molecular causes and consequences of genetic *instability* is an important research objective to which the study of Fanconi anemia, as portrayed in this thesis, is likely to make significant contributions.

This thesis is divided into four main parts. First, the reader will be introduced to the subject of Fanconi anemia and its many clinical, cellular and molecular features. The introduction is followed by the main body of the thesis. This consists of a series of original papers which present the results of my experimental work. The third part is a brief discussion of the implications of my research, including the many open questions that remain to be solved in future studies. In the last part I will summarize the various aspects of my thesis work in both English and German.

Since the scientific literature on Fanconi anemia has grown exponentially in recent years, it will be impossible to cover all relevant publications within the framework of this thesis. For the purpose of introduction to this rather complex field of research, I will concentrate on a number of key papers, including the review types of publications listed below. As first or co-author of this particular series of publications, I take the proprietary liberty to incorporate their main messages into the introduction section. Copies of these publications are provided as supplements to this thesis.

1. Schindler D and Neveling K. Fanconi Anemia. In: Schwab M (ed) Encyclopedia of Cancer. Springer, in press
2. Neveling K, Bechtold A, Hoehn H (2007). Genetic instability syndromes with progeroid features. Z Gerontol Geriat 40:339-348
3. Neveling K, Kalb R, Schindler D (2007). Cancer in Fanconi Anemia and Fanconi Anemia Genes in Cancer. In: Schindler D, Hoehn H (eds) Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, vol 15, pp 59-78
4. Kalb R, Neveling K, Herterich S, Schindler D (2007): Fanconi Anemia Genes: Structure, Mutations, and Genotype-Phenotype Correlations. In: Schindler D, Hoehn H (eds) Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, vol 15, pp 39-58
5. Hoehn H, Kalb R, Neveling K,..., Schindler D (2007): Revertant Mosaicism in

Fanconi Anemia: Natural Gene Therapie at Work. In: Schindler D, Hoehn H (eds) Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, vol 15, pp 149-172

6. Schindler D,..., Neveling K,..., Hoehn (2007). Applications of Cell Cycle Testing in Fanconi Anemia. In: Schindler D, Hoehn H (eds) Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Ge net. Basel, Karger, vol 15, pp 110-130
7. Kalb R, Neveling K, Nanda I, Schindler D, Hoehn H (2006). Fanconi Anemia: Causes and Consequences of Genetic Instability. In: Volff (ed) Genome and Di sease. Genome Dynamics, Basel, Karger, vol 1, pp 218-242

1

Introduction

1.1 Fanconi Anemia - Definition

Fanconi anemia (FA; synonyms: Fanconi's anemia, Familial (constitutional) panmyelocytopathy Fanconi, Familial hypoplastic anemia Fanconi) is a human disease clinically characterized by congenital anomalies, bone marrow failure and cancer susceptibility, and cellular features of chromosomal instability and hypersensitivity to DNA-crosslinking agents. It is a mostly autosomal and occasionally X-chromosomal recessive disorder with an overall prevalence of about 1 in 300.000 (Schindler and Neveling, in press).

1.2 The FA Clinical Phenotype

Fanconi anemia is a multisystem disorder with pleiotropic clinical manifestations (reviewed in Neveling et al., 2007a). Approximately 70% of FA patients display variable presence of (intrauterine) growth retardation, hyper- and hypopigmentations of the skin, radial ray and external ear defects, microcephaly, microphthalmia, and malformations of the inner organs including most frequently kidney, gastrointestinal tract, heart and brain (Kutler et al., 2003). Up to 80% of FA patients exhibit disturbances of the endocrine system, including growth hormone and thyroid hormone deficiency, type 2 diabetes and premature gonadal failure (Wajnrajch et al., 2001). Nearly all patients experience bone marrow failure during childhood, and at all ages there is a strongly elevated risk of neoplasias. A minority of FA patients are of normal stature and display few or no congenital malformations such that the diagnosis may be missed until they present with bone marrow failure or young adult-onset neoplasias. Average life expectancy of FA patients is around 20 years, and patients reaching the age of 50 are exceedingly rare (Kwee et al., 1997; reviewed in Kalb et al., 2006). 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 pancytopenia in the first two decades of life. However, clinical course and clinical phenotype are highly variable both within and between families. Treatment is mostly supportive and includes blood product substitution and stimulation of bone marrow cell growth by androgens, hydrocortisone and cytokines; the only curative therapy of bone marrow failure is hematopoetic stem cell transplantation, but a high proportion of successfully transplanted patients develop solid cancers later in life (Rosenberg et al., 2005).

1.3 The FA Cellular Phenotype

The FA cellular phenotype is characterized by increased chromosomal instability, both spontaneous and induced (Fig. 1A) (reviewed in Neveling et al., 2007a). FA cells are uniformly sensitive towards DNA-crosslinking agents such as diepoxybutane (DEB), mitomycin C (MMC), cisplatinum (CDDP) or nitrogen mustard (NM). In response to these agents, FA cells show elevated rates of chromatid breaks and chromatid exchanges (Sasaki and Tonomura, 1973; Auerbach and Wolman, 1978). Challenging patient cells in vitro with any of these agents and measuring the resulting chromosome breakage rate serves as diagnostic test. As a consequence of damaged and/or misrepaired DNA, FA cells accumulate with 4c DNA content at the S/G2 phase checkpoint of the cell cycle (Dutrillaux et al., 1982; Kubbies et al., 1985; Seyschab et al., 1993; Akkari et al., 2001). As a convenient alternative to chromosome breakage studies, the accumulations of cells with 4c DNA content in response to crosslinking agents can be assessed via flowcytometry (reviewed in Schindler et al., 2007a). As illustrated in figure 1B, cell cycle analysis yields a clear distinction between crosslink-sensitive (= FA) and crosslink-resistant (= non-FA) cells. These diagnostic tests are routinely performed with peripheral blood mononuclear cells, skin fibroblasts or amniocytes, the only prerequisite being a certain degree of proliferative activity of the tested cell types. An important conceptual aspect of the cell cycle test involves the unequivocal demonstration of an altered cell cycle distribution of FA cells as a consequence of accumulated DNA damage.

Unlike any other genetic instability syndrome, FA cells are hypersensitive towards oxygen and grow poorly in conventional cell culture incubators with ambient oxygen tension (reviewed in Kalb et al., 2006). Under ambient-air cell culture conditions FA cells show elevated spontaneous chromosomal breakage rates with a predominance of chromatid-type lesions (Joenje et al., 1981). However, in vitro growth and cloning of FA cells can be restored to near normal under hypoxic cell culture conditions (Schindler and Hoehn, 1988). 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 (Kalb et al., 2004).

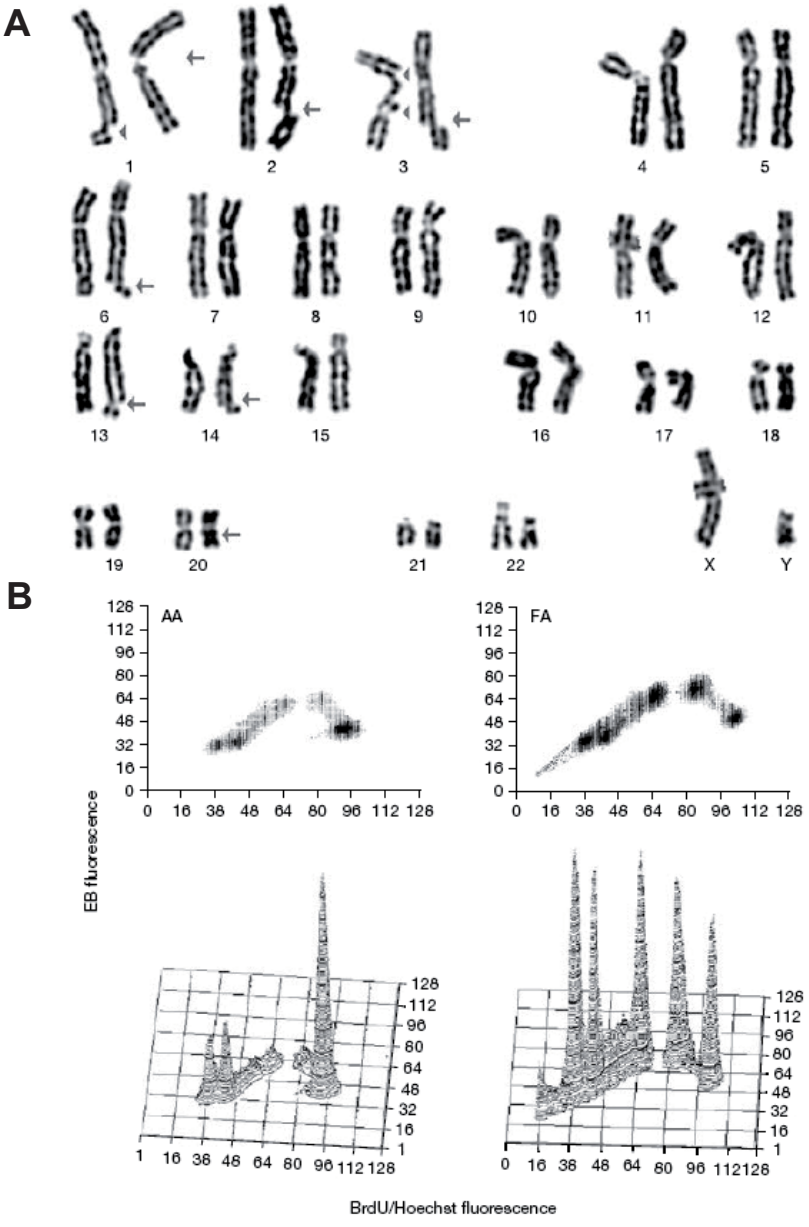


Figure 1
a) Chromatid-type chromosome lesions (arrows/arrowheads) as cytogenetic hallmarks of spontaneous and induced chromosome fragility in Fanconi anemia (FA). Blood lymphocyte karyotype stained with Giemsa. b) Cell cycle alterations as flowcytometric hallmarks of FA. The upper panels show bivariate 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 (EB) (Y-axis) and the Hoechst 33258 dye (X-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 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 (derived from Kalb et al., 2006).

1.4 FA Genes and Proteins

There are 13 genes to date whose biallelic (or in case of the X-chromosomal group FA-B hemizygous) mutations cause FA or FA-like disease: *FANCA, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M* and *-N*. The FA genes are scattered widely throughout the human genome (Fig. 2, Tab. 1) and vary considerably in both size and structure. 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 (*FANCD1*, *FANCD2*, *FANCJ, FANCL, FANCM*)

that are more ancient and highly conserved, having orthologs in lower organisms as far back as drosophila, c. elegans, and yeast (Patel and Joenje, 2007). In the following paragraphs, the FA genes and their proteins will be discussed in alphabetical order (reviewed in Kalb et al., 2007a).

FANCA

The *FANCA* gene is located on chromosome 16q24.3 and consists of 43 exons. The corresponding FANCA protein has a size of 163 kDa and contains a leucine zipper-like motif, two overlapping bipartite nuclear localization signals (NLS) (Lightfoot et al., 1999) and five functional leucine-rich nuclear export sequences (NES) that contribute to CRM-dependent nuclear export (Ferrer et al., 2005). Since the first identification in 1996 more than 200 different mutations of all possible types have been described (reviewed in Levran et al., 2005b). Especially large deletions are 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 (Centra et al., 1998; Levran et al., 1998; Morgan et al., 1999). 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 (Levran et al., 1997). Base substitutions account for less than half of the mutations in *FANCA* and cause premature termination, amino acid exchanges, or are affecting translation initiation or normal exon recognition.

FANCB

FANCB is the only X-linked FA gene. It is located on chromosome Xp22.31 and comprises 10 exons.The FANCB protein is of 95 kDa. To date, mutations in *FANCB* have been reported for 7 male FA patients including a deletion affecting the promotor region and portions of the 5´ untranslated regions, three recurrent microdeletions/-insertions, and a single splice site mutation (Meetei et al., 2004; Holden et al., 2006; Ameziane et al., 2007). 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 (Meetei et al., 2004).

FANCC

FANCC (chromosome 9q22.3) was the first known FA gene, identified in 1992 (Strathdee et al., 1992). 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* (Liebetrau et al., 1995). Mutations in *FANCC* are responsible for around 10% of all FA cases, but surprisingly few private mutations have been reported. 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 *FANC*C (Rischewski et al., unpublished data).

FANCD1

FANCD1 (chromosome 13q12.3) is identical to the gene coding for the breast cancer associated protein 2 (BRCA2) (Howlett et al., 2002). Biallelic mutations in *FANCD1/BRCA2* result in a severe form of FA with a high and very early risk for the development of malignancy (Wagner et al., 2004; Reid et al., 2005; Alter, 2006). At the mRNA level, *FANCD1/BRCA2* is the largest of the FA genes with an open reading frame of 10254 nucleotides. The FANCD1/BRCA2 protein has a molecular weight of 380 kDa. There are numerous functional domains which have been extensively reviewed (West, 2003; Shivji and Venkitaraman, 2004). 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 FANCD1/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 (Hussain et al., 2003; Tarsounas

et al., 2003; Hussain et al., 2004). FANCD1/BRCA2 appears to function in multiple and diverse cellular processes including stabilization of stalled replication forks, homologous recombination (via interaction with the RAD51 recombinase), and regulation of cytokinesis (Daniels et al., 2004). 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 (Popp et al., 2003; Reid et al., 2005).

FANCD2

FANCD2 (chromosome 3p25.3) 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. It is expressed in two alternatively spliced variants, one with a large continuous exon 43, and one with an additional exon 44 (Timmers et al., 2001). FANCD2 protein is recruited to chromatin after DNA damage-induced monoubiquitination at lysine 561 (Garcia-Higuera et al., 2001; Montes de Oca et al., 2005) where it colocalizes with other proteins involved in DNA repair including BRCA2, RAD51 and BRCA1 (Hussain et al., 2003; Hussain et al., 2004). 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, supporting its active participation in DNA repair pathways (Park et al., 2005). 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. In fact, more than 50% of the mutations observed in *FANCD2* affect splicing, which 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 underlines the functional importance of FANCD2 (Kalb et al., 2007b).

FANCE

The complementation group FA-E was defined in 1995 by cell fusion studies (Joenje et al., 1995) and the first patient was described shortly thereafter (Wegner et al., 1996). Using linkage analysis and homozygosity mapping the *FANCE* gene was mapped in 1999 to chromosome 6p21-22 (Waisfisz et al., 1999b). The corresponding RNA of the *FANCE* gene was isolated one year later (de Winter et al., 2000a). *FANCE* consists of 10 exons coding for a 58 kDa nuclear protein of 536 aminoacids. Other than a bipartite nuclear localisation signal (NLS) and a newly described “FANC” domain, there are no other known protein motifs (de Winter et al., 2000a; Pace et al., 2002; Nookala et al., 2007). Owing to its NLS, FANCE has a strong tendency for nuclear localization. The protein is required for the nuclear accumulation of FANCC (Pace et al., 2002; Taniguchi and D‘Andrea, 2002; Leveille et al., 2006). As part of the FA core complex, FANCE is also essential for FANCD2 monoubiquitination (Taniguchi and D‘Andrea, 2002). Direct interactions with FANCC and FANCD2 have been described. This has led to the speculation that FANCE serves as a critical bridge molecule connecting the FA core complex to FANCD2 (Pace et al., 2002; Gordon et al., 2005; Leveille et al., 2006). FA-E patients are relatively rare, amounting to less than 2% of all FA patients. The first comprehensive clinical, molecular and functional characterization of a larger cohort of FA-E patients is presented as part of this thesis.

FANCF

FANCF is located on chromosome 11p15 and consists of only a single exon. The encoded protein (42 kDa) shares homologies to the prokaryotic RNA-binding protein (ROM), but this is without known significance (de Winter et al., 2000b; Leveille et al., 2004). In the core complex, FANCF serves as a adaptor, linking the subcomplexes A/G and C/E. It has a C-terminal helical repeat structure similar to the Cand1 regulator of the Cul1-Rbx1-Skp1-Fbox (Skp2) ubiquitin ligase complex and mutants with base substitutions in this C-terminal region fail to interact with other components of the FA core complex (Kowal et al., 2007). To date, only 5 nonsense mutations and microdeletions (de Winter et al., 2000b) and one mutation resulting in a predicted splicing error

(Ameziane et al., 2007) have been found in FA-F patients. However, *FANCF* has gained special prominence as a gene that is frequently silenced by hypermethylation of its promotor region in various types of malignancies (reviewed in Neveling et al., 2007c), including bladder carcinomas which is reported in this thesis (Neveling et al., 2007b).

FANCG

FANCG, which is located on chromosome 9p13, is identical to XRCC9 which was identified in Chinese hamster cells as a gene involved in DNA repair (Liu et al., 1997; de Winter et al., 1998). The FANCG protein (68 kDa) has seven tetratricopeptide repeat motifs (TRPs) thought to function as a scaffold for mediating protein-protein interaction (Blom et al., 2004). 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 (Hussain et al., 2006). 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.

FANCL

FANCL (chromosome 2p16.1) was identified by mass spectroscopy of a previously isolated 43 kDa FA-associated polypeptide (FAAP43, Meetei et al., 2003a). 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 published 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 (Meetei et al., 2003a). However, further *FANCL* patients were identified recently and their description is part of this thesis.

FANCM

FANCM (located on chromosome 14q21.3) also belongs to the highly conserved FA genes (Meetei et al., 2005). Its protein shows similarities to the archael protein Hef which displays a helicase- and an endonuclease- 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 protein was discovered in a single cell line from a patient previously excluded from most of the known complementation groups (Meetei et al., 2005). Genetic work up revealed compound heterozygous mutations, a maternal nonsense mutation in exon 13 and a paternal deletion within exon 15.

FANCI, FANCJ and *FANCN*

The FA genes *FANCJ, FANCN* and *FANCI* were identified in 2005, 2006 and 2007, respectively (Levran et al., 2005a; Reid et al., 2007; Sims et al., 2007). The characterization of these genes was part of my work as a graduate student. The papers describing the identification of these genes are included in the results part of this thesis.

Depending on the underlying gene defect, FA patients are classified into different FA subtypes, also referred to as complementation groups. Complementation studies using somatic cell fusion or retroviral transduction have assigned around 66% of FA patients to complementation group A, and 8-10% each to groups C and G. The other complementation groups account for around 2% or less of the patients (FA-A: 66%, FA-B: 2%, FA-C: 10%, FA-D1: 2%, FA-D2: 2%, FA-E: 2%, FA-F: 2%, FA-G: 9%, FA-I: 2%, FA-J: 2%, FA-L: < 0.2%, FA-M: < 0.2%, FA-N: 2% (Wang, 2007).

FA candidate genes

Although 13 FA genes are known, there still are a number of FA patients without recognizable defects in one of the known FA genes. This implies that additional FA genes will be discovered. These missing genes are tracked down in several laboratories using a plethora of different ap-

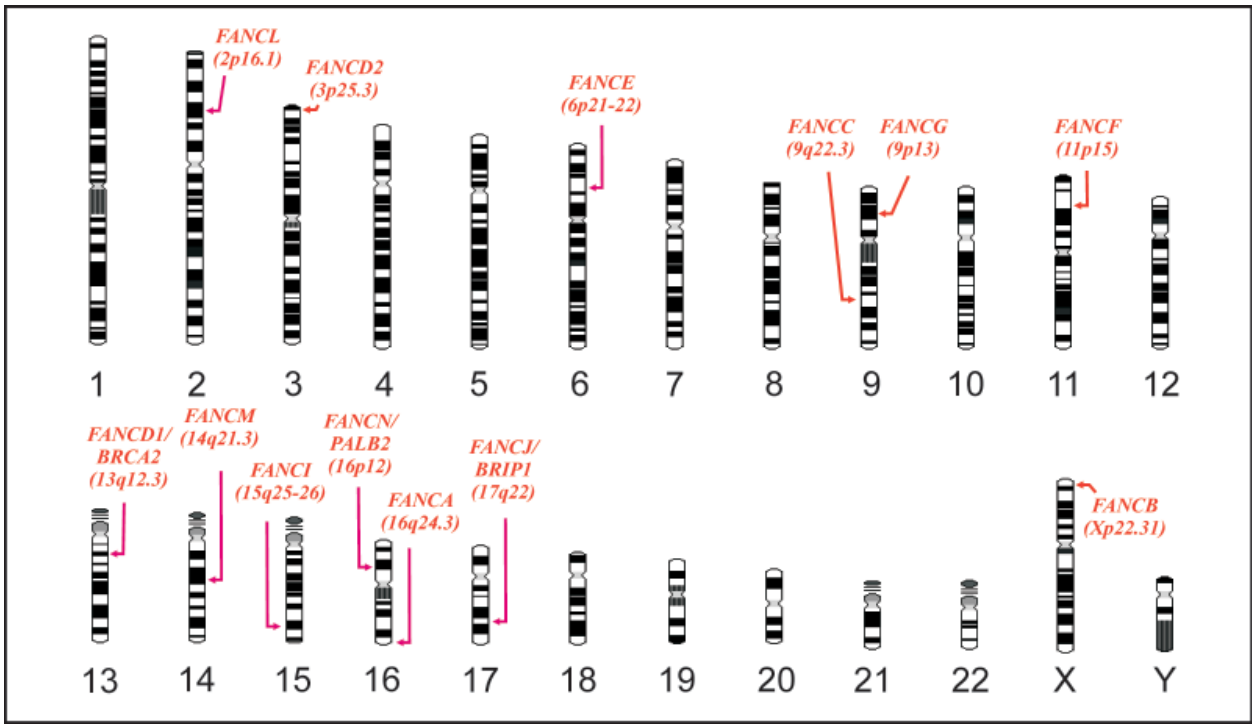


Figure 2 Human karyotype map with locations of the 13 Fanconi anemia (FA) genes known to date. FA genes are denoted with the prefix 'FANC' and alphabetical letters. Note that there are two types of FANCD genes: *FANCD1* (corresponding to *BRCA2*) and *FANCD2*. *FANCB* is the only X-chromosomal FA gene. Modified from Kalb et al., 2006.

Table 1 Characterisation of the FA complementation groups, genes and proteins

Complementation group	FA gene	Prevalence in FA	Chromosomal localisation	Exons	Protein size (kDa)	Mono-ubiquitination of D2
FA-A	*FANCA*	66%	16q24.3	43	163	no
FA-B	*FANCB*	~2%	Xp22.31	10	95	no
FA-C	*FANCC*	10%	9q22.3	15	63	no
FA-D1	*FANCD1/ BRCA2*	~2%	13q12.3	28	380	yes
FA-D2	*FANCD2*	~2%	3p25.3	44	155, 162	-
FA-E	*FANCE*	~2%	6p21-22	10	58	no
FA-F	*FANCF*	~2%	11p15	1	42	no
FA-G	*FANCG XRCC9*	9%	9p13	14	68	no
FA-I	*FANCI*	~2%	15q25-26	38	140, 147	no
FA-J	*FANCJ/ BRIP1/ BACH1*	~2%	17q22	20	140	yes
FA-L	*FANCL/ PHF9*	<0.2%	2p16.1	14	43	no
FA-M	*FANCM/ HEF*	<0.2%	14q21.3	13	250	no
FA-N	*FANCN/ PALB2*	~2%	16p12	13	140	yes

***Prevalence of FA complementation groups according to Wang, 2007**

proaches, including candidate gene analysis, expression cloning, positional cloning or physical isolation of interacting proteins. Each gene that might be an FA gene remains a mere “candidate” as long as there are no FA patients with proven mutational inactivation of the respective gene. A highly successful approach for finding new FA genes was carried out in Weidong Wangs‘ laboratory at the US National Institute of Aging. Wang´s group used co-immunoprecipitation experiments with an FANCA antibody to investigate whether there are proteins which would bind to the “bait” FANCA protein. Proteins interacting with FANCA were called “FAAP” for “FANCA-associated polypeptide”. They were numbered according to their respective molecular weights (Meetei et al., 2003b). By this approach, Weidong Wangs’ group identified many of the members of the FA core complex. For example, FAAP95 later became FANCB (Meetei et al., 2004), FAAP43 became FANCL (Meetei et al., 2003a) and FAAP250 is now FANCM (Meetei et al., 2005). To date, there still are FAAPs that interact with FA core complex members, but there are no corresponding patients such that their identification as bona fide FA genes has not been achieved. These putative but not proven FA genes have been named FAAP10, FAAP16, FAAP24 (Ciccia et al., 2007), and FAAP100 (Ling et al., 2007).

1.5 The FA-Pathway

The products of the FA genes interact with themselves and with a number of other proteins in a common pathway. The FA pathway for genomic maintenance, also called FA/BRCA pathway, is a DNA damage-activated signaling pathway, important for the recognition of DNA-interstrand crosslinks and the initiation of repair of these lesions by translesion synthesis and homologous recombination (see below “FA and DNA Repair”). According to their presumptive functions, the FA genes and their proteins can be divided into three major groups (reviewed in Neveling et al., 2007a and Wang, 2007) (Fig. 3). The first group comprises proteins FANCA, B, C, E, F, G, L, and M. These “group 1 proteins” form a multiprotein complex, labelled “FA core complex”. In response to DNA damage, this FA core complex is sequentially assembled into different subcomplexes. The first subcomplex is the heterodimer FANCA/FANCG, which is usually found in the cytoplasm. It is thought that binding of FANCG blocks the bipartite nuclear localisation signal (NLS) of FANCA, thus retaining FANCA in the cytoplasm. In response to DNA damage, FANCA is phosphorylated, possibly leading to a conformational change and translocation of FANCG to another region of FANCA, initiating its nuclear translocation (Medhurst et al., 2006). A second subcomplex located in the cytoplasm consists of FANCB, FANCL and FAAP100 (Medhurst et al., 2006; Ling et al., 2007). The nuclear localization of this subcomplex depends on FANCA and FANCM (Ling et al., 2007). Inside the nucleus, the subcomplexes FANCA/FANCG and FANCB/FANCL/FAAP100 assemble around FANCM, which has a predominant nuclear localization. Depending on FANCM, FANCA/FANCG and FANCB/FANCL/FAAP100 interact with each other and form another stable subcomplex. This subcomplex is independent of FANCC, FANCE and FANCF, suggesting that these proteins act „downstream“ with regard to core complex formation (Medhurst et al., 2006). FANCC and FANCE are part of yet another subcomplex, while FANCF serves as a adaptor between this heterodimer and the proteins around FANCM. The subcomplex FANCC/FANCE appears to recruit FANCD2 to the core complex, while FANCF stabilizes the entire complex by interactions with FANCA/FANCG and FANCC/FANCE (Medhurst et al., 2006).

FANCM binds directly to a protein called FAAP24, which is a likely candidate protein of the FA core complex (Ciccia et al., 2007). FANCM and FAAP24 are the only components of the core complex shown to have DNA interacting domains and activities (Ciccia et al., 2007; Wang, 2007). The primary role of FANCM might be the detection of ICLs. FANCM and FAAP24 thus might target the core complex to stalled replication forks, where activation through phosphorylation by ATR takes place. There are several components of the FA core complex that are hyperphosphorylated in response to DNA damage, including FANCM, FANCA, FANCE and FANCG. However, these proteins are not only phosphorylated by ATR, but phosphorylation also depends on the downstream checkpoint kinase 1 (CHK1), and possibly other kinases (Wang, 2007).

It is suggested that the core complex has at least three important roles in the FA/BRCA network (Wang, 2007). One of these functions is the interaction with DNA through FANCM and FAAP24 which anchors the core complex to sites of DNA damage. Processing DNA for subsequent repair might be another function. However, the main function of the group 1 proteins seems to be the posttranslational modification of the two proteins that form “group 2”. This occurs via monoubiquitination of group 2 proteins (FANCD2 and FANCI, also called “ID complex”) at their highly conserved lysine residues. This posttranslational modification is carried out by UBE2T as the E2 ligase and FANCL as the E3 catalytic subunit. Monoubiquitinated FANCD2 and FANCI are recruited to nuclear foci as presumptive sites of DNA repair. Interestingly, the formation of these foci depends not only on the FA core complex, but also on BRCA1, ATR and the phosphorylated histone H2A variant γH2AX which specifically associates with damaged DNA (Wang, 2007).

Once activated in response to DNA damage or stalled replication forks, the active ID complex, supported by BRCA1, recruits downstream effector proteins. These remaining three FA proteins (group 3) are not involved in the posttranslational modification of the ID complex itself. Group 3 proteins include FANCD1 (= BRCA2), FANCN (= PALB2, i.e. partner and localizer of BRCA2), and FANCJ (= BRIP1, i.e. BRCA1 interacting protein 1, also known as BACH1). While FANCJ/BRIP1 is thought to stabilize the replication fork and enable translesion synthesis, FANCD1/BRCA2 in combination with FANCN/PALB2 uploads the RAD51 recombinase. These proteins may also function in restarting the replication fork after homologous recombination. All of the latter proteins also colocalize within repair foci.

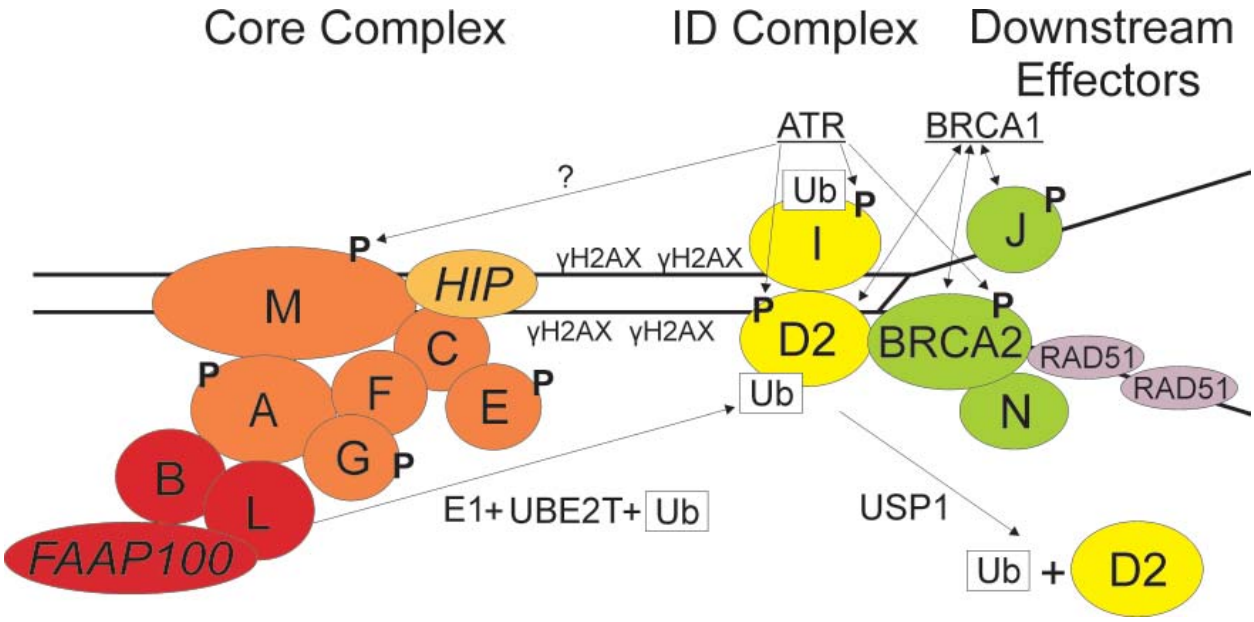


Figure 3 Model of the FA/BRCA signaling network. An ICL stalls a replication fork. ATR is activated and phosphorylates FANCM, FANCD2, FANCI and FANCD1/BRCA2. The FA core complex (FANCA, -B, -C, -E, -F, G, -L and -M, orange to red) is assembled and loaded onto dsDNA through FANCM and the Hef-interacting protein, HIP (FAAP24). The active FA core complex translocates along DNA and, as a E3 ligase, monoubiquitinates FANCD2 and FANCI by means of UBE2T as the E2 ligase and FANCL as the E3 catalytic subunit. The ID complex (FANCI and -D2, yellow) is directed to chromatin at the stalled replication fork via γH2AX. The active ID complex, supported by BRCA1, recruits downstream effector proteins (green). While FANCJ/BRIP1 is thought to stabilize the replication fork and enable translesion synthesis, FANCD1/BRCA2 in combination with FANCN/PALB2 uploads RAD51 (violet) and is attributed a role in restart of the replication fork after homologous recombination. From Schindler and Neveling, in press.

1.6 FA and DNA Repair

As already mentioned, FA cells are hypersensitive towards DNA crosslinking agents, implying a defect in the repair of DNA interstrand crosslinks (ICLs). ICLs inhibit essential processes such as DNA replication and transcription. They therefore must be repaired or bypassed for cells to survive (Wang, 2007). However, in eukaryotes the pathway that repairs ICLs is not well understood (Dronkert and Kanaar, 2001). A current concept of ICL repair implies that DSBs (which are intermediates during repair of ICLs) are probably not required for the activation of the FA pathway. Rather, the FA pathway may instead be activated by stalled replication forks that result from ICLs. It is thought that FANCM and FAAP24 might target the FA core complex to stalled replication forks, where it is phosphorylated by ATR. ATR also phosphorylates other proteins such as the H2AX, which is incorporated in the chromatin surrounding the damaged DNA. The ICL is unhooked by two endonuclease-heterodimers MUS81-EME1 and XPF-ERCC1. Unhooking of the ICL results in a DNA double strand break (DSB). The activated FA core complex then monoubiquitinates both members of the ID complex, FANCD2 and FANCI, via its catalytic subunit FANCL. The activated ID complex is recruited to chromatin and retained there by yH2AX and BRCA1. The complex consisting of BRCA2/PALB2/RAD51 is likewise recruited, promoting homologous recombination as error free repair of the DSB. The ID complex is also thought to recruit the translesion polymerases REV1 and REV3, possibly similar to monoubiquitinated PCNA that allows translesion bypass by polymerase switching. Loading of the translesion polymerases might be facilitated by the helicase BRIP1 (= FANCJ). Owing to their DNA-processing qualities, other proteins like TOPBP1, MLH1 or PMS2 may participate in the complex network of DNA repair proteins (Wang, 2007). Cells with defective core complex genes show only modest reductions of homologous recombination repair. In contrast, homologous recombination is severly affected in cells deficient for *BRCA2* or *PALB2* (= *FANCN*).

1.7 FA and Cell Cycle

Since FA cells are defective in the removal of ICLs, persisting stalled replication forks activate cell cycle checkpoint controls (reviewed in Schindler et al., 2007a). These signaling cascades ensure that cell cycle progression occurs only after successful bypass or repair of a given lesion. While cells in G0/G1 have a 2c DNA content, cells in G2/M have completed semiconservative DNA replication. These cells therefore have a 4c DNA content. FA cells accumulate at the S/G2 border content after exposure to DNA damaging agents. Since cells in both G2 and M have a 4c DNA content, G2 and M phase cells cannot be discriminated solely on the basis of DNA content. However, a series of experiments using phosphohistone 3 (3HP) as a cellular marker (whose expression is limited to the M phase of the cell cycle) proved that the MMC-induced accumulation of cells occurs prior to the M-phase but after completion or near completion of DNA-replication. These observations localize the putative crosslink-sensitive checkpoint into the early portion of the G2 phase, presumably close to the S/G2 border. Early cell kinetic studies by Kubbies et al. (Kubbies et al., 1985) clearly showed delay and arrest during both, the S and G2 phases of the cell cycle. Since cell cycle studies uniformly prove the 4c DNA content of cells that accumulate in response to crosslinking agents, is comes down to a more or less semantic argument whether to call these accumulations late S or early G2. These observations leave little doubt that the G2 phase checkpoint functions normally in FA cells, as previously demonstrated by the careful studies of Heinrich et al. (Heinrich et al., 1998) and Freie et al. (Freie et al., 2004). As pointed out by these authors, spontaneous or crosslink-induced accumulations of FA cells in the G2 phase of the cell cycle do not reflect an abnormal cell cycle response per se, but rather represent a completely normal cellular response to unresolved DNA damage.

1.8 FA and Revertant Mosaicism

Revertant mosaicism is a rather frequent phenomenon in FA (Lo Ten Foe et al., 1997; Gregory et al., 2001; Gross et al., 2002; reviewed in Hoehn et al., 2007). One out of four to five patients with FA experience a reversion or attenuation of their constitutional mutations during their lifetime. If the reversion event takes place in a bone marrow stem cell or in an early precursor cell of hematopoiesis, peripheral blood cell counts may gradually recover, leading to improved quality of life.

Depending on the mechanism of somatic reversion, the function of the affected cell lineage may be partly or completely restored (reviewed in Kalb et al., 2006). Complete restoration of a cellular phenotype to wildtype usually results from mechanisms such as intragenic recombination, back-mutation (reverse point mutation), or gene conversion (Gregory et al., 2001; Hirschhorn, 2003). Partial restoration of protein function has been observed with socalled compensating or second site mutations (Waisfisz et al., 1999a). All four principal mechanisms have been implicated in revertant mosaicism in FA (reviewed in Hoehn et al., 2007): *Intragenic crossover* is a conservative mechanism of mitotic recombination, leading for example to haplotype differences between MMC-sensitive fibroblasts and MMC-resistent lymphoblasts suggesting a mitotic recombination event as explanation for the phenotypic reversion to MMC-resistance (Lo Ten Foe et al., 1997) or leading to the simultaneous presence of different alleles in the reverted lymphoid cells, the wild-type alleles and alleles carrying both mutations (Gross et al., 2002). *Gene conversion* is another conservative mechanism of mitotic recombination without, however, mutual strand exchange. An intact homologous copy of the mutated region is required in order to serve as template for the conversion of the mutated to wildtype sequence. Gene conversion can only function in compound heterozygous patients, and it does not function if the homologous gene region on the other allele is deleted. *Back mutation* is another possible mechanism underlying revertant mosaicism. In compound heterozygous FA patients, back mutation has been assumed as probable mechanism of reversion since (1) the homologous gene region opposite to the reverted mutation was deleted in some of these patients, and (2) the reverted allele displayed the original wildtype rather than any random sequence (Gross et al., 2002). Most presumptive cases of back mutation reported were observed in *FANCA*. *Compensatory or second site mutations in cis* appear to be the most frequent type of mechanism underlying revertant mosaicism in FA. Even though this type of reversion may not result in complete restoration of protein function, it leads to at least partial functional rescue. The compensating second site mutations can lead to partial or complete mRNA rescue without the necessity for elimination of the constitutional mutation. Even though the resultant protein may differ from wildtype, its function often turns out to be sufficient for phenotypic reversion (Waisfisz et al., 1999a).

Evidence for each of these mechanisms has been obtained in mosaic FA patients, but their molecular details are not fully understood. Patients belonging to subtypes FA-A, FA-C, FA-D1, FA-D2 and FA-L have developed revertant mosaicism, with subtypes FA-A and FA-D2 being most frequently involved.

1.9 FA and Oxidative Stress

In addition to their increased sensitivity towards DNA-crosslinking agents, FA cells are exceedingly sensitive to reactive oxygen species (ROS) which is unique for any of the human caretaker gene syndromes (reviewed in Neveling et al., 2007a). FA cells grow poorly in conditions of ambient oxygen concentration (Schindler and Hoehn, 1988) and demonstrate a clear oxygen-dependence of chromosomal aberrations (Joenje et al., 1981). Growth and cell cycle transit of FA fibroblasts can be restored to near normal by growing these cells under hypoxic cell culture conditions (5 instead of 21% v/v oxygen). There are speculations that oxidative stress might account for many of the clinical and cellular abnormalities in FA, including malformations, bone marrow failure, malignancies, pigmentary changes and diabetes (Pagano et al., 2005). Experiments with murine FA bone

marrow cells lead to the hypothesis that the accumulation of DNA damage resulting from ROS provides a plausible explanation for bone marrow failure in FA. This hypothesis assumes that (1) DNA damage in FA is caused by ROS and oxidative stress, and (2) mutational inactivation of any of the FA genes causes inability to properly recognize and/or repair ROS-induced DNA damage (Fig. 4). So far, there is no direct evidence for any of these assumptions.

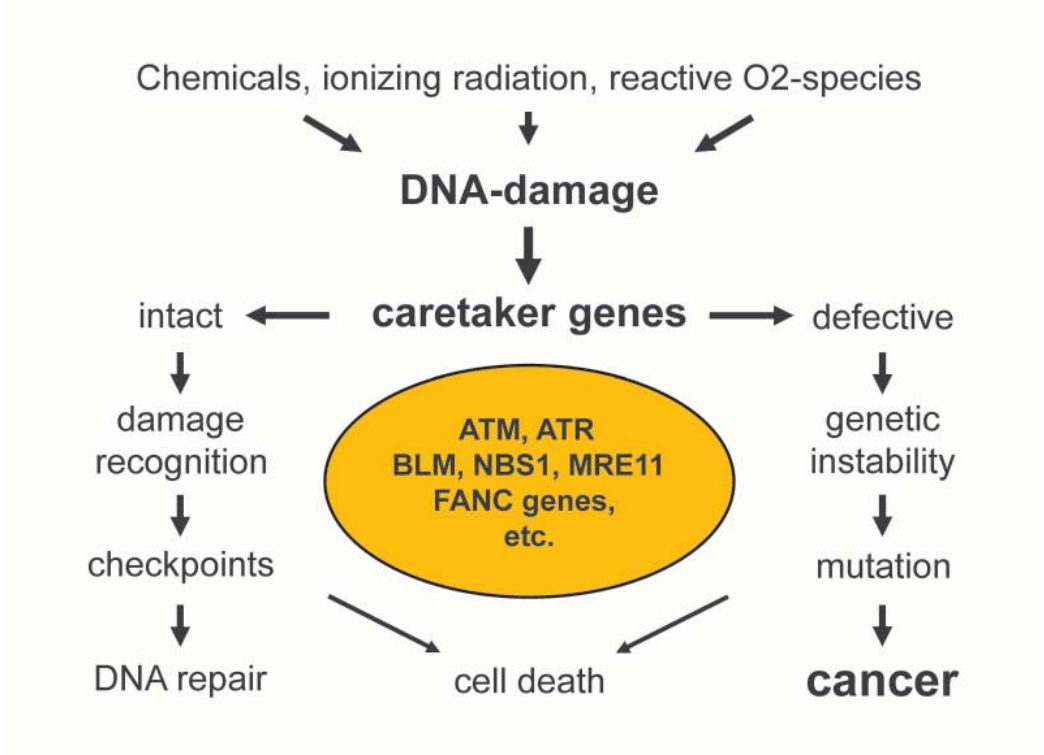


Figure 4 Hypothetical role of human caretaker genes in the DNA damage response. DNA damage can be induced in different ways, for example by chemicals or ionizing radiation or, endogenously, by reactive oxygen species. As long as caretaker genes are intact, DNA damage is recognized and, dependent of the kind of damage, checkpoints are activated, and either DNA repair or elimination via apoptosis will occur. In the case of defective caretaker genes, a regulated and orderly cell response is not guaranteed. DNA damage recognition and repair might be impaired, not occur at all, or be error-prone, resulting in genetic instability. As a result, mutations accumulate, ultimately leading either to cell death or to the emergence of malignant cell growth. From Neveling et al., 2007c.

1.10 FA and Cancer

As most detrimental manifestation of genetic instability, FA patients share a high risk for the occurrence of characteristic malignancies at relatively young age (reviewed in Neveling et al., 2007c and Schindler and Neveling, in press). Approximately 23% of FA patients develop one kind of neoplasm or more during their lifetime. In most cases, there are sharply increased risks for acute myeloid leukemia (AML) during childhood and squamous cell carcinomas (SCC) becoming effective during young adulthood. These risks are associated with defects of any of the FA genes except *FANCD1* and *-N*. *FANCD1* is also known as *BRCA2* and *FANCN* as *PALB2*. Biallelic mutations in either of these genes consistently result in a type of FA with very early onset of cancers such as medulloblastomas, nephroblastomas (Wilms tumor), and AML. Defects of *FANCD1* and *-N* include a number of cases affected by multiple tumors. FA-D1 and -N patients invariably succumb to their malignancies at early age. Given the high degree of genomic instability and elevated mutation rates seen in FA cells, there is little doubt about a causal relationship between the FA-specific defect of genomic maintenance and the emergence of cancer in FA patients.

1.11 Thesis Aims

The first question I tried to answer was whether the hypersensitivity of bladder cancers from non-FA patients towards the chemotherapeutic agent cisplatinum might be due to disruption of the FA/BRCA pathway. Bladder cancers show frequent deletions of chromosomal regions including *FANCC* and *FANCG* such that lack or alteration of these proteins might explain sensitivity towards cisplatinum as a DNA-crosslinking agent. However, the experiments showed that the only instance of disruption of the FA/BRCA pathway occurred via hypermethylation of *FANCF*, and this seemed to be rather by chance. Thus, epigenetic silencing of *FANCF* in many types of cancers is a likely consequence and not a cause of tumor initiation.

The important question of genotype-phenotype correlations in FA has so far not been sufficiently answered. I therefore studied two of the rarer FA genes and their resulting phenotypes in detail, hoping to establish a pattern of genetic changes that would explain and predict the clinical manifestations of the disease. The first gene I investigated was *FANCE*. At the beginning of this study, not much was known about FANCE, except that it was thought to form a bridge between FANCL and FANCD2 by bringing the substrate protein FANCD2 to the catalytic protein FANCL. FA-E belongs to the rare FA subgroups with only six patients described. I was able to collect a worldwide cohort of fifteen additional FA-E patients (collaborating laboratories were in Germany, Spain, the Netherlands and the USA). Other than typical FA core complex genes, the pattern of genetic alterations in *FANCE* turned out to be rather unusual with mostly truncating and only a single, albeit recurrent, missense mutation. This unusual pattern is reminiscent of that found in *FANCF*, another rare core complex gene. By performing an extensive series of complementation studies employing retroviral vectors I was able to prove the innocuous nature of a large number of *FANCE* missense mutations. c.1111C>T remained as the only missense alteration that impairs protein function. These findings suggest that genetic variability is well tolerated and can be rather high in genes whose products functions as mere adaptor proteins.

The second gene investigated in the context of putative genotype-phenotype correlations was *FANCL*. FANCL is thought to be the catalytic subunit of the E3 ubiquitin ligase represented by the FA core complex and necessary for monoubiquitination of FANCD2 and FANCI. I succeeded in identifying and analyzing the worldwide second, third and fourth *FANCL* patients. In one of these patients a somatic reversion event had occurred, adding *FANCL* to the list of FA genes that experience somatic reversions (“natural gene therapy”). Although based on only few patients, the results of my study show that FA-L patients have a similar spectrum of mutations and clinical phenotypes to most other FA core complex genes. Prior to the present work only a single FA-L patient had been published. This patient had a severe phenotype and died in early infancy. This single observation has led to the speculation that FA-L patients might be so rare because the majority of *FANCL* mutations would not be compatible with survival. The data presented in paper 3 of my thesis are at clear variance to this theory. The study of *FANCL* was initiated by my graduate student colleague Reinhard Kalb, now Cambridge UK, who discussed one of these patients in his PhD thesis (“Fanconi anemia and RAD50 deficiency: genetic and functional analysis”, Reinhard Kalb, 2007). Together, we also worked on the molecular analysis and functional characterization of *FANCD2* by examining a cohort of 33 patients. We were able to show that all FA-D2 patients, although severly affected, carry at least one hypomorphic mutation allele which obviously assures their survival. This is an important finding which underlines the central role of the FANCD2 protein in the FA/BRCA network of caretaker genes.

As ongoing effort throughout the entire time of my graduate work, I participated in the worldwide search for novel FA genes. In order to make progress in this highly competitive area of gene discovery, I collaborated with a number of laboratories in Germany, UK, Spain, the Netherlands and the USA. I was very fortunate to be part of the discovery of three new FA genes: *FANCJ/BRIP1*, *FANCN/PALB2* and *FANCI*. *FANCJ/BRIP1* was found by positional cloning using consanguineous Inuit families. The principle investigator in this collaboration was Arleen Auerbach from the Ro-

ckefeller University in New York. *FANCN/PALB2* was identified under the leadership of Nazneen Rahman (Sutton, Surrey, UK) by a candidate gene approach. *FANCI* was detected in the laboratory of Tony Huang (New York University, USA) by FANCD2 protein sequence- based homology search. My contributions to these gene discoveries were in the area of complementation and cell cycle studies, candidate gene evaluations, mutation analysis and protein expression studies.

2

Results

2.1 Disruption of the FA/BRCA pathway in bladder cancer

2.2 Recurrent disruptive mutations and forebearable substitutions inhere in the substrate binding protein of the Fanconi anemia core complex, FANCE

2.3 Mutation and Reversion in *FANCL*, the Gene Encoding the E3 Ligase Catalytic Subunit of the Fanconi Anemia Nuclear Core Complex

2.4 Hypomorphic Mutations in the Gene Enkoding a Key Fanconi Anemia Protein, FANCD2, Sustain a Significant Group of FA-D2 Patients with Severe Phenotype

2.5 The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia

2.6 Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer

2.7 FANCI is a second monoubiquitinated member of the Fanconi anemia pathway

2

Results

2.1 Disruption of the FA/BRCA pathway in bladder cancer

2.2 Recurrent disruptive mutations and forebearable substitutions inhere in the substrate binding protein of the Fanconi anemia core complex, FANCE

2.3 Mutation and Reversion in *FANCL*, the Gene Encoding the E3 Ligase Catalytic Subunit of the Fanconi Anemia Nuclear Core Complex

2.4 Hypomorphic Mutations in the Gene Enkoding a Key Fanconi Anemia Protein, FANCD2, Sustain a Significant Group of FA-D2 Patients with Severe Phenotype

2.5 The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia

2.6 Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer

2.7 FANCI is a second monoubiquitinated member of the Fanconi anemia pathway

Cytogenet Genome Res 118:166–176 (2007)
DOI: 10.1159/000108297

Cytogenetic and Genome Research

Disruption of the FA/BRCA pathway in bladder cancer

K. Neveling[a] R. Kalb[a] A.R. Florl[b] S. Herterich[a] R. Friedl[a] H. Hoehn[a] C. Hader[b] F.H. Hartmann[b] I. Nanda[a] C. Steinlein[a] M. Schmid[a] H. Tönnies[d] C.D. Hurst[e] M.A. Knowles[e] H. Hanenberg[c] W.A. Schulz[a] D. Schindler[a]

[a]Department of Human Genetics, Biocenter, University of Würzburg, Würzburg; Departments of [b]Urology and [c]Pediatric Hematology and Oncology, Heinrich Heine University of Düsseldorf, Düsseldorf; [d]Institute of Human Genetics, Charité, Universitätsmedizin Berlin, Berlin (Germany); [e]Cancer Research UK Clinical Centre, St James's University Hospital, Leeds (UK)

Manuscript received 15 January 2007; accepted in revised form for publication by L. Cannizzaro, 23 February 2007.

Abstract. Bladder carcinomas frequently show extensive deletions of chromosomes 9p and/or 9q, potentially including the loci of the Fanconi anemia (FA) genes *FANCC* and *FANCG*. FA is a rare recessive disease due to defects in anyone of 13 FANC genes manifesting with genetic instability and increased risk of neoplasia. FA cells are hypersensitive towards DNA crosslinking agents such as mitomycin C and cisplatin that are commonly employed in the chemotherapy of bladder cancers. These observations suggest the possibility of disruption of the FA/BRCA DNA repair pathway in bladder tumors. However, mutations in *FANCC* or *FANCG* could not be detected in any of 23 bladder carcinoma cell lines and ten surgical tumor specimens by LOH analysis or by FANCD2 immunoblotting assessing proficiency of the pathway. Only a single cell line, BFTC909, proved defective for FANCD2 monoubiquitination and was highly sensitive towards mitomycin C. This increased sensitivity was restored specifically by transfer of the *FANCF* gene. Sequencing of *FANCF* in BFTC909 failed to identify mutations, but methylation of cytosine residues in the *FANCF* promoter region was demonstrated by methylation-specific PCR, *Hpa*II restriction and bisulfite DNA sequencing. Methylation-specific PCR uncovered only a single instance of *FANCF* promoter hypermethylation in surgical specimens of further 41 bladder carcinomas. These low proportions suggest that in contrast to other types of tumors silencing of *FANCF* is a rare event in bladder cancer and that an intact FA/BRCA pathway might be advantageous for tumor progression.

Bladder cancer is a disease heterogeneous in morphology, tumor biology, natural history and prognosis (Borden et al., 2005; Knowles, 2006). It belongs to the ten leading types of diagnosed malignancies with a documented number of 63,210 new cases in the US in 2005. Males are three times more frequently affected than females with major risk factors being advanced age and exposure to arylamines, mostly due to cigarette smoking (Borden et al., 2005). There are two major types of bladder cancer: around 80% present as superficial papillary tumors (pTa or pT1), only 10–15% of which may later invade the muscle wall during tumor progression. Non-invasive tumors can successfully be treated by transurethral resection (TUR), often followed by adjuvant intravesical immunotherapy or chemotherapy using mitomycin C (MMC). In contrast, 20% of bladder cancers show muscle invasion at initial presentation (pT2–pT4). The prognosis of these patients is poor despite aggressive treatment with radical cystectomy, radiation and chemotherapy

This work was supported by the DFG grant LI 1038/3-1 to A.R.F. and W.A.S.

Request reprints from Detlev Schindler
Department of Human Genetics, University of Würzburg
Biozentrum, B107, Am Hubland, DE–97074 Würzburg (Germany)
telephone: +49 931 888 4089; fax: +49 931 888 4069
e-mail: schindler@biozentrum.uni-wuerzburg.de

1424–8581/07/1184–0166$23.50/0

Accessible online at:
www.karger.com/cgr

with cisplatin-based regimes (Knowles, 2001; Borden et al., 2005). A number of chromosomal changes have been associated with bladder tumor development and tumor progression. While oncogenic mutations of *FGFR3* and loss of heterozygosity (LOH) at chromosome 9 are the most common alterations in non-invasive tumors, numerous genetic alterations including *TP53* mutations and LOH at 3p, 8p, 9p/q, 13q, 14q and 17p are detectable in invasive urothelial carcinomas (UC) (Knowles, 2006; Schulz, 2006). Loss of genetic material on chromosome 9 is the most frequent alteration in UC and is found in more than 50% of all bladder tumors (Knowles, 1999; Kimura et al., 2001; Williams et al., 2002). Frequently deleted regions include 9p13→p12, 9p21, 9q12, 9q13→q31, 9q22.3, 9q32→33, 9q33→34 and 9q34 (Keen and Knowles, 1994; Knowles, 1999; Kimura et al., 2001; Florl and Schulz, 2003).

Since deletions on chromosome 9 are found throughout all stages and grades of bladder cancer, it is believed that inactivation of genes on chromosome 9 represents an early event in tumor progression. One established target of deletions is *CDKN2A* at 9p21. However, as several other regions on 9p and 9q are consistently affected by LOH, the involvement of further, as yet unidentified tumor suppressor genes must be considered.

Two prominent human caretaker genes are located in the frequently deleted regions of chromosome 9. These genes, *FANCC* (9q22.3) and *FANCG* (9p13), belong to a family of at least 13 genes *(FANCA, -B, -C, -D1/BRCA2, -D2, -E, -F, -G, -I, -J/BRIP1, -L, -M/HEF* and *-N/PALB2)* which act in the FA/BRCA pathway of DNA damage recognition and repair (Reid et al., 2007; Xia et al., 2007). The products of eight of these genes *(FANCA, -B, -C, -E, -F, -G, -L* and *-M)* form a nuclear complex required for monoubiquitination of FANCD2 during S-phase and in response to replication fork-stalling DNA damage. Monoubiquitinated FANCD2 is targeted to chromatin and assembles in nuclear foci representing regions of DNA repair. FANCD2 directly interacts with BRCA2, and co-localizes with DNA repair proteins such as BRCA1 and RAD51 involved in translesion synthesis and homologous recombination (Joenje and Patel, 2001; Kennedy and D'Andrea, 2005; Niedernhofer et al., 2005; Thompson et al., 2005). Three of the FA proteins, FANCD1, FANCJ, and FANCN, are not required for FANCD2 monoubiquitination (working downstream), and these three proteins are thought to be involved in tumorigenesis in heterozygous state: *FANCD1* is identical to the breast cancer susceptibility gene *BRCA2* (Howlett et al., 2002), FANCJ is also known as BRCA1-interacting protein (BRIP1) (Levran et al., 2005) and FANCN is a partner and localizer of BRCA2 (Xia et al., 2006). Cells deficient in any of the FA proteins show chromosomal instability, increased sensitivity towards DNA damaging agents such as cisplatin, MMC or diepoxybutane (DEB) and hypersensitivity towards oxygen (Sasaki and Tonomura, 1973; Joenje et al., 1981). Biallelic, or hemizygous in case of *FANCB*, germline mutations in any one of the FA genes cause the recessive hereditary disease FA which is associated with a greatly increased risk for neoplasia (Alter, 2003).

There are three main reasons for investigating a putative connection between FA genes and bladder cancer. First, the cellular and clinical phenotypes of FA indicate a connection between the FA proteins, DNA repair and tumorigenesis. Bladder cancer cells from invasive tumors are known to be genomically unstable. Second, cisplatin is a widespread component of all current chemotherapy regimes for invasive bladder cancer, and MMC is the most commonly used drug for adjuvant treatment of papillary bladder cancers. FA cells are highly sensitive towards these two DNA crosslinking agents. Third, deletions of chromosome 9 often include the loci of two of the FA genes *(FANCC* and *FANCG).* In order to establish whether disruption of FA genes might be involved in bladder cancer tumorigenesis or tumor progression, we investigated DNA from ten bladder cancers for LOH at *FANCC* and *FANCG*. Further 41 tumor tissues were investigated for methylation of the *FANCF* promoter region and 23 bladder cancer cell lines for defects in the FA/BRCA pathway.

Materials and methods

Cancer tissues

DNA from 41 bladder carcinoma tissues was used which had been analyzed for methylation of multiple other genes in a previous study (Neuhausen et al., 2006). Of the 41 carcinomas, two were staged as pTa, five as pT1, 13 as pT2, 16 as pT3 and five as pT4. One case was graded as G1, two as G2, 37 as G3, and one as G4, respectively. Use of patient tissues was approved by the Institutional Review Board at the faculty of medicine of the Heinrich Heine University Düsseldorf.

Cell lines and cell culture

The following cell lines derived from human bladder carcinomas were investigated in this study (see Table 1 for references): 5637, BFTC905, HT1376, SD, SW1710, VMCub1 and VMCub2 were obtained from the DSMZ (Braunschweig, Germany), the cell lines 253J, 639V, 647V, BFTC909, EJ, J82, RT112, RT4, T24, Umuc3 and VMCub3 were from Dr. J. Fogh (New York, NY). HIA was a gift of H. Hameister (Ulm, Germany) and MGHU4 of W. Beecken (Frankfurt, Germany). DSH1 was established by one of the authors (M.A.K., reported in Williams et al., 2002). In addition, we investigated the only known cell line derived from a squamous cell carcinoma of the bladder (Scaber) and a novel cell line derived from an invasive bladder carcinoma (BC44; Seifert et al., unpublished). Primary urothelial strains (UP124, UP125) were used as controls.

Bladder cancer cell lines were cultured in Eagle's minimal essential medium (MEM) (Gibco, Karlsruhe, Germany) supplemented with 16% fetal bovine serum (FBS) (Sigma, Taufkirchen, Germany). All cell cultures were kept in high humidity incubators equipped with CO_2 sensors in an atmosphere of 5% (v/v) CO_2 by replacing ambient air with nitrogen. Primary urothelial cells were cultured with the specifics described (Swiatkowski et al., 2003).

Cell cycle analysis

Approximately 5×10^5 cells each were cultured for 48 h without or with 5 or 10 ng/ml MMC, respectively. Cells were harvested by trypsinization and centrifuged. The cell pellets were resuspended in 1 ml staining buffer/1 million cells (staining buffer: 154 mM NaCl, 0.1 M Tris pH 7.4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.2% BSA, 0.1% NP40) and 1 μg/ml DAPI. Following incubation for 30 min at 4°C, DNA histograms were recorded using an analytical flow cytometer of conventional design (LSR, Becton Dickinson, Heidelberg, Germany).

Table 1. Bladder cancer cell lines used in this study, modified from Williams et al. (2005)

Cell line	Grade/stage of tumor	Sex	Other information	Reference
5637	UC	M	Primary tumor, bladder	Williams, 1980
253J	UC, G4 T4	M	Metastatic tumor, lymph node	Elliott et al., 1974; Masters, 2000
639V	UC, G3	M	Primary tumor, ureter	Elliot et al., 1976
647V	UC, G2	M	Primary tumor, bladder	Williams, 1980
BFTC905	UC, G3 papillary	F	Primary tumor, bladder	Tzeng et al., 1996
BFTC909	UC, Tx G3	M	Primary tumor, renal pelvis	Tzeng et al., 1996
DSH1	UC, T1a G2	M	Recurrence, untreated, bladder	Williams et al., 2002
EJ	UC, G3	F	Primary tumor, untreated, bladder	Williams, 1980
HIA	UC	not recorded	not recorded	Bruch et al., 1999
HT1376	UC, G3	F	Untreated, bladder	Rasheed et al., 1977
J82	UC, poorly differentiated, papillary	M	Primary tumor, treated, bladder	O'Toole et al., 1978
MGHU4	UC	M	Urothelial atypia	Lin et al., 1985
RT4	UC, G1 T2	M	Recurrence, treated, bladder	Rigby and Franks, 1970
RT112	UC, G2 papillary	F	Primary tumor, untreated, bladder	Masters, 2000
SD	UC	not recorded	Primary tumor, bladder	Paulie et al., 1983
SW1710	UC, G3 papillary	F	Bladder	Kyriazis et al., 1984
T24	UC, G3	F	Primary tumor, untreated, bladder	Williams, 1980
Umuc3	UC	M	Bladder	Grossmann et al., 1986
VMCub1	UC	M	Primary tumor, bladder	Williams, 1980
VMCub2	UC	M	Metastatic tumor, lymph nodes	Williams, 1980
VMCub3	UC	M	Primary tumor, bladder	Williams, 1980
Scaber	Squamous cell carcinoma		Bladder	O'Toole et al., 1976
BC44	UC, T4 G3	F	Bladder	Seifert et al., unpublished
UP124	Normal urothelium, control	M	Ureter	Swiatkowski et al., 2003
UP125	Normal urothelium, control	F	Ureter	Swiatkowski et al., 2003

Complementation

One of the bladder cancer cell lines was transduced with retroviral constructs containing full-length cDNAs of *FANCA, -C, -E, -F, -G* or *-L* and analyzed for MMC sensitivity using cell cycle analysis as described (Hanenberg et al., 2002).

FANCD2 immunoblotting

FANCD2 immunoblotting was performed as described (Garcia-Higuera et al., 2001) with minor modifications: cells were left untreated or were treated with 1 μg/ml cisplatin for 6 or 16 h. Lysis was achieved with 1× lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.3% NP40, 0.2% Triton X-100, proteinase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany)). 50 μg of protein extract was loaded on 7% Tris-acetate gels (Invitrogen, Karlsruhe, Germany) and electrophoresis was performed at 120 V for 6 h. Protein transfer was overnight at 4°C and 20 V onto PVDF membranes (HybondP, Amersham Biosciences, Little Chalfont, UK). Immunoblots were blocked with 5% non-fat dry milk (Hu et al., 2002) in 1× PBS, 0.05% Tween (PBS-T). As primary antibody, the mouse monoclonal FANCD2 antibody FL-17 (Santa Cruz Biotechnology Inc., Heidelberg, Germany) was used at a dilution of 1:800. As secondary antibody we used the anti-mouse IgG horseradish-peroxidase-linked $F(ab')_2$ from sheep (Amersham Biosciences, Little Chalfont, UK, dilution 1:2000). For chemiluminescence detection, a standard ECL reagent (Amersham Biosciences, Little Chalfont, UK) was employed.

Fluorescence in situ hybridization (FISH)

FISH was performed as described elsewhere (Lichter and Cremer, 1992). The bacterial artificial chromosome (BAC) clone RP11-139M16 containing the entire *FANCF* gene was obtained from the Wellcome Trust Sanger Institute (Cambridge, UK). 1 μg of BAC DNA was nick-translated using biotin-16-dUTP and used as a probe on chromosome spreads prepared from the BFTC909 cell line. 100 ng of labeled probe dissolved in hybridization mixture per slide was hybridized to denatured metaphase chromosomes for 48 h at 37°C. Prior to hybridization, the labeled probe was annealed with a 100-fold excess of Cot-1 DNA at 37°C. Post-hybridization washing, detection of the probe with fluorescein isothiocyanate (FITC)-conjugated avidin, biotinylated anti-avidin antibody and FITC-avidin and counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) were accomplished according to a standard protocol (Lichter and Cremer, 1992). Images of FITC and DAPI fluorescence were recorded separately using a Zeiss epifluorescence microscope equipped with appropriate excitation filters and a CCD camera. Digitalized images of the FITC and DAPI signals were overlaid using the Easy FISH 1.0 software (Applied Spectral Imaging, Edingen-Neckarhausen, Germany). A minimum of 15 metaphases per hybridization were examined to assess the location of the probe on specific chromosomes.

In addition, human chromosome 11 paint obtained from a commercial source (HPW Diagnostics, Rabenau, Germany) was used to hybridize on metaphases of the BFTC909 cell line according to the manufacturer's protocol.

PCR and sequencing

High molecular weight genomic DNA was prepared using a salting-out technique. Amplification of *FANCC, FANCG* and *FANCF* exons was performed using Taq polymerase and, where available, published primer sets (*FANCC*: Gibson et al., 1993; *FANCG*: Auerbach et al., 2003). For *FANCF* primers see Table 2. PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, Little Chalfont, UK). DNA sequencing of PCR products was performed using ABI-PRISM big-dye terminator chemistry on the ABI 310 instrument (Applied Biosystems, Darmstadt, Germany).

Denaturing HPLC (dHPLC)

Ten different DNAs from blood cells and the corresponding ten tumor-derived DNA preparations were analyzed. Heteroduplex formation of *FANCC* and *FANCG* PCR products was achieved by denaturating for 2 min at 96°C and cooling down to 4°C at 2°C/s. dHPLC

Table 2. Oligonucleotide primers used for genomic amplification and sequencing of *FANCF*

Primers for	Designation	Sequence (5′→3′)
Amplification	FA-F, –50 for	GCG GAT GTT CCA ATC AGT ACG
	FA-F, +50 rev	CAC GAA GGC ATA TAT TTG GTG AGA
Sequencing	FA-F, –50 for	GCG GAT GTT CCA ATC AGT ACG
	FA-F, 123 for	GCG CCA CAT CCA TCG GCG
	FA-F, 681 rev	GTG GAT GCC GGG TTC CAA CTC
	FA-F, 944 rev	CAG AGG CTT TGA AAC CTA TTG TGC
	FA-F, +50 rev	CAC GAA GGC ATA TAT TTG GTG AGA

analysis was performed on a Transgenomic WAVE system using the DNASep™ column (Transgenomic, Flein, Germany). The melting characteristics and separation modus of the DNA fragments were predicted by use of the wavemaker™ software (version 4.1.44). 5 μl of the PCR products were injected and separated. If PCR products of DNAs from patients' blood cells suggested heteroduplex formation, the corresponding tumor-derived DNAs were also analyzed.

DNA methylation analysis

The *Hpa*II-restriction assay, as well as methylation-specific PCR (MS-PCR) were performed as described previously (Taniguchi et al., 2003). Bisulfite modification of genomic DNA employed the CpGenome™ DNA Modification Kit (Q-Biogene, Heidelberg, Germany). For bisulfite sequencing, bisulfite-treated DNA was amplified as previously described (Florl et al., 2004) with primers FF345B (nt –345 to –315; 5′-GTTTAGAAAATTTTTATTTAAGGATA-3′) and FR27B (nt –6 to +24; 5′-ATCCAAATACTACAAAAAAAATTCCATAAA-3′) for 37 cycles at 52°C annealing temperature. PCR products were separated by agarose gel electrophoresis and cloned into the TA-vector pCR4-TOPO (Invitrogen, Karlsruhe, Germany). Several clones from each sample were sequenced by standard methods.

Comparative genomic hybridization (CGH)

CGH was performed as described previously (Tönnies et al., 2003) with slight modifications. In brief, test and control DNA were differently labeled by nick translation using SpectrumGreen®-dUTP and SpectrumOrange®-dUTP (Vysis, Wiesbaden, Germany). 200 ng of labeled test DNA, 200 ng reference DNA, and 12.5 μg Cot-1 DNA were co-precipitated, denatured, and hybridized to denatured normal male metaphase spreads. After incubation for 3 days at 37°C, standard post-hybridization washes were performed. Metaphase images were evaluated as with the FISH studies. Image analysis and karyotyping were performed using the ISIS analysis system (Metasystems, Altlussheim, Germany).

Spectral karyotyping (SKY) analysis

SKY was performed on chromosome preparations prepared from the BFTC909 cell line. The human SKY-Paint DNA kit from Applied Spectral Imaging (Migdal Ha'Eemek, Israel) was hybridized to denatured metaphase spreads. After hybridization for 2 days at 37°C, slides were washed and haptenized probe sequences were detected following the manufacturer's protocol. Slides were counterstained with DAPI and embedded in an antifade reagent (para-phenylenediamine). The multicolor hybridizations were visualized with the Spectral Cube system (SD200). Spectral analysis and classification was done with the SKY-View 1.6 software (Applied Spectral Imaging).

Results

Bladder carcinomas often show deletions of chromosome 9, apparently including regions that harbor the FA genes *FANCC* and *FANCG*. High sensitivity of these tumors to MMC and cisplatin raised the questions whether these FA genes are indeed subject to allelic loss in such cases and if so, whether the remaining alleles contain inactivating mutations. According to a previous study using microsatellite markers between D9S168 and D9S158 (Kimura et al., 2001), DNA from ten bladder carcinoma tissues showed LOH involving the regions of interest of chromosome 9 in the vicinity of *FANCC* and *FANCG*. In our initial experiments, this tumor DNA and DNA from corresponding peripheral blood mononuclear cells was investigated for LOH at *FANCC* and *FANCG* by comparing germline and tumor DNA on direct sequencing (see Table 3). All base substitutions observed within *FANCG* were polymorphisms rather than bona fide mutations. These polymorphisms included IVS4 –18T/G, IVS5 +58C/T (rs17885726) and IVS12 +7A/G (rs17882272). Our study revealed LOH at these sites in five tumors (T1, T2, T5, T6 and T7), while one case showed retention of heterozygosity (T10). No LOH at *FANCG* was detectable in the other four tumors: two were non-informative cases (T8, T9), and in the two remaining cases (T3, T4), the markers could not be analyzed because of inadequate amounts of tumor DNA (Table 3). *FANCG* is located between the 9p STR markers D9S171 and D9S1862. All five tumors with LOH at *FANCG* showed also LOH of D9S171. Two of them (T2, T7) also exhibited LOH of D9S1862; in the three remaining (T1, T5, T6) this marker was not determined.

Sequencing of *FANCC* in the DNA from the ten patients showed one case with retention of heterozygosity (IVS11 –25A/C in T2), while all the remaining tumors were non-informative cases (Table 3). *FANCC* is located between markers D9S196 and D9S287 on 9q. Two tumors (T2, T7) displayed LOH involving both these markers, while a single tumor had LOH involving D9S196 but was non-informative for D9S287 (T4).

Since our pilot study confirmed frequent LOH on chromosome 9 in bladder cancer involving at least the *FANCG* gene locus, as a next step functional testing of the FA/BRCA pathway in bladder cancer cell lines was performed via FANCD2 immunoblotting. Defects in any FA core complex gene (to which both *FANCC* and *FANCG* belong) result in lack of FANCD2 monoubiquitination after exposure to MMC or cisplatin. All 23 bladder cancer cell lines tested are known to carry extensive chromosomal aberrations which in the majority of cases involve chromosome 9 (Williams et al., 2002, 2005; Florl and Schulz, 2003). With the exception

Table 3. LOH analysis in bladder carcinoma tissues

Marker	Position	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
D9S168	10.578K	LOH	LOH	LOH	LOH	LOH	LOH	n.i.	LOH	MI	LOH
D9S157	17.618K	LOH	RET	LOH	n.i.	LOH	LOH	n.i.	n.i.	RET	n.i.
D9S162	19.669K	LOH	LOH	LOH	n.i.	LOH	LOH	RET	LOH	n.i.	LOH
D9S942	21.980K	RET	n.i.	RET	LOH	RET	RET	RET	RET	LOH	RET
D9S171	24.524K	LOH	LOH	LOH	LOH	LOH	LOH	LOH	LOH	LOH	LOH
FANCG	**35.063K**	**LOH**	**LOH**	**n.d.**	**n.d.**	**LOH**	**LOH**	**LOH**	**n.i.**	**n.i.**	**RET**
D9S1862	68.373K	n.d.	LOH	n.d.	LOH	n.d.	n.d.	LOH	n.d.	n.d.	n.d.
D9S15	69.571K	LOH	LOH	n.i.	n.i.	n.i.	LOH	n.i.	LOH	n.i.	LOH
D9S273	69.768K	LOH	LOH	LOH	n.d.	LOH	LOH	LOH	LOH	LOH	LOH
D9S153	78.810K	n.d.	LOH	n.d.	LOH	n.d.	n.d.	LOH	n.d.	n.d.	n.d.
D9S283	89.643K	LOH	n.d.	LOH	LOH	LOH	LOH	n.i.	LOH	LOH	LOH
D9S196	93.553K	n.d.	LOH	n.d.	LOH	n.d.	n.d.	LOH	n.d.	n.d.	n.d.
FANCC	**94.490K**	**n.i.**	**RET**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**	**n.i.**
D9S287	95.545K	n.d.	LOH	n.d.	n.i.	n.d.	n.d.	LOH	n.d.	n.d.	n.d.
D9S180	97.729K	n.d.	n.d.	n.d.	LOH	n.d.	n.d.	LOH	n.d.	n.d.	n.d.
D9S176	99.137K	n.i.	LOH	LOH	n.i.	LOH	LOH	n.i.	LOH	n.i.	n.i.
D9S53	104.641K	LOH	n.d.	n.i.	n.i.	LOH	n.i.	LOH	LOH	n.i.	n.i.
D9S1872	118.869K	LOH	LOH	RET	LOH	LOH	LOH	n.i.	LOH	LOH	LOH
D9S63	129.734K	n.d.	n.i.	n.d.	LOH	n.d.	n.d.	LOH	n.d.	n.d.	n.d.
D9S1847	132.466K	n.d.	n.d.	n.d.	LOH	n.d.	n.d.	n.i.	n.d.	n.d.	n.d.
D9S158	136.325K	LOH	n.i.	RET	n.i.	LOH	LOH	n.i.	LOH	LOH	LOH

Microsatellite markers were from Kimura et al. (2001). LOH: loss of heterozygosity; n.d.: not determined; n.i.: not informative; RET: retention of heterozygosity; MI: microsatellite instability.

of a single cell line (BFTC909), all cell lines tested responded normally to cisplatin treatment with the appearance of monoubiquitinated FANCD2 (FANCD2-L) in addition to the native isoform (FANCD2-S), indicating a fully functional FA core complex (Fig. 1A). Furthermore, prolonged cisplatin incubation times (16 h vs. 6 h) resulted in an increase of the monoubiquitinated FANCD2 isoform, suggesting regular function. In these cell lines, abrogation of the FA/BRCA pathway by mutations in *FANCD1/BRCA2* or *FANCJ/BRIP1*, located downstream of *FANCD2* (and therefore not necessary for FANCD2 monoubiquitination), were excluded via immunoblotting (data not shown). *FANCN* mutations were not excluded since the gene had not been reported at the time of this study. The single exceptional cell line BFTC909 displayed only the non-ubiquitinated FANCD2-S isoform suggesting a defect in one of the FA core complex genes.

Since defective FA genes cause cellular sensitivity towards DNA damaging agents, the FANCD2 monoubiquitination-deficient cell line BFTC909 and six randomly chosen proficient bladder cancer cell lines were tested for MMC sensitivity resulting in G2 phase arrest. Figure 1B shows the cell cycle distribution of BFTC909 in comparison to the two proficient cell lines BFTC905 and RT112. BFTC909 proved higher sensitivity towards MMC as evidenced by a disproportionate elevation of the G2 phase cell cycle fraction. A paired t-test indicated statistical significance of this hypersensitivity, when the ratios of percentages of cells in G2 phase relative to cells in S phase were compared with and without MMC treatment ($P = 0.014$). In contrast to BFTC909, all other cell lines tested showed only minor increases of the G2:S ratio in response to MMC (Fig. 1C).

Lack of the FANCD2-L isoform combined with increased sensitivity towards MMC is characteristic of a defect in one of the FA core complex genes. In order to determine which FA core complex gene might disrupt the pathway in BFTC909, the cell line was transduced with six known FA core complex genes *(FANCA, -C, -E, -F, -G* and *-L)*. Surprisingly, transduction with the vector expressing *FANCF* in BFTC909 rather than *FANCG* or *FANCC* restored a normal MMC response on cell cycle analysis (Fig. 2A). Complementation of BFTC909 exclusively by *FANCF* was confirmed by FANCD2 immunoblotting (Fig. 2B). Only the *FANCF*-transduced cells showed both, the native and the monoubiquitinated isoforms of FANCD2, while transduction with all other FA genes failed to restore FANCD2 monoubiquitination.

Sequencing of *FANCF* in BFTC909 failed to detect any mutation. Since silencing of *FANCF* via hypermethylation of the promoter region had been reported in other types of neoplasias (reviewed in Taniguchi and D'Andrea, 2006), we investigated the methylation status of *FANCF* in BFTC909, initially using a standard *Hpa*II-restriction assay (Fig. 3A). In this line, *FANCF* was found to be resistant to digestion by *Hpa*II, but sensitive to *Msp*I. This suggested at least partial hypermethylation of *FANCF*. Methylation-specific PCR confirmed methylation of the BFTC909 *FANCF* promoter which turned out to be almost complete (Fig. 3B). With the same technique, two further cell lines, VMCub1 and 639V, and one out of 41 surgical bladder tumor specimens (TCC8),

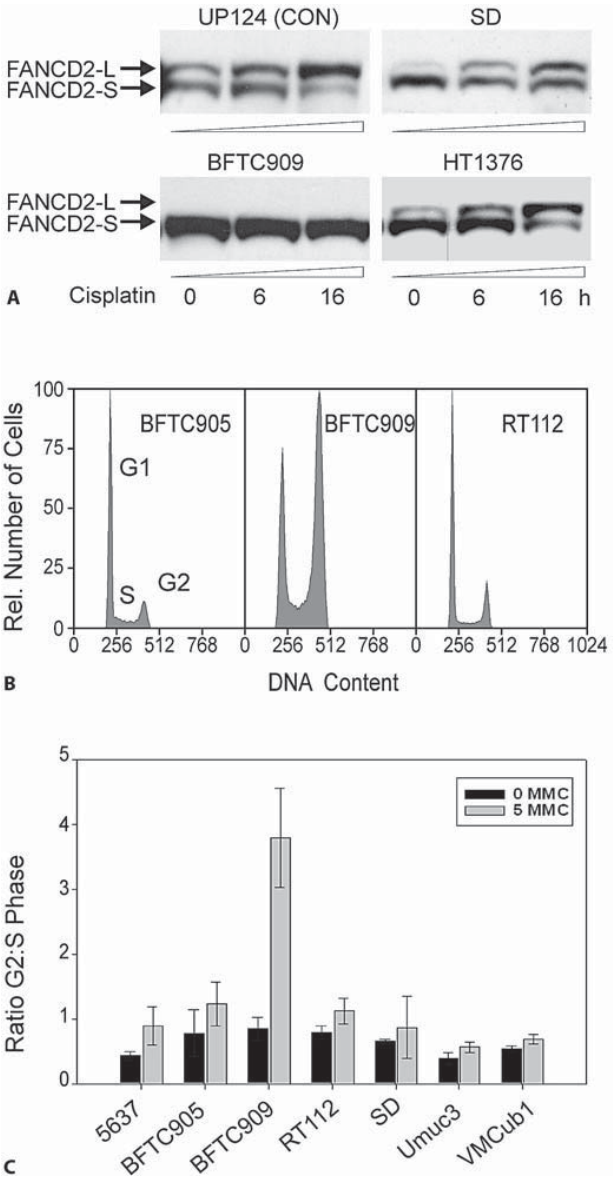


Fig. 1. Functional analysis of the FA/BRCA pathway. (**A**) FANCD2 immunoblotting of a primary urothelial cell culture (UP124) and the bladder carcinoma cell lines SD, BFTC909 and HT1376. Cells were treated with 1 μg/ml cisplatin for 6 or 16 h. The blots reveal both non-ubiquitinated (S) and monoubiquitinated (L) FANCD2 protein in the control cells UP124 as well as in bladder carcinoma cell lines SD and HT1376, with increasing levels of FANCD2-L upon longer treatment. In contrast, BFTC909 displays only the FANCD2-S band. (**B**) Flow cytometric analysis (DNA histograms) of three bladder cancer cell lines (BFTC905, BFTC909 and RT112) following incubation with 5 ng/ml MMC. In contrast to the cell lines BFTC905 and RT112, proficient for FANCD2 monoubiquitination, BFTC909 responded to MMC with a pronounced G2 phase arrest indicating hypersensitivity towards the crosslinking agent. (**C**) Bar graph showing the sensitivity of seven bladder cancer cell lines towards MMC, represented by the ratios of percentages of cells in G2 phase to cells in S phase after treatment with 5 ng/ml MMC (48 h).

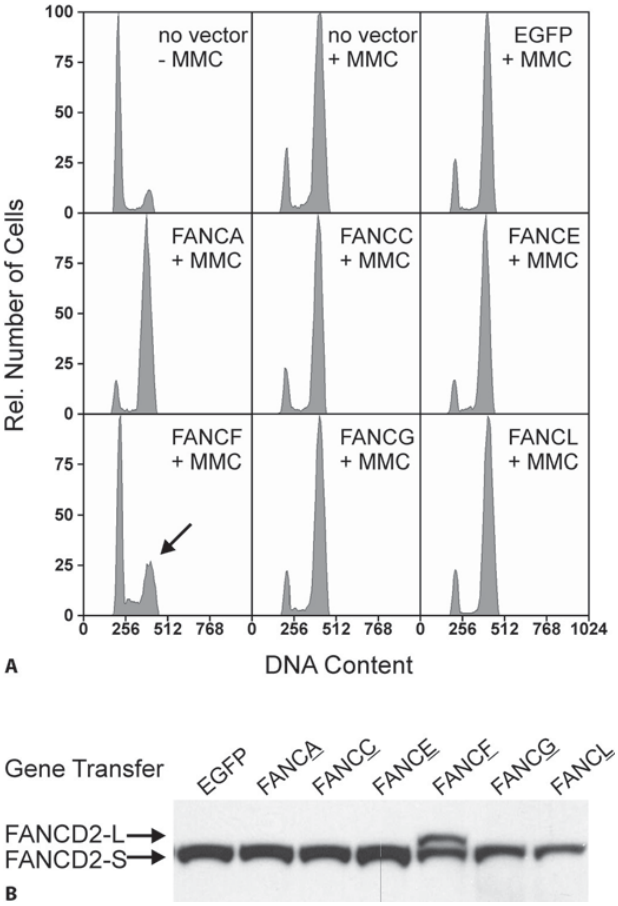


Fig. 2. Retroviral complementation of BFTC909. (**A**) Flow cytometric analysis (DNA histograms). Cells were native or transduced with retroviral vectors separately expressing the FA genes *FANCA*, *-C*, *-E*, *-F*, *-G* or *-L*. Subsequently, cells were left untreated or treated with 10 ng/ml MMC for 16 h. Treatment of BFTC909 with MMC results in a prominent G2 phase arrest, indicating that this cell line is hypersensitive towards MMC. Only the transfer of *FANCF* was able to restore the G2 phase arrest to normal. (**B**) FANCD2 immunoblotting of the cell line BFTC909 after transduction with retroviral vectors separately expressing six FA genes of the nuclear core complex. Only *FANCF* was able to complement the defective FANCD2 monoubiquination as evidenced by the appearance of the FANCD2-L band.

revealed partial hypermethylation. However, there was no concomitant evidence for defective FANCD2 monoubiquitination in the two cell lines (data not shown). The hypermethylated BFTC909 cell line, one of the partially methylated cell lines (639V) and a non-methylated cell line (J82) were chosen for bisulfite sequencing in addition to normal peripheral blood mononuclear cells as control. As shown in Fig. 3C, nearly all CpG sites of the promoter region of *FANCF* in cell line BFTC909 were methylated. Partial hypermethylation of 639V and the unmethylated status of J82 were confirmed.

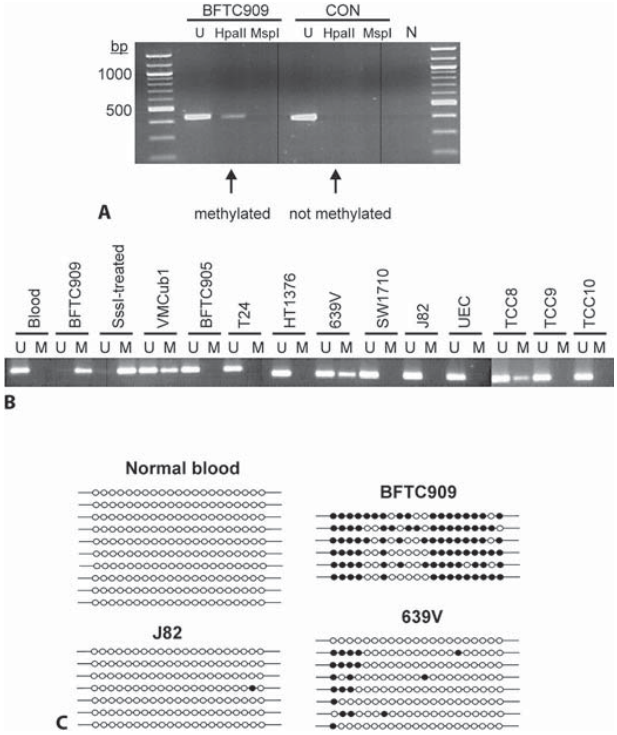


Fig. 3. Methylation analysis. (**A**) *Hpa*II restriction assay of BFTC909 DNA. The *Hpa*II restriction assay shows that the fragment +280 to +432 of *FANCF* is not restricted by *Hpa*II in BFTC909, while it is restricted in a control DNA. U: untreated DNA; N: negative control. (**B**) MS-PCR analysis of *FANCF*. Bladder carcinoma cell lines and tumor tissues were analyzed for *FANCF* hypermethylation. Among the cell lines, only BFTC909 appeared almost completely methylated, whereas VMCub1 and 639V showed partial methylation. BFTC905, T24, HT1376, SW1710 and J82 are unmethylated. Leukocytes (Blood) and normal urothelial cells (UEC) served as controls. Among the three tumor tissues shown here (TCC8, 9 and 10), one (TCC8) showed weak methylation. Note that the figure is composed from three different gel runs. U: unmethylated, M: methylated, SssI: Blood DNA methylated in vitro with CpG-methyltransferase from *Spiroplasma* species. (**C**) Bisulfite sequencing analysis of the CpG island in the *FANCF* promoter (from –345 to +24; 21 CpGs). The methylation status in different bladder cancer cell lines was investigated. A control DNA derived from normal human blood showed no methylation in the *FANCF* CpG island, whereas the FA-deficient cell line BFTC909 was hypermethylated. Consistent with the results of methylation-specific PCR, the bladder cancer cell line 639V showed partial methylation and J82 essentially lacked methylated sites. Open circle: non-methylated CpG, filled circle: methylated CpG.

Chromosome 11, where *FANCF* is located, is represented in BFTC909 by one intact copy and one copy involved in a translocation, as demonstrated by chromosome 11 painting (Fig. 4A). Spectral karyotyping of BFTC909 revealed a hypo-tetraploid karyotype with a large number of chromosomal aberrations including a translocation between chromosomes 11 and 3 (Fig. 4B). CGH revealed an underrepresentation of 11p and of distal 11q relative to overall ploidy status, but retention of proximal 11q (Fig. 4C). Although there is no obvious breakpoint in 11p15, we additionally excluded microdeletions affecting *FANCF* at 11p15 by FISH using a *FANCF* probe. As shown in Fig. 4D, two signals each were detected for *FANCF* in control cells as well as in BFTC909 cells, indicating disomy for *FANCF* in BFTC909. In the NCBI single nucleotide polymorphism (SNP) database, four SNPs are described for the 1125-nt coding region of *FANCF* (rs11556562, rs11026706, rs7103674, rs7103293). In BFTC909, all of these are homozygous, suggesting loss of one allele early in tumor progression or evolution of the cell line and subsequent reduplication of the other allele.

In order to investigate the prevalence of *FANCF* promoter hypermethylation in native bladder cancer tissues, DNA from 41 bladder cancer tissues previously studied for a range of methylation alterations (Neuhausen et al., 2006) was investigated by methylation-specific PCR of *FANCF*. While all samples yielded strong bands using primers specific for the unmethylated sequence, only a single case was weakly positive with primers specific for the methylated sequence (TCC8, see above). In this particular tumor, hypermethylation in several other genes together with pronounced global hypomethylation had been observed in the previous study (Neuhausen et al., 2006).

Discussion

This is the first study of the FA/BRCA pathway in bladder cancer cells. Crosslinking compounds such as cisplatin or mitomycin C are frequent components of current chemotherapy regimens for bladder cancer. Their use is based on empirical clinical data, while an understanding of the biological basis of their efficacy is lacking (Lehmann et al., 2003). Since many bladder carcinomas display extensive deletions of chromosome 9 carrying the FA genes *FANCC* and *FANCG*, loss of these genes might have been responsible for hypersensitivity towards DNA crosslinking agents. In particular, *FANCC* is located at 9q22.3 within one of the most frequently deleted regions in bladder cancer (Knowles, 1999; Kimura et al., 2001). We therefore expected to detect genetic alterations of *FANCC* or *FANCG* in bladder carcinomas. Contrary to our expectation, neither LOH analysis and sequencing of the two genes in tumor tissues with LOH, nor FANCD2 immunoblotting in cell lines revealed consistent defects in *FANCC* or *FANCG*.

Following treatment with DNA damaging agents, cells with defects in any of the FA core complex genes, including *FANCC* and *FANCG,* typically show prominent G2 phase accumulation and lack of monoubiquitination of FANCD2 (Garcia-Higuera et al., 2001; Shimamura et al., 2002). When tested for these FA-typical cell cycle and immunoblotting phenotypes, only a single cell line out of 23 bladder carcinoma cell lines displayed such a typical FA cellular feature. Again contrary to our expectation, neither *FANCC* nor *FANCG* were found to be defective in this cell line BFTC909. Instead, its FA-like phenotype was caused by silencing of the *FANCF* gene due to promoter hypermethyl-

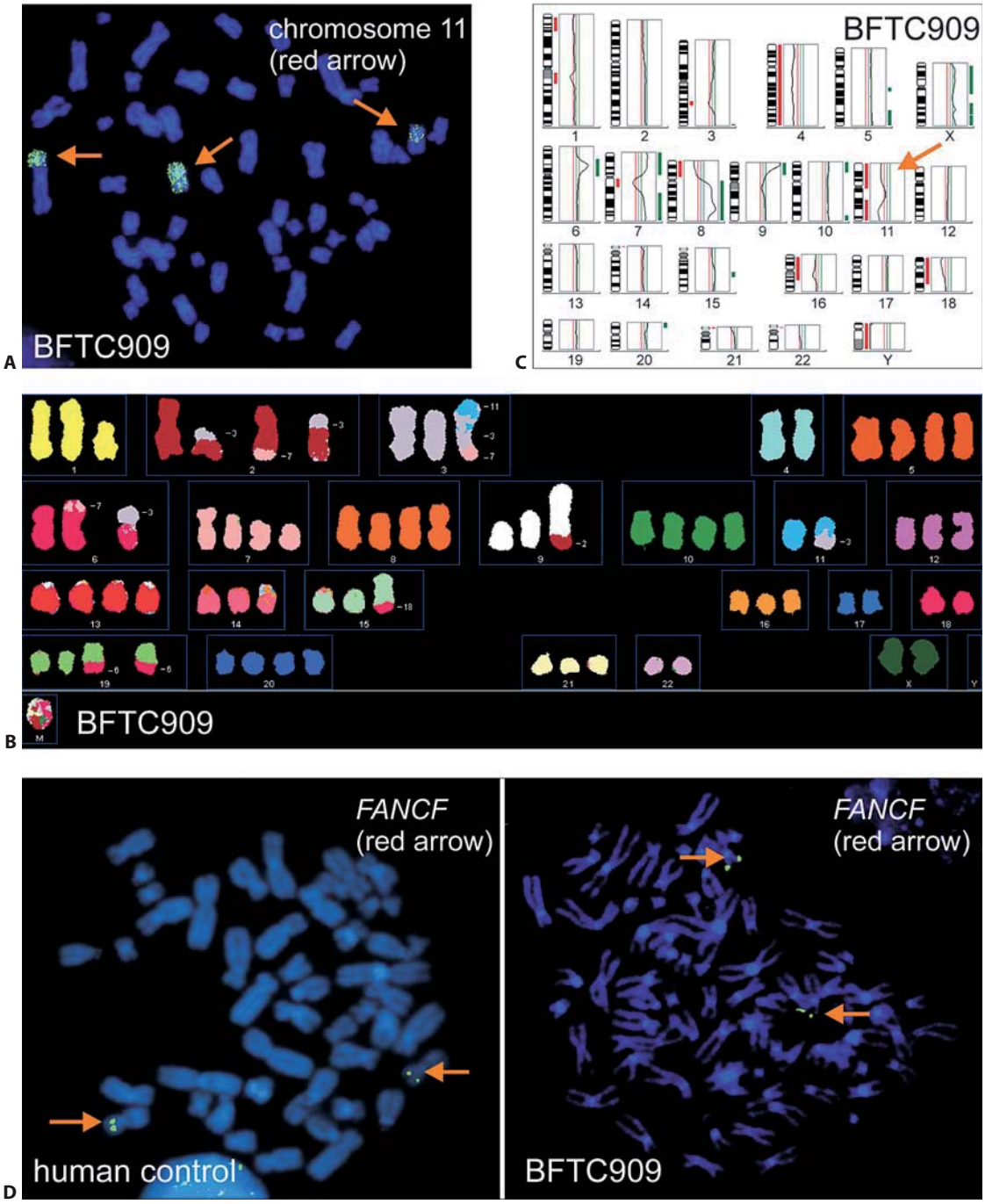


Fig. 4. Cytogenetic analysis of BFTC909. (**A**) Chromosome 11 painting. Chromosome 11 painting of the cell line BFTC909 showed only one intact chromosome 11. A second chromosome 11 was involved in a translocation. (**B**) Spectral karyotyping of BFTC909. The cell line revealed a hypo-tetraploid karyotype with a large number of chromosomal aberrations including a translocation between chromosomes 11 and 3. For chromosome 11, the cell line is disomic with no structural aberrations of 11p15, the gene locus of *FANCF*. (**C**) CGH ratio profile of BFTC909. CGH analysis shows underrepresentation of 11p and distal 11q relative to hypo-tetraploid status of the bladder cancer cell line with multiple structural aberrations. (**D**) Fluorescence in situ hybridization (FISH). In situ hybridization using a probe containing *FANCF* resulted in two specific signals on the short arms of the two chromosome 11 homologs (red arrows), showing that BFTC909 is disomic for *FANCF*.

ation. Hypermethylation of *FANCF* has been described for several kinds of tumors, including cervical cancer (Narayan et al., 2004), ovarian cancer (Olopade and Wei, 2003; Taniguchi et al., 2003; Dhillon et al., 2004; Teodoridis et al., 2005; Wang et al., 2006), AML (Tischkowitz et al., 2003), head and neck squamous cell carcinoma (HNSCC) and non-small-cell lung cancer (NSCLC) (Marsit et al., 2004). Even though *FANCF* hypermethylation has been observed in a large variety of tumors, the proportion of cases affected by hypermethylation is relatively low in each of these tumors.

FANCF is located on chromosome 11p15 adjacent to a region known to represent a hot-spot for hypermethylation (de Bustros et al., 1988; Feinberg, 1999). Dysregulation of imprinted genes in this region can be found in several tumors. A prominent example is the altered expression of *CDKN1C* resulting from promoter hypermethylation in the Beckwith-Wiedemann syndrome and in several other human cancers, including bladder cancer (Hoffmann et al., 2005). In this context, it is interesting that the only instance of *FANCF* hypermethylation in a bladder cancer tissue found in this study occurred in a tumor with multiple other hypermethylation events.

These considerations raise the question whether hypermethylation of *FANCF* reflects a 'bystander' effect resulting from spreading of epigenetic modification of genes located in such hot spot regions, or whether the disruption of the FA/BRCA pathway via *FANCF* silencing is a specific event during tumorigenesis. Inactivation of *FANCF* causes genetic instability and might therefore be an early step in cancerogenesis, preferentially in FA-typical cancers that occur in non-FA patients (Tischkowitz et al., 2003). This concept has received strong support by the recent observation of frequent epigenetic silencing of another caretaker gene, *WRN*, in human tumors (Agrelo et al., 2006). However, other than squamous cell carcinoma of the upper digestive tract or the genital organs, bladder cancer is not a common type of cancer in FA (Alter, 2003). Our results show that *FANCF* hypermethylation is a rare event in bladder tumors since it was found in only one out of 23 established tumor cell lines, and rarely in native tumor tissues. Taniguchi et al. (2003) suggested that *FANCF* methylation may be found less than expected because secondary demethylation may render tumor cells resistant to DNA damaging agents. This hypothesis implies that hypermethylation accompanies tumor initiation. More typically, however, partial hypermethylation accompanies the early stages of tumor formation and methylation increases as the tumor progresses. This is particularly well documented in bladder cancer, where aberrant but weak hypermethylation is common in morphologically normal urothelial tissue of bladders carrying tumors (Dhawan et al., 2006; Neuhausen et al., 2006). The partial methylation of *FANCF* in two bladder carcinoma cell lines (VMCub1 and 639V) might reflect this standard situation. Secondary demethylation appears to be an unlikely explanation for the findings in bladder cancer, since the tumor samples studied were typically acquired prior to the initiation of chemotherapy.

The physical and functional preservation of *FANCC* and *FANCG* in bladder cancer suggests that these two genes might be essential for survival of the tumor. This notion would fit with the general observation that both constitutional and somatic FA gene mutations are relatively rare in human neoplasia (reviewed in Lyakhovich and Surralles, 2006). Only a small minority of non-FA tumors were found to harbor mutations in FA genes, apart from *BRCA2/FANCD1* and the low-penetrance breast cancer genes *BRIP1/FANCJ* and *PALB2/FANCN* (Seal et al., 2006; Rahman et al., 2007). Possible exceptions are AML with described mutations in *FANCA* and *FANCC* as well as pancreatic cancer where also several sequence changes in *FANCA, FANCC* and *FANCG* have been described (van der Heijden et al., 2003; Couch et al., 2005). However, other researchers found no convincing evidence for frequent occurrence of FA gene mutations in non-FA AML (reviewed in Neveling et al., 2007). In addition, it seems that germline *FANCA* mutations do not contribute to familial pancreatic cancer susceptibility and that *FANCC* and *FANCG* mutations may overall have a comparatively low penetrance, if any, for the pancreatic cancer phenotype.

The paucity of involvement of FA genes in human neoplastic disease suggests that an intact FA pathway might be important for tumor cell survival. Our data shows that although tumors typically exhibit a high rate of mutations, in all but a single bladder cancer cell line the FA genes were found to be functional, including those FA genes that are located within the frequently deleted regions of chromosome 9. Moreover, if disruption of the FA pathway were a first and general step during tumor development, we would expect a much wider range of tumor types in FA patients as is actually observed. Conversely, if disruption of the FA core complex should favor the development of rather specific tumor types, hypermethylation of *FANCF* should be restricted to these particular tumors. However, hypermethylation of *FANCF* has been observed in a wide variety of tumors other than those typically seen in FA (Taniguchi and D'Andrea, 2006).

Taken together, these arguments lead to the conclusion that although reduction or elimination of a DNA repair system of a given cell may facilitate the generation of mutations in tumor suppressor genes, disruption of the FA/BRCA pathway may be disadvantageous for tumor cells by rendering them vulnerable to the adverse effects of DNA damaging agents and oxidative stress. In contrast, retention of a functional FA/BRCA pathway might be important for tumor cells to retain a certain human cell character despite chromosomal rearrangements, gains and losses and the resulting consequences. We suggest that silencing of the *FANCF* gene via hypermethylation is a secondary and non-causal event in tumorigenesis, resulting primarily from the location of *FANCF* within a known hotspot region for methylation at 11p15. This would also imply that the therapeutic effectiveness of crosslinking agents used for bladder cancer chemotherapy does not result from defects in the FA DNA repair pathway in most of these tumors. However, if disrup-

tion of the FA pathway is present even though in a low proportion of bladder carcinomas, future clinical studies must show if this would influence prognosis or open additional therapeutic options.

Acknowledgements

We thank Birgit Gottwald, Würzburg, for excellent cell culture work, Alessandra Baumer, Zurich, for helpful comments and assistance concerning methylation analysis and Dr. Irit Bar-Am, Applied Spectral Imaging, Migdal Ha'Eemek, Israel for excellent help and cooperation with the spectral karyotyping analysis.

References

Agrelo R, Cheng WH, Setien F, Ropero S, Espada J, et al: Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. Proc Natl Acad Sci USA 103:8822–8827 (2006).

Alter BP: Cancer in Fanconi anemia, 1927–2001. Cancer 97:425–440 (2003).

Auerbach AD, Greenbaum J, Pujara K, Batish SD, Bitencourt MA, et al: Spectrum of sequence variation in the *FANCG* gene: an International Fanconi Anemia Registry (IFAR) study. Hum Mutat 21:158–168 (2003).

Borden LS Jr, Clark PE, Hall MC: Bladder cancer. Curr Opin Oncol 17:275–280 (2005).

Bruch J, Wohr G, Bruderlein S, Barbi G, Wolter H, et al: Detailed marker chromosome analysis in cell line U-BLC1, established from transitional-cell carcinoma of the bladder. Int J Cancer 80: 903–910 (1999).

Couch FJ, Johnson MR, Rabe K, Boardman L, McWilliams R, et al: Germ line Fanconi anemia complementation group C mutations and pancreatic cancer. Cancer Res 65:383–386 (2005).

de Bustros A, Nelkin BD, Silverman A, Ehrlich G, Poiesz B, Baylin SB: The short arm of chromosome 11 is a 'hot spot' for hypermethylation in human neoplasia. Proc Natl Acad Sci USA 85: 5693–5697 (1988).

Dhawan D, Hamdy F, Rehman I, Patterson J, Cross SS, et al: Evidence for the early onset of aberrant promoter methylation in urothelial carcinoma. J Pathol 209:336–343 (2006).

Dhillon VS, Shahid M, Husain SA: CpG methylation of the *FHIT, FANCF, cyclin-D2, BRCA2* and *RUNX3* genes in granulosa cell tumors (GCTs) of ovarian origin. Mol Cancer 3:33 (2004).

Elliott AY, Cleveland P, Cervenka J, Castro AE, Stein N, et al: Characterization of a cell line from human transitional cell cancer of the urinary tract. J Natl Cancer Inst 53:1341–1349 (1974).

Elliott AY, Bronson DL, Stein N, Fraley EE: In vitro cultivation of epithelial cells derived from tumors of the human urinary tract. Cancer Res 36:365–369 (1976).

Feinberg AP: Imprinting of a genomic domain of 11p15 and loss of imprinting in cancer: an introduction. Cancer Res 59:1743–1746 (1999).

Florl AR, Schulz WA: Peculiar structure and location of 9p21 homozygous deletion breakpoints in human cancer cells. Genes Chromosomes Cancer 37:141–148 (2003).

Florl AR, Steinhoff C, Muller M, Seifert HH, Hader C, Engers R, et al: Coordinate hypermethylation at specific genes in prostate carcinoma precedes LINE-1 hypomethylation. Br J Cancer 91:985–994 (2004).

Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, et al: Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell 7:249–262 (2001).

Gibson RA, Buchwald M, Roberts RG, Mathew CG: Characterisation of the exon structure of the Fanconi anaemia group C gene by vectorette PCR. Hum Mol Genet 2:35–38 (1993).

Grossman HB, Wedemeyer G, Ren L, Wilson GN, Cox B: Improved growth of human urothelial carcinoma cell cultures. J Urol 136:953–959 (1986).

Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, et al: Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol 30: 410–420 (2002).

Hoffmann MJ, Florl AR, Seifert HH, Schulz WA: Multiple mechanisms downregulate *CDKN1C* in human bladder cancer. Int J Cancer 114:406–413 (2005).

Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, et al: Biallelic inactivation of *BRCA2* in Fanconi anemia. Science 297:606–609 (2002).

Hu N, Li G, Li WJ, Wang C, Goldstein AM, et al: Infrequent mutation in the *BRCA2* gene in esophageal squamous cell carcinoma. Clin Cancer Res 8:1121–1126 (2002).

Joenje H, Patel KJ: The emerging genetic and molecular basis of Fanconi anaemia. Nat Rev Genet 2:446–457 (2001).

Joenje H, Arwert F, Eriksson AW, de Koning H, Oostra AB: Oxygen-dependence of chromosomal aberrations in Fanconi's anaemia. Nature 290:142–143 (1981).

Keen AJ, Knowles MA: Definition of two regions of deletion on chromosome 9 in carcinoma of the bladder. Oncogene 9:2083–2088 (1994).

Kennedy RD, D'Andrea AD: The Fanconi anemia/BRCA pathway: new faces in the crowd. Genes Dev 19:2925–2940 (2005).

Kimura F, Florl AR, Seifert HH, Louhelainen J, Maas S, et al: Destabilization of chromosome 9 in transitional cell carcinoma of the urinary bladder. Br J Cancer 85:1887–1893 (2001).

Knowles MA: The genetics of transitional cell carcinoma: progress and potential clinical application. BJU Int 84:412–427 (1999).

Knowles MA: What we could do now: molecular pathology of bladder cancer. Mol Pathol 54: 215–221 (2001).

Knowles MA: Molecular subtypes of bladder cancer: Jekyll and Hyde or chalk and cheese? Carcinogenesis 27:361–373 (2006).

Kyriazis AA, Kyriazis AP, McCombs WB, 3rd, Peterson WD Jr: Morphological, biological, and biochemical characteristics of human bladder transitional cell carcinomas grown in tissue culture and in nude mice. Cancer Res 44:3997–4005 (1984).

Lehmann J, Retz M, Stockle M: Is there standard chemotherapy for metastatic bladder cancer? Quality of life and medical resources utilization based on largest to date randomized trial. Crit Rev Oncol Hematol 47:171–179 (2003).

Levran O, Attwooll C, Henry RT, Milton KL, Neveling K, et al: The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet 37:931–933 (2005).

Lichter P, Cremer T: Chromosome analysis by nonisotopic in situ hybridization, in Rooney DE, Czepulkowski BH (eds): Human Cytogenetics, A Practical Approach, vol I, pp 157–192 (IRL Press, Oxford 1992).

Lin CW, Lin JC, Prout GR: Establishment and characterization of four human bladder tumor cell lines and sublines with different degrees of malignancy. Cancer Res 45:5070–5079 (1985).

Lyakhovich A, Surralles J: Disruption of the Fanconi anemia/BRCA pathway in sporadic cancer. Cancer Lett 232:99–106 (2006).

Marsit CJ, Liu M, Nelson HH, Posner M, Suzuki M, Kelsey KT: Inactivation of the Fanconi anemia/BRCA pathway in lung and oral cancers: implications for treatment and survival. Oncogene 23:1000–1004 (2004).

Masters JR: Human cancer cell lines: fact and fantasy. Nat Rev Mol Cell Biol 1:233–236 (2000).

Narayan G, Arias-Pulido H, Nandula SV, Basso K, Sugirtharaj DD, et al: Promoter hypermethylation of *FANCF*: disruption of Fanconi anemia-BRCA pathway in cervical cancer. Cancer Res 64:2994–2997 (2004).

Neuhausen A, Florl AR, Grimm MO, Schulz WA: DNA methylation alterations in urothelial carcinoma. Cancer Biol Ther 5:993–1001 (2006).

Neveling K, Kalb R, Schindler D: Cancer in Fanconi anemia and Fanconi anemia genes in cancer, in Schindler D, Hoehn H (eds): Monographs in Human Genetics, vol. 15, pp 59–78 (S. Karger, Basel 2007).

Niedernhofer LJ, Lalai AS, Hoeijmakers JH: Fanconi anemia (cross)linked to DNA repair. Cell 123:1191–1198 (2005).

Olopade OI, Wei M: *FANCF* methylation contributes to chemoselectivity in ovarian cancer. Cancer Cell 3:417–420 (2003).

O'Toole C, Nayak S, Price Z, Gilbert WH, Waisman J: A cell line (SCABER) derived from squamous cell carcinoma of the human urinary bladder. Int J Cancer 17:707–714 (1976).

O'Toole C, Price ZH, Ohnuki Y, Unsgaard B: Ultrastructure, karyology and immunology of a cell line originated from a human transitional-cell carcinoma. Br J Cancer 38:64–76 (1978).

Paulie S, Hansson Y, Lundblad ML, Perlmann P: Lectins as probes for identification of tumor-associated antigens on urothelial and colonic carcinoma cell lines. Int J Cancer 31:297–303 (1983).

Rahman N, Seal S, Thompson D, Kelly P, Renwick A, et al: *PALB2*, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat Genet 39:165–167 (2007).

Rasheed S, Gardner MB, Rongey RW, Nelson-Rees WA, Arnstein P: Human bladder carcinoma: characterization of two new tumor cell lines and search for tumor viruses. J Natl Cancer Inst 58:881–890 (1977).

Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, et al: Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet 39:162–164 (2007).

Rigby CC, Franks LM: A human tissue culture cell line from a transitional cell tumour of the urinary bladder: growth, chromosome pattern and ultrastructure. Br J Cancer 24:746–754 (1970).

Sasaki MS, Tonomura A: A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res 33: 1829–1836 (1973).

Schulz WA: Understanding urothelial carcinoma through cancer pathways. Int J Cancer 119: 1513–1518 (2006).

Seal S, Thompson D, Renwick A, Elliott A, Kelly P, et al: Truncating mutations in the Fanconi anemia J gene *BRIP1* are low-penetrance breast cancer susceptibility alleles. Nat Genet 38: 1239–1241 (2006).

Shimamura A, de Oca RM, Svenson JL, Haining N, Moreau LA, et al: A novel diagnostic screen for defects in the Fanconi anemia pathway. Blood 100:4649–4654 (2002).

Swiatkowski S, Seifert HH, Steinhoff C, Prior A, Thievessen I, et al: Activities of MAP-kinase pathways in normal uroepithelial cells and urothelial carcinoma cell lines. Exp Cell Res 282: 48–57 (2003).

Taniguchi T, D'Andrea AD: The molecular pathogenesis of Fanconi anemia: recent progress. Blood 107:4223–4233 (2006).

Taniguchi T, Tischkowitz M, Ameziane N, Hodgson SV, Mathew CG, et al: Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. Nat Med 9:568–574 (2003).

Teodoridis JM, Hall J, Marsh S, Kannall HD, Smyth C, et al: CpG island methylation of DNA damage response genes in advanced ovarian cancer. Cancer Res 65:8961–8967 (2005).

Thompson LH, Hinz JM, Yamada NA, Jones NJ: How Fanconi anemia proteins promote the four Rs: replication, recombination, repair, and recovery. Environ Mol Mutagen 45:128–142 (2005).

Tischkowitz M, Ameziane N, Waisfisz Q, De Winter JP, Harris R, et al: Bi-allelic silencing of the Fanconi anaemia gene *FANCF* in acute myeloid leukaemia. Br J Haematol 123:469–471 (2003).

Tönnies H, Poland J, Sinha P, Lage H: Association of genomic imbalances with drug resistance and thermoresistance in human gastric carcinoma cells. Int J Cancer 103:752–758 (2003).

Tzeng CC, Liu HS, Li C, Jin YT, Chen RM, et al: Characterization of two urothelium cancer cell lines derived from a blackfoot disease endemic area in Taiwan. Anticancer Res 16:179–1804 (1996).

van der Heijden MS, Yeo CJ, Hruban RH, Kern SE: Fanconi anemia gene mutations in young-onset pancreatic cancer. Cancer Res 63:2585–2588 (2003).

Wang Z, Li M, Lu S, Zhang Y, Wang H: Promoter hypermethylation of *FANCF* plays an important role in the occurrence of ovarian cancer through disrupting Fanconi anemia-BRCA pathway. Cancer Biol Ther 5:256–260 (2006).

Williams RD: Human urologic cancer cell lines. Invest Urol 17:359–363 (1980).

Williams SV, Sibley KD, Davies AM, Nishiyama H, Hornigold N, et al: Molecular genetic analysis of chromosome 9 candidate tumor-suppressor loci in bladder cancer cell lines. Genes Chromosomes Cancer 34:86–96 (2002).

Williams SV, Adams J, Coulter J, Summersgill BM, Shipley J, Knowles MA: Assessment by M-FISH of karyotypic complexity and cytogenetic evolution in bladder cancer in vitro. Genes Chromosomes Cancer 43:315–328 (2005).

Xia B, Sheng Q, Nakanishi K, Ohashi A, Wu J, et al: Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. Mol Cell 22: 719–729 (2006).

Xia B, Dorsman JC, Ameziane N, de Vries Y, Rooimans MA, et al: Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. Nat Genet 39:159–161 (2007).

2

Results

Recurrent disruptive mutations and forebearable substitutions inhere in the substrate binding protein of the Fanconi anemia core complex, FANCE

Kornelia Neveling[1], Reinhard Kalb[1], Helmut Hanenberg[2,3], Maria Castella[4], Juan A. Casado[5], France Léveillé[6], Johan P. de Winter[6], Sat D. Batish[7], Francis P. Lach[7], Juan Bueren[5], Jordi Surralles[4], Arleen D. Auerbach[7] and Detlev Schindler[1] §

[1] Department. of Human Genetics, University of Wurzburg, Germany;
[2] Department of Pediatric Oncology, Hematology and Immunology, University of Dusseldorf, Germany;
[3] Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN
[4] Group of Mutagenesis, Dept. of Genetics and Microbiology, Universitat Autonoma de Barcelona, Spain;
[5] Hematopoiesis and Gene Therapy Division, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain;
[6] Department of Clinical Genetics and Human Genetics, Vrije Universiteit Medical Center, Amsterdam, The Netherlands;
[7] Laboratory of Human Genetics and Hematology, Rockefeller University, New York, NY

§ Correspondence: Dr. Detlev Schindler, Dept. of Human Genetics, Biozentrum, Am Hubland, D-97074 Wurzburg, Germany. Phone: +49 931 888 4089; Fax: +49 931 888 4069; Email: schindler@biozentrum.uni-wuerzburg.de

This work was supported, in part, by grants from the Deutsche Fanconi-Anämie-Hilfe (to D.S.), the Aktionskreis Fanconi-Anämie (to D.S.), the Schroeder-Kurth Fund (to R.K.and K.N.) and the Senator Kurt und Inge Schuster Foundation (to R.K.).

to be submitted to: Nucleic Acids Research

Abstract

FANCE is one of at least eight Fanconi anemia (FA) proteins required for monoubiquitination of the ID complex during S phase or in response to DNA damage. Together with FANCC and FANCD2, FANCE forms a ternary subcomplex that may provide a critical bridge function between the FA core complex proteins and the ID complex. *FANCE* belongs to the group of recently in evolution emerged FA genes with paralogs limited to the vertebrate lineage. Because of the rare occurrence of patients belonging to the FA-E subtype, there is only limited information on the pattern of genetic alterations in *FANCE*. We assigned 15 patients from 12 families to subtype FA-E and determined their remarkably limited spectrum of mutations. With the exception of a single recurrent amino acid substitution, R371W, only alterations causing premature protein truncation were found. R371W was present in more than 50% of our patients. Other types of *FANCE* missense substitutions leading to amino acid changes and disruption of phosphorylation sites have been expressed and functionally tested, but did not correct MMC sensitivity. We conclude that only mutations leading to premature protein truncation or instability of protein structure are detrimental in *FANCE*, while many other types of alterations qualify as innocuous variants.

Introduction

Fanconi anemia (FA) is a rare recessive inherited disease with variable congenital malformations, cancer susceptibility, and progressive bone marrow failure. At the cellular level, hypersensitivity to bifunctional alkylating agents such as mitomycin C (MMC), diepoxybutane (DEB) or cisplatin (CDDP) results in increased chromosomal breakage, reduced cell survival, and cell cycle arrest at the S/G2 transition. These parameters are used to confirm or rule out the clinical suspicion of FA (Schindler D, 2007). At present 13 genes (*FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N*) have been identified whose mutational inactivation cause corresponding complementation groups of FA (reviewed in (Wang, 2007).

The protein products of the FA genes interact in a common pathway involved in the cellular response to DNA damage (Wang and D'Andrea, 2004; Kennedy and D'Andrea, 2005; Niedernhofer et al., 2005; Wang, 2007). During S phase and in response to DNA damage, at least eight of the FA proteins assemble in a nuclear core complex. This complex is necessary to monoubiquitinate and thereby activate the key FA proteins FANCD2 (Garcia-Higuera et al., 2001; Medhurst et al., 2001; Meetei et al., 2003) and FANCI (Dorsman et al., 2007; Sims et al., 2007; Smogorzewska et al., 2007) . Monoubiquitinated FANCD2 and FANCI colocalize in nuclear foci with proteins such as BRCA2 and RAD51 known to be involved in DNA repair by homologous recombination (Garcia-Higuera et al., 2001; Taniguchi et al., 2002; Hussain et al., 2004; Wang et al., 2004; Montes de Oca et al., 2005). The FA pathway is important to sustain genomic stability and to prevent cancer.

FA-E was first reported in 1995 as the fifth FA subgroup (Joenje et al., 1995), and the corresponding clinical and cytogenetic data of the first patient were published one year later (Wegner et al., 1996). In 1999, Waisfisz and colleagues used cell fusion and complementation analysis to assign three families to complementation group FA-E. Using linkage analysis and homozygosity mapping, they subsequently assigned the *FANCE* gene to chromosome 6p21-22 (Waisfisz et al., 1999). Shortly thereafter, the Amsterdam group succeeded in isolating a cDNA representing *FANCE* and identified the first mutations (de Winter et al., 2000a). *FANCE* contains 10 exons encoding a 58 kDa nuclear protein that consists of 536 amino acids. Other than a bipartite nuclear localisation signal (NLS) and the recently described FANC repeats, there are no known protein motifs (de Winter et al., 2000a; Pace et al., 2002; Nookala et al., 2007). Owing to its NLS, FANCE has a strong tendency for nuclear localization and appears to be required for the nuclear accumulation of FANCC (Pace

et al., 2002; Taniguchi and D'Andrea, 2002; Leveille et al., 2006). As one of the components of the FA core complex, FANCE is essential for FANCD2 monoubiquitination (Taniguchi and D'Andrea, 2002). Direct interactions with FANCC and FANCD2 have been described such that FANCE may function as a critical bridge molecule connecting the FA core complex to FANCD2 (Pace et al., 2002; Gordon et al., 2005; Leveille et al., 2006). It is currently thought that the FA core complex serves as E3 ligase with FANCE mediating the close proximity of the FA core complex to its substrates FANCD2 and FANCI, thereby facilitating monoubiquitination of these proteins.

In addition to the three FA-E patients whose mutations were described in 2000 (de Winter et al., 2000a), three other patients were recently reported (Ameziane et al., 2007), although the status of one of the latter remained arguable. We here present clinical and molecular data of 15 further patients from 12 families assigned to subgroup FA-E. Whereas these patients display a typical FA clinical phenotype, we show that the pattern of mutations in *FANCE* deviates from that of most other FA genes. By functional testing we provide a possible explanation for the rareness of this FA subgroup and the limited pattern of mutations observed in these patients.

Materials and Methods

Patient's statistics

The present studies were performed with the informed consent of the patients and/or their families and approval of the local IRBs. A total of fifteen FA-E patients (patients 1-15) from 12 different families (family 4 has two affected children, patients 4 and 5, and family 6 has three affected children, patients 6, 7 and 8) were assigned to complementation group FA-E and mutation analysis was performed in all of them. Clinical data were not available from patient 15, therefore this patients is not part of phenotype analysis. The only fetal case (patient 1) is included in the phenotype analysis, but excluded from data concerning median age of diagnosis, median age of death and hematological manifestations. However, results concerning the mutations and haplotypes of both patients are included in the text, figures and tables. Haplotype analysis was limited to FA-E families with c.1111 C>T.

Cell culture

Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) were established using cyclosporin A (Neitzel, 1986). They were maintained, like whole blood and primary lymphocyte cultures, in RPMI 1640 medium supplemented with GlutaMAX (Gibco, Karlsruhe, Germany) and 15% fetal bovine serum (FBS; Sigma, Taufkirchen, Germany). Fibroblast strains were established using standard cell culture protocols. They were propagated in Eagle's MEM with GlutaMAX (Gibco, Karlsruhe, Germany) supplemented with 15% FBS. All cultures were kept in high humidity incubators equipped with CO2 and O2 sensors, in an atmosphere of 5% (v/v) CO2. In case of fibroblasts, hypoxic cell culture conditions were used (5% (v/v) O2) by replacing ambient air with nitrogen (Schindler and Hoehn, 1988).

Diagnostic procedures

The clinical diagnosis of FA was confirmed by the detection of cellular hypersensitivity to DNA-crosslinking agents by chromosome breakage or cell survival studies or by cell cycle analysis. For chromosome breakage studies, baseline and MMC- or DEB-induced chromosomal breakage in phytohemagglutinin (PHA)-stimulated cultured peripheral blood lymphocytes were analyzed as described (Joenje H, 1997; Auerbach et al., 1989). Cell survival was determined using CD3/CD28/IL2-stimulated lymphocytes or lymphoblasts. 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 (Hanenberg et al., 2002). For cell cycle analysis, PHA-

stimulated blood lymphocytes isolated via Ficoll-Paque PLUS (Amersham Biosciences, Little Chalfont, UK) separation, lymphoblasts or fibroblasts were grown for 48 or 72 h and subjected to mono- or bivariate (BrdU-Hoechst/Ethidium bromide) cell cycle analysis (Schindler and Hoehn, 1999). Cell cycle distributions reflecting DNA content and cell cycle progression were quantitated using the MPLUS AV software package (Phoenix Flow Systems, San Diego, CA). The cellular FA phenotype was recognized by the detection of typical G2 phase accumulations. Cell cycle testing for G2 phase arrest has previously been shown to arrive at the same diagnostic conclusions as standard chromosome breakage analysis (Seyschab et al., 1995).

Retroviral complementation

For complementation studies, cell lines were transduced with retroviral vectors containing full-length cDNA of *FANCE* (S11FEIEG2, S11FEIN) (figure 1). S11FEIEG2 has been previously described (Huck et al., 2006), S11FEIN was cloned by restriction of S11FEIEG2 with *Not*I-*Dra*III and ligating the *FANCE*-containing fragment into S11IN cut identically. S11IN contains the neomycin resistance gene (*NEO*) instead of *eGFP* (Kalb et al., 2007). The empty vectors or those with other *FANC* cDNAs served as controls. S11 vectors are based on the spleen focus-forming virus and are derived from the GR plasmid (Hildinger et al., 1999). Selection of cells transduced with S11FEIN, S11IN or other vectors containing the neomycin resistance gene 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 (Hanenberg et al., 2002; Chandra et al., 2005; Casado et al., 2007). Positive complementation was indicated by loss of G2 arrest, indicating correction of MMC sensitivity. The *FANCE*-negative cell line EUFA622 (de Winter et al., 2000a) and an LCL from patient 2 with biallelic nonsense mutations in *FANCE* were used for testing the effect of expressed sequence alterations using the site-mutated vector S11FEIEG2.

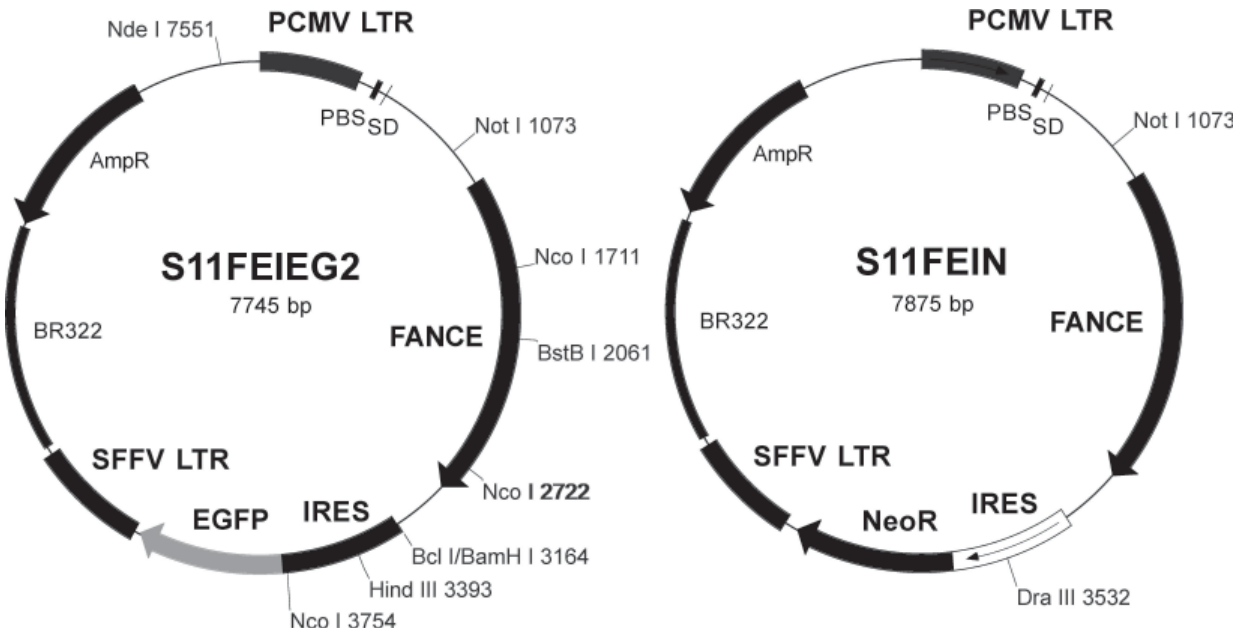


Figure 1 Circular maps of the vectors S11FEIEG2 and S11FEIN that were used for retroviral complementation. The S11FEIEG2 vector is an S11 vector based on spleen focus-forming virus. It contains a bicistronic construct of *FANCE* and *eGFP* cDNAs connected via an internal ribosomal entry site (IRES) (Huck et al, 2006). The S11FEIN vector was cloned by restriction of S11FEIEG2 with *Not*I-*Dra*III and ligating the *FANCE*-containing fragment into S11IN cut identically. It contains the neomycin resistance gene (*NEO*) instead of *eGFP*. Long terminal repeats (LTRs) and restriction sites used for construction of the vectors and site-directed mutagenesis are indicated. Bacterial resistance is *Amp*R.

FANCD2 immunoblotting

FANCD2 immunoblotting was performed as described (Garcia-Higuera et al., 2001) with minor modifications: cells were treated with 50 ng/ml MMC for 16 hours. Lysis was achieved 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 β-glycerophosphate; 0.1 mM Na3VO4; 0.3% nonidet P-40; 0.2% tritone X, protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). 50 µg of whole protein extract were loaded on 7% tris-acetate gels (Invitrogen, Karlsruhe, Germany) and electrophoresis was performed at 120 V for 6 hours. Protein transfer was overnight at 4°C and 20 V onto PVDF membranes (HybondP, Amersham Biosciences, Little Chalfont, UK). Immunoblots were blocked with 5% non-fat dry milk (Roth, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) in PBS/0.05% tween (PBS-T). As primary antibody, the mouse monoclonal FANCD2 antibody FL-17 (Santa Cruz Biotechnology Inc., Heidelberg, Germany) was used at a dilution of 1:800. As secondary antibody, we used the anti-mouse IgG horseradish peroxidase linked F(ab‘)2 from sheep (Amersham Biosciences, Little Chalfont, UK, dilution 1:2000). For chemiluminescence detection, a standard ECL reagent (Amersham Biosciences, Little Chalfont, UK) was employed.

FANCE immunoblotting

Cell lysates (~400.000 cells) from lymphoblastoid cell lines were obtained as previously described (Leveille et al., 2004) and proteins were separated on SDS-polyacrylamide gels. Proteins were transferred to a PVDF membrane and immunoblotted with FANCE antibody.

PCR and sequencing

Genomic DNA (gDNA) was prepared using a modified salting-out technique (Miller et al., 1988). Amplification of *FANCE* exons was performed using Pfx polymerase (exons 1+2) (Invitrogen, Karlsruhe, Germany) and Taq polymerase (exons 3-10). Primers were obtained from TibMolBiol (TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany). PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare; Munich, Germany). DNA sequencing of PCR products was performed using ABI-PRISM big-dye terminator chemistry and the ABI 310 instrument (Applied Biosystems, Darmstadt, Germany). gDNA amplification and sequencing primers are provided in supplementary tables S1A and S1B.

For analysis of splice site mutations, RNA was purified using the Micro-to-Midi Total RNA Purification System (Invitrogen, Karlsruhe, Germany). Isolated RNA was reverse transcribed using SuperScript II Reverse Transcriptase and Oligo(dT)20 (both Invitrogen, Karlsruhe, Germany) according to the manufacturers instructions. *FANCE* cDNA was amplified in 2 overlapping fragments. Amplification was performed using FailSafe™ PCR System with buffers G or K (EPICENTRE® Biotechnologies, Madison, WI). Purification of PCR products and sequencing was as described for gDNA. cDNA amplification and sequencing primers are provided in supplementary tables S2A and S2B.

Site-directed mutagenesis

For site-directed mutagenesis, the 3’ portion of *FANCE* (containing the nucleotides to be mutated) was subcloned into a smaller vector. To this end, the vector S11FEIEG2 was cut with *BstB*I (inside *FANCE*) and *Hind*III. The resulting 1331-nt fragment was cloned into the multiple cloning site of pETM30 (EMBL, Heidelberg, Germany). pETM30 containing the *FANCE*-insert was cut by *Hind*III and *Xba*I. Using these sites, the fragment containing *FANCE* was cloned into pUC18 (Invitrogen, Karlsruhe, Germany). Site-directed mutagenesis was performed using this pUC18-*FANCE* construct with the QuikChange® Site-Directed Mutagenesis Kit and Pfu polymerase (both Stratagene, La Jolla, CA). Site-mutated plasmids were isolated by plasmid mini preparation (Qiagen, Hilden, Germany) and the substitutions introduced were checked by direct sequencing. After successful mutagenesis, the corresponding fragment was cut out of pUC18-*FANCE* by *Hind*III and *BstB*I and cloned back into S11FEIEG2. The following plasmids were generated by this technique: S11FEIEG2-T346A, S11FEIEG2-S356G, S11FEIEG2-R365K, S11FEIEG2-

R371W, S11FEIEG2-S374A, S11FEIEG2-S486A, S11FEIEG2-S486T, and S11FEIEG2-A502T. For construction of plasmid S11FEIEG2-P184Q, S11FEIEG2 was cut by *Nco*I and *BstB*I (both inside *FANCE*). The resulting 350-nt fragment was cloned into pETM30 (EMBL, Heidelberg, Germany) and cut out by *Hind*III and *Xba*I. Using these sites, the fragment was cloned into pUC18 (Invitrogen, Karlsruhe, Germany) (see above). Site- directed mutagenesis was performed as described above. After successful mutagenesis, the corresponding fragment was cut out of pUC18-*FANCE* by *Nco*I and *BstB*I and cloned back into S11FEIEG2 using *BstB*I-*Nde*I and *Nde*I-*Nco*I fragments in a two-insert ligation. Primers for site-directed mutagenesis are provided in supplementary table S3.

Haplotype analysis

For haplotype studies, we used Haploview 4.0 to create a LD Plot of a 100 kb region containing *FANCE* +/- 50 kb (Barrett et al., 2005). Data from 300 normal individuals analyzed with a 550K Illumina-Chip were used as source of this LD Plot. SNPs in *FANCE* are located in block 4. Block 4 contains 5 tagSNPs (2 inside and 3 outside of *FANCE*). These SNPs were used for haplotype analysis by direct sequencing. SNP amplification and sequencing primers are provided in supplementary tables S4A and S4B.

Results

Assignment to subgroup FA-E

The diagnosis of FA was confirmed either by chromosome breakage studies (not shown) or via cell cycle analysis after exposure of cells to DNA damaging agents (table S5 and figures 2 A and B). Absence of monoubiquitinated FANCD2 isoform FANCD2-L was confirmed by FANCD2 immunoblotting (data not shown). Assignment to subgroup FA-E was by retroviral complementation using the vectors S11FEIEG2 and/or S11FEIN (see materials and methods) or via direct sequencing (patients 14 and 15). Successful retroviral complementation is shown by reduction of G2-phase accumulations of MMC-treated FA-E lymphoblasts following transduction with the vector S11FEIN (figure 2C) or by graphic presentation of representative survival curves (figure 2D). FANCE immunoblotting showed absence of FANCE protein in patient 14 (=EUFA279) and two previously reported FA-E patients (Ameziane et al., 2007) (figure 2E). Seven FA patients (patients 4-11) listed in the International Fanconi Anemia Registry (IFAR) were assigned to complementation group FA-E. Three other FA-E patients emerged in a cohort of 372 confirmed FA patients diagnosed in two German laboratories (patients 1-3). Two FA-E patients each were identified by the Spanish FA network (patients 12+13) and the Dutch FA groups (patients 14+15).

Clinical data

As shown in table S6, the clinical phenotype of our cohort of FA-E patients reflects the entire spectrum of developmental defects that tend to occur in the majority of FA patients. Most patients had growth retardation (11) and radial ray defects (10). Six patients each had microcephaly, microphtalmia, cardiac malformations and café-au-laits spots. Five patients had absence or malformations of the kidney. Two patients had anal atresia (patients 10 and 13) and single patients were affected by brain malformations (patient 1), esophageal atresia (patient 2), abnormal genitalia (patient 9), microtia (patient 9), skeletal malformations other than radial ray defects (patient 12) and jaundice at birth (patient 13). Hematological manifestations including thrombocytopenia and anemia were recorded in eight of the patients. Two patients (patient 6 and 7) underwent bone marrow transplantation (see below). Another (patient 9) died prior to onset of hematological changes. Among our cohort, hematological manifestations occurred between 2.5 and 10.5 years of age. The median age of diagnosis was 2.8 years (n=13) ranging from birth to 10 years of age.

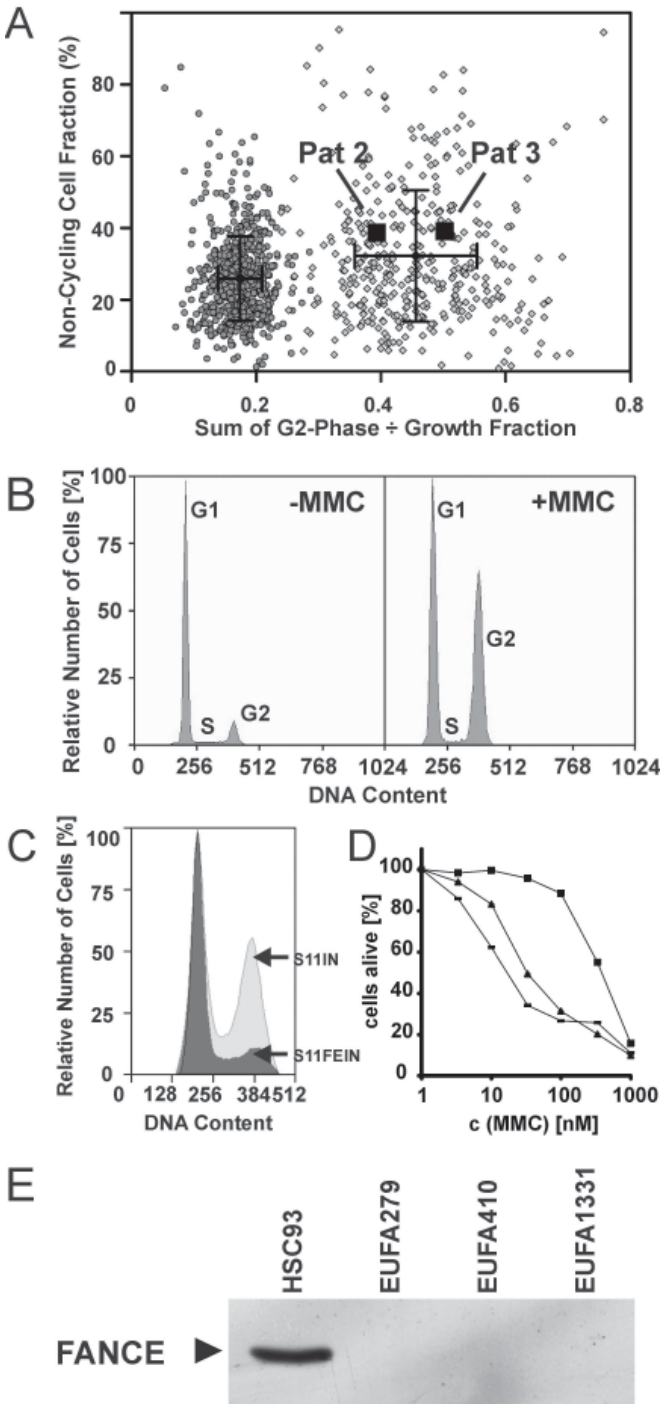


Figure 2 A) Increased spontaneous G2-phase accumulations in primary phytohaemagglutinin (PHA)-stimulated lymphocytes from two FA-E patients (patient 2 and patient 3). Black squares show the proportions of cells in the G2 phase relative to the growth fraction of a 72h culture without MMC (0.393 for patient 2 and 0.502 for patient 3). The G2-phase/ growth fraction ratios are increased when compared to 630 controls (0.174 ± 0.035, dark grey circles) and fall into the range of 394 FA-positive cultures (0.456 ± 0.098, light grey squares). The non-cycling cell fractions are 38.7% for patient 2 and 39.1% for patient 3 (means ± SD are 25.19 ± 11.72 for the controls and 32.32 ± 18.30 for the FA cultures). B) Cell cycle distributions of an LCL of FA-E patient 1. Exposure to MMC causes accumulation of cells in the G2 phase of the cell cycle. Relative number of cells in G2 without MMC: 11.3%, relative number of cells in G2 after exposure to MMC: 46.1%. C) Retroviral transduction of an FA-E patients' fibroblasts (patient 9) using vector S11FEIN complements the cellular MMC sensitivity and reduces the G2 phase to near nomal (from 33.8% to 9.5%). D) Survival curves of FA-E LCL (patient 4/II) transduced with the *FANCE* containing vector S11FEIN (■), *eGFP* (—) or *FANCD2* (▲). The curves show MMC dose-dependent cell survival, which is increased after transduction with the vector S11FEIN containing a functional *FANCE* cDNA. There is no change with any of the other vectors. E) FANCE immunoblotting shows the total absence of FANCE protein in three FA-E patients (EUFA279, EUFA410, EUFA1331). Patient EUFA279 is part of this study (patient 14), while patients EUFA410 and EUFA1331 were published in Ameziane et al (2007). Non-FA cell line HSC93 serves as control.

Five of the 13 fully informative patients are deceased, with age of death ranging from 8 month to 10 years. Median age at death was 6.1 years (n=13). There were no cancer clusters in these families or tumor types other than those believed to be influenced environmentally. The family of patient 1 had a history of spontaneous abortions and previous affecteds with FA: there was one spontaneous abortion during the 8th week of pregnancy, one previous stillborn with FA, and a stillborn. Two of our patients (patients 6/I and 6/II) underwent bone marrow transplantations (BMTs). Patient 6/I had alternative donor BMT at age 8 years. This patient died two years later. Patient 6/II underwent a BMT at age 2½ before the occurrence of a bone marrow failure. In this

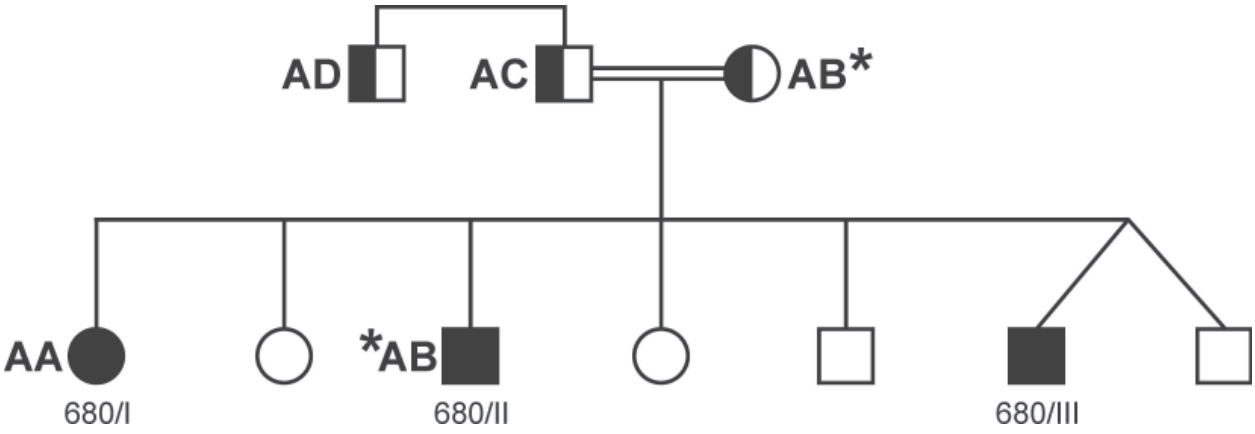


Figure 3 Pedigree of FA-E family 6, a consanguineous family of Arabian origin. The parents of our proband (patient 6/I) are second cousins and have seven offspring. Three of the children are affected, one female (patient 6/I) and two male (patients 6/II and 6/III). Patient 6/II was diagnosed at age 1.3. This patient underwent bone marrow transplantation at age 2½ prior to the occurrence of bone marrow failure. The mother served as an 8/8 matched donor. HLA alleles are given next to the pedigree symbols. Allele A must be coupled with the mutation, since it is common to all carriers and affected individuals. Patient 6/II is a likely recombinant, since this patient has the opposite maternal HLA alleles but is homozygous for the *FANCE* mutation. Black asterisk indicates the recombination event.

case, the mother of the patient served as an 8/8 matched donor for her son. At the last follow-up, this boy survived at 5 years of age (figure 3).

Mutations and polymorphisms in *FANCE*

Table 1 lists genetic alterations and their predicted consequences at the protein level, whereas mutations in combination with other diagnostic data are presented in table S5.

Two nonsense mutations, c.265 C>T (p.R89X) and c.355 C>T (p.Q119X), were homozygous, the latter was also reported by de Winter et al. (de Winter et al., 2000a).

One patient (patient 13) showed the duplication c.1418dupG. At the protein level, this leads to a premature stop codon six amino acids downstream (p.M473NfsX6). This patient is the only compound heterozygote in our cohort (the corresponding second mutation being c.1111 C>T, see below).

Three splice site mutations included c.248+1 G>A (IVS1+1 G>A). Sequencing of cDNA from the respective LCLs revealed that splice site disruption results in the activation of two cryptic splice sites, located at positions IVS1+30 and IVS1+204. Usage of these two cryptic splice donor sites instead of the authentic GT leads to read-through of the open reading frame into the following intron, resulting in both cases in the same premature stop codon (p.K84X). The second splice site mutation was c.1114-8 G>A (IVS5-8 G>A). This particular change does not disrupt a canonical but generates a new splice site, with G>A at position -8 causing a GG to AG change at positions -7 and -8. The newly created AG is used as splice acceptor which leads to the inclusion of six nucleotides into the open reading frame (c.1113insCTGTAG) and results in a premature stop (p.R371insLX). The third splice mutation was c.1510-1 G>A (IVS9-1 G>A). In this case, the splice acceptor of exon 10 is disrupted (AG to AA). Splicing from exon 9 to 10 was defective. Downstream of exon 9, there were at least three different overlaying sequences suggesting the usage of multiple aberrant splice acceptors.

There was only a single type of missense substitution (c.1111 C>T (R371W)). While all other mutations were private, c.1111 C>T was found in eight of the 15 patients representing seven of the 12 families (see tables 1 and S5). With the exception of the patient 13, all c.1111C>T substitutions were homozygous. The only compound heterozygous patient (patient 13) carried the maternal mutation c.1418dupG (p.M473NfsX6) in addition to the paternal c.1111 C>T. Altogether, the observed spectrum of *FANCE* mutations appears relatively limited (figure 4).

Table 1. Identified *FANCE* mutations and their effects

Exon/ Intron no.	Patient no.	Mutation		Consequence protein
		DNA	RNA	
IVS 1	9	c.248 +1 G>A (IVS1 +1 G>A)	- r.248_249ins30 - r.248_249ins204	- p.K84X - p.K84X
Exon 2	1	c.265 C>T	r.265 C>T	p.R89X
	2	c.355 C>T	r.355 C>T	p.Q119X
Exon 5	3, 4, 5, 11-15 (13 het.)	c.1111 C>T	r.1111 C>T	p.R371W
IVS 5	6-8	c.1114 -8 G>A (IVS5 -8 G>A)	r.1113_1114 ins6	p.R371_I372 insLX
Exon 9	13 (het.)	c.1418 dupG	r.1418 dupG	p.M473NfsX6
IVS 9	10	c.1510 -1 G>A (IVS9 -1 G>A)	multiple aberrant splicing	presumably premature termination

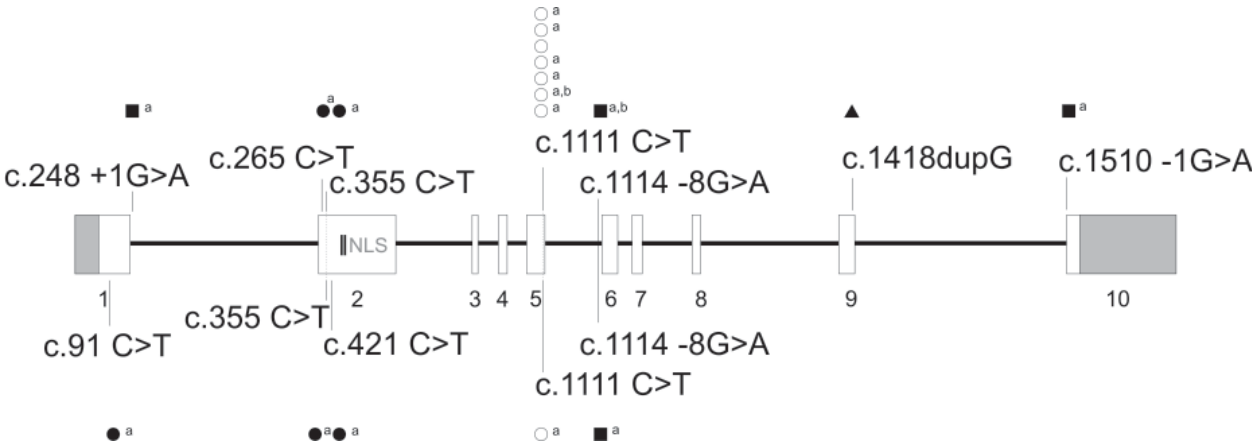


Figure 4 Schematic depiction of the *FANCE* gene and corresponding mutations. Alterations identified in this study are shown above, while mutations described by de Winter et al (2000) and Ameziane et al (2007) are shown underneath the gene. Nonsense mutations are indicated by black circles (●), missense substitutions by open circles (○). Black squares (■) show splice site mutations. A black triangle (▲) indicates duplication. Superscript a denotes homozygous mutations (2 alleles) and superscript b denotes siblings.

Homozygous mutations in 14 of the 15 patients suggest a high degree of consanguinity. Such evidence existed in nine of our families (see table S6). The two families with the nonsense mutations c.265 C>T (p.R89X) and c.355 C>T (p.Q119X) are consanguineous families of Turkish origin (patients 1 and 2). The parents of patients 6, 7 and 8 (mutation c.1114-8 G>A) are second cousins of Arabian ancestry. Patients 4 and 5 (siblings), 11, 12, 14 and 15 all carry the homozygous mutation c.1111 C>T. These patients are from consanguineous families with Mexican, Brazilian, Spanish, German and Dutch ancestry. Patient 10 (homozygous mutation c.1510-1 G>A) is from a German family with confirmed consanguinity. Of the remaining two patients with homozygous mutations, the family of patient 3 (mutation c.1111 C>T) comes from Dagestan, with insufficient pedigree data. Patient 9 (c.248+1 G>A) is from Guatemala without known consanguinity.

Detected polymorphisms included rs2395626 (IVS1-770 T/G), rs4713866 (ATG–56 C/T), rs9470029 (IVS1+58 A/C), rs7757405 (IVS1-35 A/G), rs17859434 (c.387 A/C (P129P)), rs4713868 (IVS2+138 A/G), and rs13214239 (IVS3+39 A/G). Not previously reported polymorphisms included IVS2+58 A/G, IVS5+23 insC, and c.1071 C>T (p.L357L).

Haplotype analysis

In order to test the genetic background of the single missense mutation c.1111 C>T, we performed haplotype analysis of all seven probands carrying this mutation, including the parents of patient 13 (being heterozygous for this mutation). An LD Plot (see materials and methods and figure 5A) was used to analyze the tagSNPs of block 4 containing *FANCE* (Barrett et al., 2005), including rs2894401, rs4713864, rs7758978, rs2395626, and rs7757405 (figure 5B). We observed three different haplotypes (figure 5C). The first consists of the tagSNPs G-A-G-T-G and occurs in 53.3% of the 300 normal individuals used for haplotype construction. This haplotype was present in patients 3, 4, 12, 13 and 15. The second haplotype consists of A-C-C-G-A and was present in 24.9% of the control population. This haplotype was detected only in patient 14. The third haplotype consists of the tagSNPs G-A-C-G-G, occurring in 6.6% of the 300 normal individuals tested, and was present in our patients 3 and 11. Six of the seven patients were homozygous for one of the respective haplotypes, including the single patient from presumably non-consanguineous parents (patient 13). The parents of this patient were also homozygous for this haplotype (data not shown). Only a single patient (patient 3) was heterozygous for two of the prevalent haplotypes (G-A-G-T-G and G-A-C-G-G), suggesting lack of consanguinity in this family despite homozygous mutation of the patient.

Investigation of additional missense substitutions

The Fanconi Anemia Mutation Database (http://www.rockefeller.edu/fanconi/mutate/) reveals three missense substitutions additional to R371W, which have all been identified in breast cancer patients. These alterations are S356G, R365K, and A502T. One further missense substitution, P184Q, was recently reported as a *FANCE* mutation (Ameziane et al., 2007). In order to test the potential pathogenic nature of these alterations, we expressed these and additional missense substitutions in the *FANCE*-defective FA-E cell line EUFA622 (de Winter et al., 2000a) and in an LCL from patient 2 of the present study with two nonsense mutations. As shown in figure 6, only the c.1111 C>T (R371W) substitution was not capable of correcting MMC sensitivity. Cells transduced with this site-mutated vector showed a pronounced G2 phase arrest in response to DNA damage (figure 6).

Employing retroviral complementation, we also tested three base substitutions involving sites critical for potential phosphorylation events. Two of them, T346A and S374A, have been reported as highly conserved phosphorylation sites (Wang et al., 2007). The third, S486A, involves a residue that was speculated to be a good candidate for phosphorylation due to proximity to the putative FANCD2 binding site (Luca Pellegrini, personal communication, 2007). S486T was tested as a control for S486A, assuming that a serine to threonine substitution would retain whereas a change to alanine would abolish phosphorylation. Our transfection experiments yielded complementation

by all of these substitutions, indicating their innocuous nature with respect to MMC sensitivity in the *FANCE* defective cell line (figure 6).

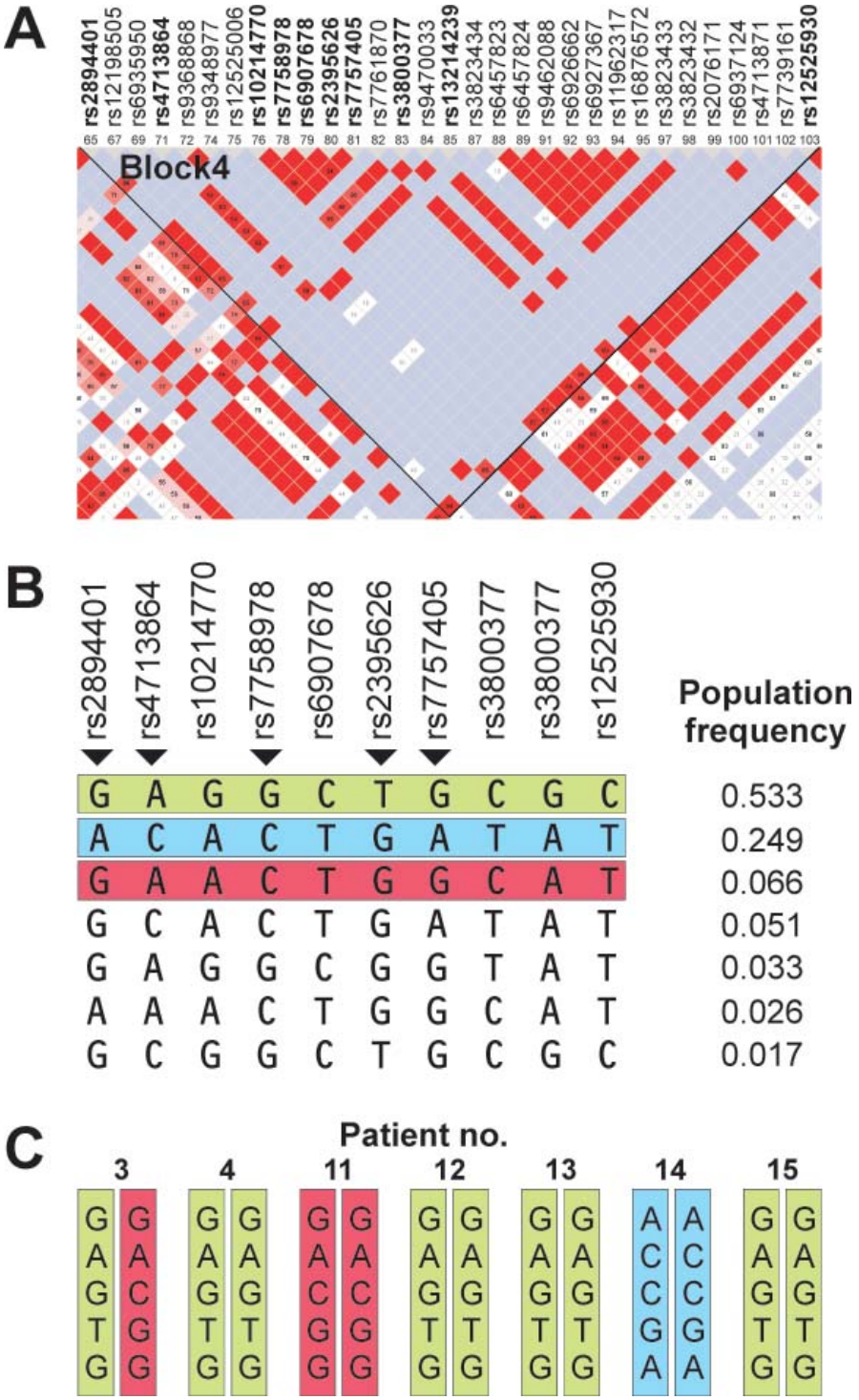


Figure 5 Haplotype analysis of all *FANCE* probands carrying the c.1111 C>T mutation. A) Using Haploview 4.0, an LD Plot of a 100 kb region containing *FANCE* +/- 50 kb was generated. Data from 300 individuals analyzed with a 550K Illumina-Chip were used as the source. SNPs in *FANCE* are located in block 4. B) Five tagSNPs in block 4 are highlighted by black triangles (▼). tagSNPs are rs2894401 (65), rs4713864 (71), rs7758978 (78), rs2395626 (80), and rs7757405 (81). Seven haplotypes for block 4 were detected in the cohort of 300 analyzed individuals, with a population frequency ranging from 1.7 to 53.3%. C) TagSNPs from block 4 were used to construct haplotypes of individuals carrying the c.1111 C>T mutation. Three different haplotypes were connected with the c.1111 C>T change, all of them previously detected in the cohort of 300 normal controls. Patient 3 was heterozygous, while all other patients were homozygous.

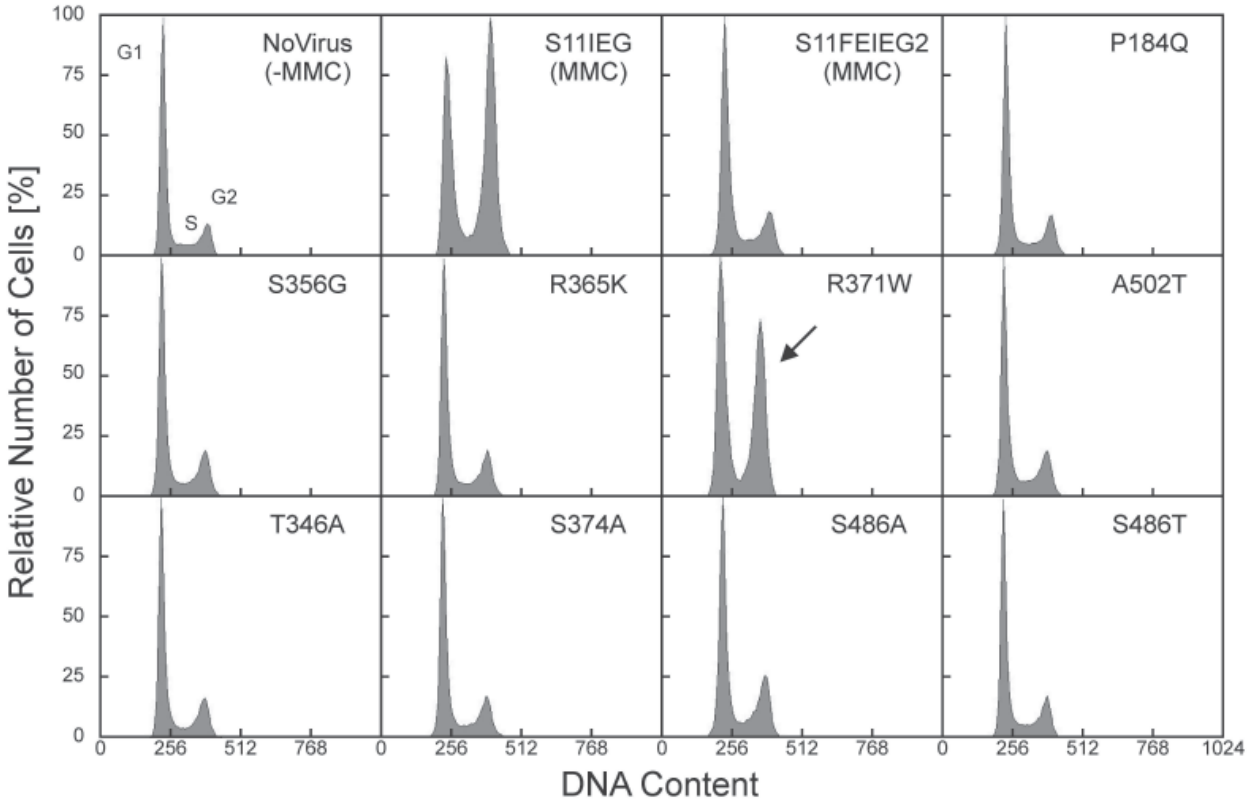


Figure 6 Cell cycle distributions of *FANCE*-defective LCL of patient 2 transduced with the site-mutated vectors S11FEIEG2 expressing various missense substitutions. Non-transduced cells or cells transduced with the empty vector show accumulation of cells in G2 after exposure to MMC (10.8 vs. 49.5%). G2 phase accumulation after exposure to MMC is reduced to near normal by transduction with the *FANCE* wildtype vector S11FEIEG2 (14.5%). Transduction with site-mutated S11FEIEG2 vectors complemented the cellular defect in all but a single case. Only the base substitution c.1111 C>T failed to restore MMC resistance (40.1%).

Discussion

It has been estimated that worldwide less than 2% of FA patients belong to subtype FA-E. From a clinical point of view, the FA-E patients in the present study show a similar spectrum of developmental defects as other FA patients carrying biallelic mutations in any of the core complex genes. There were no exceptional clinical features, and there was no evidence for clusters of neoplasia in their families. Growth retardation was present in 79%, and radial ray defects in 71% of our patients. Microcephaly, microphtalmia, heart malformations and café-au-laits spots were detected in 43% of our patients. Malformations or absence of the kidney were noted in 36% of our FA-E patients. 14% had anal atresia and single patients suffered from esophageal atresia, skeletal malformations other than radial ray defects, abnormal genitalia, microtia, and brain malformations, but none of the patients displayed a complete VACTERL phenotype. Only one of the 13 patients with fully informative clinical data presented without any malformation. Hematological problems were reported in 62% of our patients with onset between 2.5 to 10.5 years. Two patients had undergone bone marrow transplantation (see below). The median age of diagnosis in the 14 FA-E patients was 2.8 years of age. Presently, five of the 13 fully informative FA-E patients succumbed to their disease at a median age of death of six years. Statistical comparisons to patients of other subgroups were not significant due to the still small size of our cohort.

A widespread belief is that preimplantation diagnosis for fully HLA-matching sibling BM donors to FA-E patients is useless due to close linkage of *FANCE* and the HLA locus. *FANCE* is located on chromosome 6p at position 35528116 to 35542859 in close vicinity to the HLA loci which span about 4Mb from *HLA-F* (29799220-29802262) to *HLA-DPB2* (33188271 to 33204868). 8/8 HLA match between patient 7 and his mother indicated that there must have been a recombination event in the ~2Mb region between the HLA loci and *FANCE*. Recombination events in the HLA locus are thought to occur with frequencies of around 0.1 to 1%. This range is due to differences in the recombination rates at different positions of the HLA loci. For HLA class II (which is the HLA class closest to *FANCE*) there are three regions where recombination events are frequent. One is between *HLA-DNA* and *BRD2* (formerly *RING3*), another between *DQB3* and *DQB1* and a third one in an 8.8 kb fragment in *TAP2* (Cullen et al., 1997). Recombination hotspots have been described involving *HLA-DMB*, *BRD2* and *HLA-DOA*, *HLA-DOA* and *HLA-DPA1*, and *HLA-DPB1* and *HLA-DPB2* (Miretti et al., 2005). Although recombinations between *FANCE* and the HLA locus might be rare, the present example shows that they are nevertheless existing. Thus, the search of healthy sibling BM donors has to be considered in FA-E patients.

c.1111 C>T (R371W) occurred in more than 50% of our FA-E patients. Haplotype analysis yielded three different haplotypes among seven patients and two parents. Most of the patients and the parents were homozygous for one of the haplotypes, and only one patient presented with two different haplotypes. Interestingly, the patient with the two different haplotypes was one of the patients for whom consanguinity was unclear. The haplotyping results would argue against consanguinity in the family of this patient. In another non-consanguineous family the patient himself but also his parents are all homozygous for the same haplotype. This might be explained by the fact that this family belongs to the most common haplotype among the tested SNPs, with 53.3% prevalence in a cohort of 300 individuals. The presence of three different haplotypes with occurrence of one and the same mutation among diverse ethnicities suggests that the missense mutation c.1111 C>T is a recurrent rather than a founder mutation. A more remote assumption would be an ancient mutation with evolution of the local haplotype. Recurrent mutations have been described in other FA genes, a prominent example being the R798X change in the *FANCJ* gene. This alteration was detected in 9 of 11 patients with partially different haplotypes (Levran et al., 2005).

Three further missense substitutions (S356G, R365K and A502T) in the Fanconi Anemia Mutation Database have never been mentioned in connection with FA but represent heterozygous substitutions found in breast cancer patients (Seal et al., 2003). As pointed out by Nookala et al, two of these alterations (S356G and R365K) disrupt hydrogen bond interactions between the side chain of the affected amino acids and neighboring main chain atoms that are important for maintaining the correct conformation of the polypeptide chain. We expressed all four missense substitutions (S356G, R365K, R371W, A502T) and in addition one with unknown pathogenic status (R184Q) (Ameziane et al., 2007) and assayed them of MMC sensitivity in *FANCE*-deficient LCLs. R371W proved the only one with pathogenic status. All other substitutions resulted in complementation, indicating their innocuous nature with respect to protein function. A similar result was obtained with four additional base substitutions, T346A, S374A, S486A and S486T. These alterations of *FANCE* are thought to represent evolutionary conserved phosphorylation sites, whose disruption might result in loss of FANCE functionality (Wang et al., 2007; Pellegrini, personal communication, 2007). However, even these base substitutions were tolerated by *FANCE* and MMC sensitivity of the *FANCE*-negative cell lines was corrected.

As a result of our complementation assay, c.1111 C>T stands out as the only missense change in *FANCE*, that appears to affect protein function, at least with respect to MMC-sensitive cellular phenotype. As described by Nookala and colleagues (Nookala et al., 2007), arg371 is located in one of the FANC repeats (repeat 1) in the C-terminal region of FANCE. The substitution of this arginine with trypthophane results in the loss of several structural hydrogen bonds. This leads

to destabilization of the ternary structure of the FANCE protein, thus explaining its pathogenic nature. Nothing was known about the nature of P184Q, since molecular modelling did not extend to the N-terminal region of FANCE. Or results exclude P184Q as an authentic mutation. The missense substitutions S356G and R365K might have been disrupting mutations, since they abrogate hydrogen bonds between the side chain of the affected amino acids and the neighboring main chain chromosomes (Nookala et al., 2007). For A502T, no such effect was postulated. We have seen no detrimental effect for none of these three mutations. Among the phosphorylation sites, T346 and S374 are phosphorylated by Chk1 (Wang et al., 2007). In their report, Wang and colleagues have already shown that single mutants such as T346A and S374A do not abolish FANCE function, whereas the double mutant T346A/S374A was not able to correct MMC sensitivity, although FANCD2 monoubiquitination and foci formation were intact in the double mutant (Wang et al., 2007). In accordance with the data by Wang et al, non-phosphorable substitutions of none of these single phosphorylation sites had a negative effect on FANCE function. S486 was predicted to be a good candidate for site-directed mutagenesis, since this residue is highly conserved and in close proximity to the site that is predicted to bind FANCD2. However, substitutions at this potentially important site to either alanine or to threonine did not re-emerge MMC sensitivity.

The 15 patients of our study have either nonsense mutations or mutations leading to frameshifts and premature protein truncation. The only missense mutation observed is known to destroy a helical motif, thereby leading to destabilization of the ternary structure of the FANCE protein. As a conclusion from our data, we find that only disruptive mutations affect FANCE function. Vice versa, many missense substitutions in the *FANCE* gene are permissive and do not present as mutations. The exclusive presence of disruptive *FANCE* mutations may also explain induced chromosome breakage rates of FA-E patients above the mean of FA patients of other groups, and the absence of residual FANCE protein from immunoblots. Tolerance of amino acid substitutions might explain the low number of FA-E patients, since only functionally “strong” mutations of *FANCE* might lead to a recognizable FA phenotype. Very similar observations exist for the *FANCF* gene, which appears to lack clinically relevant missense mutations (de Winter et al., 2000b; Ameziane et al., 2007). Permutions of different amino acids at different sites fail to affect FANCF function (de Winter et al., 2000b; Kowal et al., 2007). FANCF has been described as a flexible adaptor protein that interacts through its C-terminus with FANCG, while its N-terminus stabilizes the heterodimer FANCA/FANCG and is essential for binding of FANCC/FANCE (Leveille et al., 2004). FANCE has been reported to bind the substrate of the FA core complex to the ubiquitin E3 ligase via an interaction of its N-terminus with FANCC and its C-terminus with FANCD2 (Nookala et al., 2007). There are also remarkable similarities in the structures of FANCE and FANCF: both exhibit repeats domains similar to HEAT or ARM repeats in their C-terminal regions. These domains have been appropriately named “FANC” repeats in the paper by Nookala and colleagues (Kowal et al., 2007) (Nookala et al., 2007). As a conclusion, the structural and functional similarities between FANCE and FANCF suggest that FANCE may also serve as a “flexible adaptor protein”.

Acknowledgements

We thank Richard Friedl and Birgit Gottwald for excellent technical assistance, Dr. Ivonne Powell from the EMBLEM Technology Transfer GmbH, (Heidelberg, Germany) for kindly providing the pETM30 plasmid, Dr. Thomas Bettecken (MPI für Psychiatrie, Munich) for help with haplotyping, Dr. K.J. Patel for kindly providing FANCE antibody, Drs. Ángeles Dasí and Javier Molina for providing clinical data of the patients, Dr. Jürgen Enczmann for informations concerning recombination rates and Dr. Luca Pellegrini for structural considerations. We thank Ralf Dietrich (Unna) for facilitating personal contact with FA-affected families. We also thank the patients, their families and their medical doctors for providing clinical information.

References

Ameziane, N., Errami, A., Leveille, F., Fontaine, C., de Vries, Y., van Spaendonk, R.M., de Winter, J.P., Pals, G. and Joenje, H. (2007) Genetic subtyping of Fanconi anemia by comprehensive mutation screening. Hum Mutat.

Auerbach, A.D., Rogatko, A. and Schroeder-Kurth, T.M. (1989) International Fanconi Anemia Registry: relation of clinical symptoms to diepoxybutane sensitivity. Blood, 73, 391-396.

Barrett, J.C., Fry, B., Maller, J. and Daly, M.J. (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics, 21, 263-265.

Casado, A.J., Callen, E., Jacome, A., Rio, P., Castella, M., Lobitz, S., Ferro, T., Munoz, A., Sevilla, J., Cantalejo, A., Cela, E., Cervera, J., Sanchez-Calero, J., Badell, I., Estella, J., Dasi, A., Olive, T., Jose Ortega, J., Rodriguez-Villa, A., Tapia, M., Molines, A., Madero, L., Segovia, J.C., Neveling, K., Kalb, R., Schindler, D., Hanenberg, H., Surralles, J. and Bueren, J.A. (2007) A comprehensive strategy for the subtyping of patients with Fanconi anaemia: conclusions from the Spanish Fanconi Anemia Research Network. J Med Genet, 44, 241-249.

Chandra, S., Levran, O., Jurickova, I., Maas, C., Kapur, R., Schindler, D., Henry, R., Milton, K., Batish, S.D., Cancelas, J.A., Hanenberg, H., Auerbach, A.D. and Williams, D.A. (2005) A rapid method for retrovirus-mediated identification of complementation groups in Fanconi anemia patients. Mol Ther, 12, 976-984.

Cullen, M., Noble, J., Erlich, H., Thorpe, K., Beck, S., Klitz, W., Trowsdale, J., Carrington, M. (1997) Characterisation of recombination in the HLA class II region. Am J Hum Genet, 60, 397-407.

de Winter, J.P., Leveille, F., van Berkel, C.G., Rooimans, M.A., van Der Weel, L., Steltenpool, J., Demuth, I., Morgan, N.V., Alon, N., Bosnoyan-Collins, L., Lightfoot, J., Leegwater, P.A., Waisfisz, Q., Komatsu, K., Arwert, F., Pronk, J.C., Mathew, C.G., Digweed, M., Buchwald, M. and Joenje, H. (2000a) Isolation of a cDNA representing the Fanconi anemia complementation group E gene. Am J Hum Genet, 67, 1306-1308.

de Winter, J.P., Rooimans, M.A., van Der Weel, L., van Berkel, C.G., Alon, N., Bosnoyan-Collins, L., de Groot, J., Zhi, Y., Waisfisz, Q., Pronk, J.C., Arwert, F., Mathew, C.G., Scheper, R.J., Hoatlin, M.E., Buchwald, M. and Joenje, H. (2000b) The Fanconi anaemia gene FANCF encodes a novel protein with homology to ROM. Nat Genet, 24, 15-16.

Dorsman, J.C., Levitus, M., Rockx, D., Rooimans, M.A., Oostra, A.B., Haitjema, A., Bakker, S.T., Steltenpool, J., Schuler, D., Mohan, S., Schindler, D., Arwert, F., Pals, G., Mathew, C.G., Waisfisz, Q., de Winter, J.P. and Joenje, H. (2007) Identification of the Fanconi anemia complementation group I gene, FANCI. Cell Oncol, 29, 211-218.

Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M. and D'Andrea, A.D. (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell, 7, 249-262.

Gordon, S.M., Alon, N. and Buchwald, M. (2005) FANCC, FANCE, and FANCD2 form a ternary complex essential to the integrity of the Fanconi anemia DNA damage response pathway. J Biol Chem, 280, 36118-36125.

Joenje H. (1997) Fanconi anemia: cytogenetic diagnosis. Free University of Amsterdam Amsterdam.

Hanenberg, H., Batish, S.D., Pollok, K.E., Vieten, L., Verlander, P.C., Leurs, C., Cooper, R.J., Gottsche, K., Haneline, L., Clapp, D.W., Lobitz, S., Williams, D.A. and Auerbach, A.D. (2002) Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol, 30, 410-420.

Hildinger, M., Abel, K.L., Ostertag, W. and Baum, C. (1999) Design of 5' untranslated sequences in retroviral vectors developed for medical use. J Virol, 73, 4083-4089.

Huck, K., Hanenberg, H., Gudowius, S., Fenk, R., Kalb, R., Neveling, K., Betz, B., Niederacher, D., Haas, R., Gobel, U., Kobbe, G. and Schindler, D. (2006) Delayed diagnosis and complications of Fanconi anaemia at advanced age--a paradigm. Br J Haematol, 133, 188-197.

Hussain, S., Wilson, J.B., Medhurst, A.L., Hejna, J., Witt, E., Ananth, S., Davies, A., Masson, J.Y., Moses, R., West, S.C., de Winter, J.P., Ashworth, A., Jones, N.J. and Mathew, C.G. (2004) Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways. Hum Mol Genet, 13, 1241-1248.

Joenje, H., Lo ten Foe, J.R., Oostra, A.B., van Berkel, C.G., Rooimans, M.A., Schroeder-Kurth, T., Wegner, R.D., Gille, J.J., Buchwald, M. and Arwert, F. (1995) Classification of Fanconi anemia patients by complementation analysis: evidence for a fifth genetic subtype. Blood, 86, 2156-2160.

Kalb, R., Neveling, K., Hoehn, H., Schneider, H., Linka, Y., Batish, S.D., Hunt, C., Berwick, M., Callen, E., Surralles, J., Casado, J.A., Bueren, J., Dasi, A., Soulier, J., Gluckman, E., Zwaan, C.M., van Spaendonk, R., Pals, G., de Winter, J.P., Joenje, H., Grompe, M., Auerbach, A.D., Hanenberg, H. and Schindler, D. (2007) Hypomorphic mutations in the gene encoding a key Fanconi anemia protein, FANCD2, sustain a significant group of FA-D2 patients with severe phenotype. Am J Hum Genet, 80, 895-910.

Kennedy, R.D. and D'Andrea, A.D. (2005) The

Fanconi Anemia/BRCA pathway: new faces in the crowd. Genes Dev, 19, 2925-2940.

Kowal, P., Gurtan, A.M., Stuckert, P., D'Andrea, A.D. and Ellenberger, T. (2007) Structural determinants of human FANCF protein that function in the assembly of a DNA damage signaling complex. J Biol Chem, 282, 2047-2055.

Leveille, F., Blom, E., Medhurst, A.L., Bier, P., Laghmani el, H., Johnson, M., Rooimans, M.A., Sobeck, A., Waisfisz, Q., Arwert, F., Patel, K.J., Hoatlin, M.E., Joenje, H. and de Winter, J.P. (2004) The Fanconi anemia gene product FANCF is a flexible adaptor protein. J Biol Chem, 279, 39421-39430.

Leveille, F., Ferrer, M., Medhurst, A.L., Laghmani el, H., Rooimans, M.A., Bier, P., Steltenpool, J., Titus, T.A., Postlethwait, J.H., Hoatlin, M.E., Joenje, H. and de Winter, J.P. (2006) The nuclear accumulation of the Fanconi anemia protein FANCE depends on FANCC. DNA Repair (Amst), 5, 556-565.

Levran, O., Attwooll, C., Henry, R.T., Milton, K.L., Neveling, K., Rio, P., Batish, S.D., Kalb, R., Velleuer, E., Barral, S., Ott, J., Petrini, J., Schindler, D., Hanenberg, H. and Auerbach, A.D. (2005) The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet, 37, 931-933.

Medhurst, A.L., Huber, P.A., Waisfisz, Q., de Winter, J.P. and Mathew, C.G. (2001) Direct interactions of the five known Fanconi anaemia proteins suggest a common functional pathway. Hum Mol Genet, 10, 423-429.

Meetei, A.R., Sechi, S., Wallisch, M., Yang, D., Young, M.K., Joenje, H., Hoatlin, M.E. and Wang, W. (2003) A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. Mol Cell Biol, 23, 3417-3426.

Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res, 16, 1215.

Miretti, M.M., Walsh, E.C., Ke, X., Delgado, M., Griffiths, M., Hunt, S., Morrison, J., Whittaker, P., Lander, E.S., Cardon, L.R., Bentley, D.R., Rioux, J.D., Beck, S. and Deloukas, P. (2005) A high-resolution linkage-disequilibrium map of the human major histocompatibility complex and first generation of tag single-nucleotide polymorphisms. Am J Hum Genet, 76, 634-646.

Montes de Oca, R., Andreassen, P.R., Margossian, S.P., Gregory, R.C., Taniguchi, T., Wang, X., Houghtaling, S., Grompe, M. and D'Andrea, A.D. (2005) Regulated interaction of the Fanconi anemia protein, FANCD2, with chromatin. Blood, 105, 1003-1009.

Neitzel, H. (1986) A routine method for the establishment of permanent growing lymphoblastoid cell lines. Hum Genet, 73, 320-326.

Niedernhofer, L.J., Lalai, A.S. and Hoeijmakers, J.H. (2005) Fanconi anemia (cross)linked to DNA repair. Cell, 123, 1191-1198.

Nookala, R.K., Hussain, S. and Pellegrini, L. (2007) Insights into Fanconi Anaemia from the structure of human FANCE. Nucleic Acids Res, 35, 1638-1648.

Pace, P., Johnson, M., Tan, W.M., Mosedale, G., Sng, C., Hoatlin, M., de Winter, J., Joenje, H., Gergely, F. and Patel, K.J. (2002) FANCE: the link between Fanconi anaemia complex assembly and activity. Embo J, 21, 3414-3423.

Schindler D, F.R., Gavvovidis I, Kalb R, Neveling K, et al (2007) Applications of cell cycle testing in Fanconi anemia. In Schindler D, H.H. (ed.), Fanconi Anemia. A paradigmatic disease for the understanding of cancer and aging. Karger, Basel, Vol. 15, pp. 110-130.

Schindler, D. and Hoehn, H. (1988) Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. Am J Hum Genet, 43, 429-435.

Seal, S., Barfoot, R., Jayatilake, H., Smith, P., Renwick, A., Bascombe, L., McGuffog, L., Evans, D.G., Eccles, D., Easton, D.F., Stratton, M.R. and Rahman, N. (2003) Evaluation of Fanconi Anemia genes in familial breast cancer predisposition. Cancer Res, 63, 8596-8599.

Seyschab, H., Friedl, R., Sun, Y., Schindler, D., Hoehn, H., Hentze, S. and Schroeder-Kurth, T. (1995) Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. Blood, 85, 2233-2237.

Sims, A.E., Spiteri, E., Sims, R.J., 3rd, Arita, A.G., Lach, F.P., Landers, T., Wurm, M., Freund, M., Neveling, K., Hanenberg, H., Auerbach, A.D. and Huang, T.T. (2007) FANCI is a second monoubiquitinated member of the Fanconi anemia pathway. Nat Struct Mol Biol, 14, 564-567.

Smogorzewska, A., Matsuoka, S., Vinciguerra, P., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Ballif, B.A., Gygi, S.P., Hofmann, K., D'Andrea, A.D. and Elledge, S.J. (2007) Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. Cell, 129, 289-301.

Taniguchi, T. and D'Andrea, A.D. (2002) The Fanconi anemia protein, FANCE, promotes the nuclear accumulation of FANCC. Blood, 100, 2457-2462.

Taniguchi, T., Garcia-Higuera, I., Andreassen, P.R., Gregory, R.C., Grompe, M. and D'Andrea, A.D. (2002) S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. Blood, 100, 2414-2420.

Waisfisz, Q., Saar, K., Morgan, N.V., Altay, C., Leegwater, P.A., de Winter, J.P., Komatsu, K., Evans, G.R., Wegner, R.D., Reis, A., Joenje, H., Arwert, F., Mathew, C.G., Pronk, J.C. and Digweed, M. (1999) The Fanconi anemia group

E gene, FANCE, maps to chromosome 6p. Am J Hum Genet, 64, 1400-1405.

Wang, W. (2007) Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nat Rev Genet, 8, 735-748.

Wang, X., Andreassen, P.R. and D'Andrea, A.D. (2004) Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. Mol Cell Biol, 24, 5850-5862.

Wang, X. and D'Andrea, A.D. (2004) The interplay of Fanconi anemia proteins in the DNA damage response. DNA Repair (Amst), 3, 1063-1069.

Wang, X., Kennedy, R.D., Ray, K., Stuckert, P., Ellenberger, T. and D'Andrea, A.D. (2007) Chk1-mediated phosphorylation of FANCE is required for the Fanconi anemia/BRCA pathway. Mol Cell Biol, 27, 3098-3108.

Wegner, R.D., Henrichs, I., Joenje, H. and Schroeder-Kurth, T. (1996) Fanconi anemia complementation group E: clinical and cytogenetic data of the first patient. Clin Genet, 50, 479-482.

Supplementary Tables

Table S1A. *FANCE* gDNA amplification and sequencing primers

Exon	Designation	Sequence (5' → 3')	Designation	Sequence (5' → 3')
1	gFA-E Ex1 for	AACGCCGAGGAGAGCTTGTAAC	gFA-E Ex1 rev	ACAGCGAACAGAAGGCGTGTG
2	gFA-E Ex2 for	CCAACATCCACTGACTCTTGCAG	gFA-E Ex2 rev	AAGAGTCTGTAGAGTGGAGGCTACA
3-5	gFA-E Ex3-5 for	CTTGGCCTCTTGACTTTCTTGAATC	gFA-E Ex3-5 rev	CTCTCAAGTACCACCCTTCTGAG
6-7	gFA-E Ex6-7 for	GGATTTGGGAACTGAGCAAGAGAG	gFA-E Ex6-7 rev	CAACACATGTGATGTCACTCTAGC
8	gFA-E Ex8 for	GGAGTTGGAGCAGCAGATAGATAC	gFA-E Ex8 rev	TGAAGTGACCATGTGCTCAGCTCT
9	gFA-E Ex9 for	GGAGGTTTAGGTTGGTTAAGTTACC	gFA-E Ex9 rev	CTGACATACATATTCATACACCCTTGTG
10	gFA-E Ex10 for	TGAGATTAGATTGCTCAGGAGTCTC	gFA-E Ex10 rev	GGAAGCCAATATTCCCTATGCAGC

Table S1B. *FANCE* gDNA sequencing primers

Exon	Designation	Sequence (5' → 3')	Exon		Sequence (5' → 3')
1	Ex1.1 for seq	GTCTGGAGCTGTAAGTCCTCG	4	Ex4 for seq	TTGAACCAAGTGTAGACTTACCATC
	Ex1.2 rev seq	CACCTCTGTGTGTGTCGTTC		Ex4 rev seq	AAGATAAGGGACAGAGAATGTAACC
2	Ex2.1 for seq	GAATTGCTGCGAAGGGATTTGG	5	Ex5 for seq	CAGCTCTCCCAGACTTTCTTTG
	Ex2.2 for seq	GAAGATCATGAGAAGGAGAGACC	6	Ex 6 rev seq	CACTGAATACCCAACTCCTGC
3	Ex3 rev seq	CAGCCAGCTTGTATATCACTGCA	7	Ex7 for seq	CAGTGATTAAAGATGCCTGCTGC

Table S2A. *FANCE* cDNA amplification and sequencing primers

Frg.	Designation	Sequence (5' → 3')	Designation	Sequence (5' → 3')
1	c.Frg1 for	AACGCCGAGGAGAGCTTGTAAC	c.Frg1 rev	GCATCCTCCAATCCCTCTAACC
2	c.Frg2 for	CTGGATGATGCTAAAGGTCTGGC	c.Frg2 rev	CTTTATCCTCAGGCTAGGGCTC

Table S2B. *FANCE* cDNA sequencing primers

Frg.	Designation	Sequence (5' → 3')	Frg.	Designation	Sequence (5' → 3')
1	Ex1.1 for seq	CTGAAACCACTGTTGCTGCGATTG	2	Ex2.1 for seq	GCTGACCAGAAGCCTCTTTCTTG
	Ex1.2 rev seq	GAAAGGACTCAGAGGAAGAGGCT		Ex2.2 for seq	GGTGTTGCAGTCACTCCTAGAG
	c.FA-E351rev	CACAGAGAGGAGCCCACTTTC		c.FA-E1273rev	TCACAAGGCAACACAGTAACTCTG
	c.FA-E376rev	CTAGGTCCTGCTGGGCAATCT		c.FA-E1377rev	CTCTAGGAGTGACTGCAACACC
	c.FA-E498rev	ACTTTGGAGCTGTCTCTGGCATC			

Table S3. Primers for site-directed mutagenesis

Mutation aa	Mutation nt	for- Sequence (5' → 3')	rev-Sequence (5' → 3')
P184Q	c.551 C>A	GTTGAAATCCCCCCAGGCTCAAGACCCTGAAGAAGAGGAGA	TCTCCTCTTCTTCAGGGTCTTGAGCCTGGGGGGATTTCAAC
T346A	c.1036A>G	TCGGTCTCCTGCGGCTCTGCGCCTGGCTGCTGGCCCTTTCA	TGAAAGGGCCAGCAGCCAGGCGCAGAGCCGCAGGAGACCGA
S356G	c.1066A>G	CCCTTTCACCTGATCTCGGCCTCAGCAATGCTACT	AGTAGCATTGCTGAGGCCGAGATCAGGTGAAAGGG
R365K	c.1094 G>A	CAATGCTACTGTGCTGACCAAAAGCCTCTTTCTTGGACGGA	TCCGTCCAAGAAAGAGGCTTTTGGTCAGCACAGTAGCATTG
R371W	c.1111 C>T	CCAGAAGCCTCTTTCTTGGATGGATCCTCTCCTTGACTTCC	GGAAGTCAAGGAGAGGATCCATCCAAGAAAGAGGCTTCTGG
S374A	c.1120 T>G	TCTTTCTTGGACGGATCCTCGCCTTGACTTCCTCAGCCTCC	GGAGGCTGAGGAAGTCAAGGCGAGGATCCGTCCAAGAAAGA
S486A	c.1456 T>G	AGGGGCTGGCAGCCACCACCGCCATGGCCTATGCCAAGCTC	GAGCTTGGCATAGGCCATGGCGGTGGTGGCTGCCAGCCCCT
S486T	c.1456 T>A	AGGGGCTGGCAGCCACCACCACCATGGCCTATGCCAAGCTC	GAGCTTGGCATAGGCCATGGTGGTGGTGGCTGCCAGCCCCT
A502T	c.1504G>A	CAGTGATGACCAAGTATCAGACTAACATCACTGAGACCCAG	CTGGGTCTCAGTGATGTTAGTCTGATACTTGGTCATCACTG

Table S4A. Primers for amplification of SNP-regions

SNP	for- Sequence (5' → 3')	rev-Sequence (5' → 3')
rs2395626	GTAGGATCTGGGTTTGTGCCTCA	GCACGCAGTATCTGCAAGAGTC
rs7757405	CCAACATCCACTGACTCTTGCAG	AAGAGTCTGTAGAGTGGAGGCTACA
rs2894401	AGTTGCACAGGGTTGGCTCTTGTC	GCTAGTGAAGATGCCAGATTGTC
rs4713864	GAACCCTTTCGGACAACATAAAGTC	CTCCATCTACACCATCTCCTATCAC
rs7758978	GAAGGTTCAAACCCTCTTGCCAG	TTTGGGTCTTCGAATGACAAGCTG

Table S4B. Primers for SNP sequencing

SNP	for- Sequence (5' → 3')	rev-Sequence (5' → 3')
rs2395626	CAAGTTTCATCTCTGGGAAGCCTT	AATACTCCAGGATGCTGTGCAG
rs7757405	CCAACATCCACTGACTCTTGCAG	--
rs2894401	CTGGAGTGCAATGGCATAATCTC	GAAGATGCCAGATTGTCAGTCACAT
rs4713864	GTATTCTTACAATAAAGTGAGCTAGAG	GCCAGTACAGTTGCTGTTGAAC
rs7758978	GCACCTAAGTTATCATTGCTGGTG	GTTCTAAACATGGCTCAAGGACC

Table S5. Laboratory diagnostic data of the 15 FA-E patients

Patient number	Kindred/ sibling	Cell type of lab diagnosis	G2-Phase arrest, G2/GF		Breaks/cell		Technique of complementation group assignment	*FANCE* mutation	
			Spon	MMC/DEB	Spon	MMC/DEB		Allele 1	Allele 2
1	1/I	fibroblasts	11.3%	46.1% (MMC)	x	x	RC of fibroblasts	c.265 C>T R89X	c.265 C>T R89X
2	2/I	PBL	39.3%	67.7% (MMC)	x	x	RC of LCL	c.355 C>T Q119X	c.355 C>T Q119X
3	3/I	fibroblasts and PBL	12.7%	62.0% (MMC)	0.26	17,3	RC of fibroblasts	c.1111 C>T R371W	c.1111 C>T R371W
4	4/I	PBL	x	x	0.27	14.0 (DEB)	Sibling	c.1111 C>T R371W	c.1111 C>T R371W
5	4/II	PBL	x	x	0.30	6.2 (DEB)	RC of LCL	c.1111 C>T R371W	c.1111 C>T R371W
6	6/I	PBL	x	x	n.d.	3.8 (DEB)	RC of T-cells	c.1114 -8 G>A (IVS5 -8 G>A)	c.1114 -8 G>A (IVS5 -8 G>A)
7	6/II	PBL	x	x	0.32	8.0 (DEB)	RC of fibroblasts	c.1114 -8 G>A (IVS5 -8 G>A)	c.1114 -8 G>A (IVS5 -8 G>A)
8	6/III	PBL	x	x	1.4	21.7 (DEB)	sibling	c.1114 -8 G>A (IVS5 -8 G>A)	c.1114 -8 G>A (IVS5 -8 G>A)
9	9/I	PBL	x	x	0.16	10.9 (DEB)	RC of LCL	c.248 +1 G>A	c.248 +1 G>A
10	10/I	PBL	x	x	0.24	15.6 (DEB)	RC of LCL	c.1510 -1 G>A (IVS9 -1 G>A)	c.1510 -1 G>A (IVS9 -1 G>A)
11	11/I	PBL	x	x	n.d.	9.2 (DEB)	RC of LCL	c.1111 C>T R371W	c.1111 C>T R371W
12	12/I	PBL	x	x	0.28	8.38 (DEB)	RC of T cells	c.1111 C>T R371W	c.1111 C>T R371W
13	13/I	PBL	x	x	0	6.5 (DEB)	RC of T cells	c.1418dupG M473NfsX6	c.1111 C>T R371W
14	14/I	PBL	x	x	0.17	9.8 (DEB)	Sequencing/cDNA transfection	c.1111 C>T R371W	c.1111 C>T R371W
15	15/I	PBL	-	-	-	-	Sequencing	c.1111 C>T R371W	c.1111 C>T R371W

NOTE.-PBL=peripheral blood lymphocytes; G2=G2 phase fraction of the cell cycle; GF=growth fraction; G2/GF=ratio G2 phase fraction over GF; MMC=mitomycin C; DEB= diepoxybutane; n.d.= not determined; x=not done; -=not available; RC= retroviral complementation; LCL= lymphoblastoid cell line

Table S6. Clinical data of the 15 FA-E patients

Patient number	Kindred/ Sibling	Consanguinity Gender	Ethnicity	Age at diagnosis	Clinical presentation	Hematologic manifestations	Survival at last follow-up	Family history
1	1/I	Yes/ F	Turkish	prenatal	stillborn, brain and heart malformations, agenesis of kidney, radial aplasia	n.d.	pregnancy interrupted	1 SAB, one previous stillborn with FA, no cancer history
2	2/I	Yes/ M	Turkish	1 year	pre- and postnatal growth retardation, microphtalmia, left thumb absent, right thumb hypoplastic, atresia of esophagus	BMF as of age 6 years, until 2007 no clonal aberrations, no therapy by 8 years	8 years	No SABs, no cancer history
3	3/I	Unknown/ F	Dagestan	8 years	growths retardation, café-au-lait spots, bird-like face, microcephaly, small eyes, kidney and heart malformations	BMF as of age 8 years	15 years	No SABs, no cancer history
4	4/I	2nd cousin/ F	Mexican	1.3 years	bilateral hanging thumb, cardiac (PDA &VSD), growth retardation, microcephaly, microphthalmia	None at death	† 4 years	proband
5	4/II	2nd cousin/ F	Mexican	birth	thumb abnormal, café-au-lait spots, growth retardation, cardiac (PDA)	None at LFU	2 years	Affected sibling
6	6/I	2nd cousin/ F	Arabic	6.3 years	café-au-lait spots, growth retardation, microcephalie, thumb and kidney malformations	6.7 years, BMT with unrelated donor at age 8	† 10 years	proband

7	6/II	2nd cousin /M	Arabic	1.3 years	growth retardation, microcephaly	BMT at age 2½ before BMF, with mother as 8/8 matched donor	5 years	2 affected sibs
8	6/III	2nd cousin/ M	Arabic	birth	absent kidney	None at LFU	1 month	2 affected sibs
9	9/I	No known consanguinity /M	Guatemalan	birth	café-au-lait spots, growth retardation, bilateral radial aplasia, abnormal genitalia, microtia, heart malformations	None at death	† 8 month	none
10	10/I	Distant cousins/ M	German	10 years	extra thumb (unilateral), anal atresia	10.5 years	11.5 years	none
11	11/I	First cousins/ F	Brazilian	3 years	bifid thumb, short stature, café-au-lait spots, microphthalmia	3 years	† 9 years	none
12	12/I	Yes/ F	Spanish	Suspected diagnosis of FA at birth due to malformations and low weight. Chromosome breakage study confirmed FA at 3 month of age	growth retardation, microcephaly, microsomy, small eyes, skeletal malformations, radial hypoplasia, hypoplastic thumbs, heart anomalies (ASD and VSD), no skin pigmentation anomalies	Thrombocytopenia at 2.5 years; thrombocytes counts: 50.000/mm3; the other lineages are unaffected; no therapy	3.9 years	MP: lung; maternal grand grand mother: gastric; no abortions
13	13/I	No/ M	Spanish	New born, neonatal thrombocytopenia and anemia. Diagnostic confirmed by chromosome fragility test	both thumbs absent, imperforate anus, left kidney missing, microphthalmia, low birth weight,growth retardation, café-au-lait spots, microcephaly, jaundice at birth.	Thrombocytopenia and anemia (neonatal). Actual blood counts: Hemoglobin= 11 gm/ dl, leukocytes=5000, platelets=150.000. No treatment received yet.	6 years	No, no abortions

14	14/I	Yes/ F	German	6 years	IUGR, no malformations	BMF beginning at 5 years, transfusions, cortisol, androgenes	† 6 years 10 month Infection (pneumonia, sepsis)	maternal great-grandfather (MMP): lung, paternal great-grandmother (PPM): stomach, grandfather (PP): esophagus; 1 SAB
15	15/I	Yes/ ?	Dutch	-	-	-	-	-

NOTE.-M=male; F=female; GR=growth retardation; IUGR=intrauterine growth retardation; BMF=bone marrow failure; BMT=bone-marrow transplantation;LFU=last follow-up; MMP=mother of the paternal grandmother; PPM= father of the maternal grandfather; PP= paternal grandfather; MP=maternal grandfather; SAB= spontaneous abortion; PDA= patent ductus arteriosus; ASD= atrium septum defect; VSD=ventricular septum defect; -= no informations available

2

Results

Mutation and Reversion in *FANCL*, the Gene Encoding the E3 Ligase Catalytic Subunit of the Fanconi Anemia Nuclear Core Complex

Kornelia Neveling*[1], Reinhard Kalb*[1], Paula Rio[2], Sarah Reid[3], Heiner Schaal[4], Francis P. Lach[5], Michael Jansen[6], Nazneen Rahman[3], David A. Williams[6], Arleen D. Auerbach[5], Helmut Hanenberg*[7],[8], Detlev Schindler*[1]§

[1] Department. of Human Genetics, University of Wurzburg, Germany;
[2] Hematopoiesis and Gene Therapy Division, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain;
[3] Section of Cancer Genetics, Institute of Cancer Research, Sutton, Surrey, UK;
[4] Department of Virology, University of Dusseldorf, Germany;
[5] Laboratory of Human Genetics and Hematology, Rockefeller University, New York, NY;
[6] Division of Experimental Hematology, University of Cincinnati College of Medicine, Cincinnati, OH;
[7] Department of Pediatric Oncology, Hematology and Immunology, University of Dusseldorf, Germany;
[8] Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN

* These authors contributed equally to the present work

§ Correspondence: Dr. Detlev Schindler, Dept. of Human Genetics, Biozentrum, Am Hubland, D-97074 Wurzburg, Germany. Phone: +49 931 888 4089; Fax: +49 931 888 4069; Email: schindler@biozentrum.uni-wuerzburg.de

This work was supported, in part, by grants from the Deutsche Fanconi-Anämie-Hilfe (to D.S.), the Aktionskreis Fanconi-Anämie (to D.S.), the Schroeder-Kurth Fund (to R.K.and K.N.) and the Senator Kurt und Inge Schuster Foundation (to R.K.).

to be submitted to: Human Mutation

Abstract

To date, thirteen Fanconi anemia (FA) genes have been identified, whose products interact in the recognition and signalling of DNA damage. Eight of the FA proteins assemble in a nuclear complex, which is necessary for the 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 report three further patients, all assigned to subgroup FA-L via retroviral complementation. Patient 1 presented with a severe phenotype, whereas patients 2 and 3 had mild to moderate, yet typical manifestations of FA. The mutations of patient 1 included heterozygosity for a 5-bp deletion and a missense substitution. Blood cells and lymphoid cell lines lacked the typical hypersensitivity to MMC, were proficient of FANCD2 monoubiquitination, and revealed a heterozygous base substitution at c.472-2A>T (IVS6-2A>T). This resulted in a complex compensatory event at the transcript level, indicating somatic reversion. Retroviral transduction of patient's fibroblasts with the revertant cDNA led to loss of MMC sensitivity, emergence of FANCD2 monoubiquitination and appearance of FANCD2 nuclear foci formation, proving functional restoration of FANCL function by the compensatory mutation. The clinical course of the patient was characterized by increasing cytopenia of all cell lineages except lymphocytes suggesting 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. The mutations of patient 2 and 3 included a homozygous large genomic deletion, a 3-bp deletion and exonization of an intron fragment. Conclusions from this study are that FA-L patients occur at a recognizable rate, have a typical spectrum of manifestations and hematological course comparable with those of other FA patients with defects in the FA nuclear core complex, and reveal the spectrum of mutation and reversion reported for other FA genes.

Keywords: Fanconi anemia; FANCL; retroviral complementation; somatic reversion; revertant mosaicism; compensatory mutation

Introduction

Fanconi anemia (FA) is a recessively inherited multisystem disease characterized by congenital malformations, progressive bone marrow failure 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 the corresponding genes have been identified (Levitus et al. 2004; Levitus et al. 2005; Levran et al. 2005; Meetei et al. 2005; Dorsman et al. 2007; Sims et al. 2007; Smogorzewska et al. 2007; Wang, 2007). The FA gene products cooperate in a DNA caretaker protein network that maintains genomic stability based on the effective resolution of DNA-interstrand crosslinks (Thompson 2005). In response to DNA damage and stalled replication forks, eight of the FA proteins (FANC-A, B, C, E, F, G, L and M) assemble in a nuclear multi-protein complex (FA core complex), whose function is required for monoubiquitination of the key proteins of the pathway, FANCD2 and FANCI (Garcia-Higuera et al. 2001; Meetei et al. 2004; Sims et al. 2007; Smogorzewska et al. 2007, Wang, 2007). Monoubiquitinated FANCD2 is targeted to nuclear foci containing proteins such as BRCA1, the BRCA1-interacting protein 1 BRIP1/FANCJ, BRCA2/FANCD1 and RAD51, which participate in DNA double-strand repair via homologous recombination (Digweed et al. 2002; Niedzwiedz et al. 2004; Godthelp et al. 2005; Nakanishi et al. 2005; Thompson et al. 2005).

The process of monoubiquitination 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) (Sigismund et al. 2004). The presumptive E3 ligase activity for monoubiquitination of FANCD2 and FANCI is provided by FANCL, which

appears to function as the catalytic subunit of the FA core complex (Meetei et al. 2003). Recently, UBE2T was found to be the corresponding E2 ligase (Machida et al. 2006). FANCL contains three WD40 repeats, known as sites of protein-protein interaction, and a C-terminal PHD (plant homeodomain) zinc finger motif (Meetei et al. 2003). Meetei et al. (Meetei et al. 2003) demonstrated that FANCL is able to ubiquitinate itself in vitro and that mutations in *FANCL* result in defective FANCD2 monoubiquitination. Both, *FANCL* and *FANCD2* belong to the small group of evolutionarily conserved FA genes.

To date, only a single patient with a *FANCL* defect has been reported in the literature, whose lymphoblasts (EUFA868) have served for the characterization of *FANCL* as an authentic FA gene (Meetei et al. 2003). The laboratories participating in the present study collaborate in ongoing efforts to assign FA patients to their complementation groups through retroviral gene transfer and DNA sequencing (Hanenberg et al. 2002; Chandra et al. 2005). Among our diagnostic cohort, we identified three additional patients belonging to complementation group FA-L. We here present a brief report of these patients and their mutations. They are the first FA-L patients on whom clinical information is being made available. Their mutations allow for recognition of an initial mutational spectrum of *FANCL* and functional consequences thereof. One of these patients 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 specimens were referred to the participating laboratories for diagnostic testing upon suspicion of FA. Diagnostics, genetic subtyping and mutation analysis were performed with informed consent according to the Declaration of Helsinki. The studies were approved by the IRBs of the participating centers.

The clinical impression 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) or diepoxybutane (DEB) sensitivity assays following published procedures (Auerbach 1993; Joenje, 1997). In the Wurzburg laboratory, peripheral blood mononuclear cells (PBL) 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, Heidelberg, Germany). Cell cycle distributions reflecting DNA content were quantitated using the MPLUS AV software package (Phoenix Flow Systems, San Diego, CA). 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 (Seyschab et al. 1995; Heinrich et al. 1998). Lymphocytes and EBV transformed lymphoblast cell lines (LCLs) were maintained in RPMI 1640 medium containing GlutaMAX (Gibco, Karlsruhe, Germany), supplemented with 16% fetal bovine serum (FBS). Fibroblast cultures were established using standard cell culture procedures and grown in FBS-supplemented Eagle's MEM medium with GlutaMAX (Invitrogen, Karlsruhe, Germany). All cell cultures were kept in high humidity incubators equipped with CO2 and O2 sensors in an atmosphere of 5% (v/v) CO2 and 5% (v/v) O2 by replacing ambient air with nitrogen.

For cell cycle analysis, cells of logarithmical growing cultures were harvested 48 h after treatment with 0 and 15 ng/ml MMC. Prior to flow cytometry, live cells were stained using the DNA-binding dye Hoechst 33342 (Molecular Probes, Eugene, OR) (10 µg/ml, 30 min, 37 °C) or alternatively, nuclei were stained with DAPI as described previously (Schindler and Hoehn, 1999).

Immunoblotting

FANCD2 immunoblotting was performed as described by Garcia-Higuera et al. (Garcia-Higuera et al. 2001) with minor modifications. About 16 h prior to the harvest of cell cultures, 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 2-glycerophosphate; 0,1 mM Na3VO4; 0.3% nonidet P-40, 0.2% Triton X-100, proteinase inhibitors complete, Roche, Mannheim, Germany). 50 µg of whole protein extract were loaded on 7% tris-acetate gels (Invitrogen, Karlsruhe, Germany) to be separated at 120 V for 6 h. Protein transfer was overnight at 4°C and 20 V onto PVDF membranes (HybondP, Amersham Biosciences, Little Chalfont, UK). Immunoblots were blocked with 5% non-fat dry milk (Roth, Karlsruhe, Germany) in PBS/0.05% Tween (PBS-T). As primary antibody, we used the mouse monoclonal FANCD2 antibody FL-17 (Santa Cruz Biotechnology, Heidelberg, Germany) at a dilution of 1:800. The secondary antibody was anti-mouse IgG horseradish peroxidase linked F(ab‘)2 from sheep (Amersham Biosciences, Little Chalfont, UK, dilution 1:2000). For chemiluminescence detection, we used a standard ECL reagent (Amersham Biosciences, Little Chalfont, UK).

Vector construction and assignment to complementation group FA-L

For construction of the FANCL-IRES(internal ribosomal entry site)-EGFP(enhanced green fluorescent protein) retroviral expression vector S11FLIEG, *FANCL* cDNA was amplified using primers with a 5’ *Not*I and a 3’ *Sal*I restriction site. Employing these restriction sites, *FANCL* cDNA was cloned into the identically cut vector S11IEG to result in a bicistronic construct of *FANCL* and *EGFP* cDNAs connected by an IRES site (Huck et al. 2006) (Fig. 1A). S11 vectors are based on the spleen focus-forming virus and are derived from the GR plasmid (Hildinger et al. 1999). Retroviral plasmid was transfected into ecotropic Phoenix cells and ecotropic pseudotyped retroviruses were used to infect PG13 cells to achieve stable retroviral packaging as described elsewhere (Hanenberg et al. 1996; Hanenberg et al. 2002). cDNAs of other *FANC* genes were separately cloned into the same vector S11IEG (Hanenberg et al. 2002) and served as controls, as did the empty vector. Retroviral transduction of cultured cells followed published protocols (Hanenberg et al. 1997; Hanenberg et al. 2002). Previously unassigned FA LCLs and cultured fibroblasts, all deficient of FANCD2 monoubiquitination, were transduced to express wtFANCL. They were analyzed for their sensitivity to MMC (15 ng/ml, 48 h), with use of flow cytometry, in order to assess survival rates and cell cycle arrest (Hanenberg et al. 2002; Chandra et al. 2005, Casado et al. 2006).

The *FANCL* mutation vector S11FLIEG891 was generated by site-directed mutagenesis (see below). The *FANCL* reversion vector S11FL*IEG was constructed with cDNA from an LCL of patient 1.

Reference sequences

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

Genetic analyses

Genomic DNA was isolated with a modified salting-out technique (Miller et al. 1988). For exon scanning sequencing, we amplified all 14 *FANCL* exons and adjacent intron regions using primers given in Supplementary table S1. PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare; Munich, Germany). Sequencing of the PCR products was performed wih ABI-PRISM big-dye terminator chemistry and different types of ABI automated sequencers (Applied Biosystems, Darmstadt, Germany) using primers shown in Supplementary table S1; Supplementary table S2 contains customized, sequence-specific primers.

For a subset of cell lines, PCR products were subjected to heteroduplex analysis (Frueh and Noyer-Weidner 2003) using the WAVE system (Transgenomic, Glasgow, UK). DHPLC profiles

were run both spiked with control DNA and unspiked in order to detect homo- and heterozygous alterations.

Total RNA was isolated using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen, Karlsruhe, Germany) and was reverse transcribed using SuperScript II Reverse Transcriptase and Oligo(dT)20 (both Invitrogen, Karlsruhe, Germany) according to the manufacturers instructions. *FANCL* cDNA was amplified with Phusion polymerase (Finnzyme, Espoo, Finnland) and purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience, Munich, Germany). Primers used for cDNA amplification and sequencing are shown in Supplementary table S3.

For site-directed mutagenesis, *FANCL* (containing the nucleotides to be mutated) was amplified from the vector S11FLIEG. The PCR product was directly subcloned into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany). Site-directed mutagenesis was performed in this vector with the QuikChange® Site-Directed Mutagenesis Kit and Pfu polymerase (both Stratagene, La Jolla, CA). Site-mutated plasmids were isolated by plasmid mini preparation (Qiagen, Hilden, Germany) and the substitutions introduced were verified by direct sequencing. Using *EcoR*I and *Sal*I restriction sites, the site-mutated fragment was ligated back into S11FLIEG. Construction of the vector S11FLIEG891 was by means of the mutation primers FANCLmut891C>Gfor, 5'-CCAGCTCGTGCTATGCTGGAAAAATCTG-3', and FANCLmut891C>Grev, 5'-CAGATTTTTCCAGCATAGCACGAGCTGG-3'.

Inverse PCR was performed exactly as described (Ochman et al. 1988). Shortly, gDNA was separately digested with *EcoR*I, *Xho*I, *BamH*I and *Not*I. The restriction products were ligated under diluted conditions that favored monomolecular circularization. Applying each crude mix as template, the intramolecular ligation products were used for inverse amplification with an antisense primer upstream of exon 6 together with a sense primer downstream of exon 7 (Supplementary tables S1 and S2).

Splice assay

A potential effect of the base substitution c.891C>G, aberrant splicing, was examined by cloning a genomic fragment extending from *FANCL* exon 9 to 12 into the vector SVcrev (Freund et al. 2003). gDNAs of patient 1 and a normal control were used as templates for amplification with the sense primer FALex9-12EcoRI, 5´-GAA**GAATTC**GTAATAATGTTTCCATAAATATAG-3', and the antisense primer FALex9-12XhoI, 5´-GAA**CTCGAG**CTCATATAAGCATATTTGATGG-3'. Both PCR products and the target vector were cut with *EcoR*I and *Xho*I (restriction sites highlighted in the primer sequences), followed by ligation at 14 °C o/n. Fidelity of the inserted sequence was verified by sequencing. Transfection was done as previously described (Freund et al. 2003).

Immunofluorescence

Cells were fixed with 3.7% paraformaldehyde in PBS (w/v) for 15 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 5 min. After blocking for 30 min (10% FBS, 0.1% NP-40 in PBS), FANCD2 protein was detected using the same mouse monoclonal FANCD2 antibody as for immunoblotting (FL-17, Santa Cruz Biotechnology, Heidelberg, Germany) at a dilution of 1:200. Subsequently, the cells were washed in TBS three times. As secondary antibody, we used Alexa Fluor 594 anti-mouse IgG(H+L) (Molecular Probes, Leiden, The Netherlands) diluted in TBS (1:1000). After incubation for 45 min at RT, the cells were washed three times and covered with DAPI-containing Vectashield mounting medium (Vector Laboratories,Burlingame, CA).

Results

Prescreen

In a pilot study, 43 DNAs of LCLs originating from well-characterized FA patients previously excluded from belonging to any of the complementation groups FA-A, B, C, D1, D2, E, F and G were screened for sequence alterations of *FANCL* by DHPLC analysis and primary sequencing in 13 fragments. A total of 11 sequence variants were identified (Table 1). These include one synonymous missense variant, S327S, and 10 intronic variants. They represent annotated or anonymous SNPs that are characterized as polymorphisms by their nature or location. They may be useful for DNA chip arrays or the construction of haplotype maps. None of the LCLs revealed a mutation. This led us to require positive complementation in a functional assay as a prerequisite for sequence analysis of *FANCL* in further FA patients.

Table 1. Sequence variants / polymorphisms detected in *FANCL*

Position	Site	Designation	Frequency
Exon 12	c.981T>C (p.S327S)	rs848291	43% (16/37)
Intron	c.217-11T>C (IVS3-11T>C)	–	19% (7/37)
	c.273+20insT (IVS4+20insT)	–	8.1% (3/37)
	c.374+35delT (IVS5+35delT)	–	8.3% (3/36)
	c.540+93C>T (IVS7+93C>T)	–	nd
	c.541-27A>G (IVS7-27A>G)	rs12624152	8.3% (3/36)
	c.691+59delTAA (IVS8+59delTAA	–	32% (12/37)
	c.691+70A>G (IVS8+70A>G)	rs848288	nd
	c.775+22C>T (IVS9+22C>T)	rs10445896	35% (13/37)
	c.775+31A>G (IVS9+31A>G)	rs10445895	25% (9/36)
	c.1021-65G>A (IVS13-65G>A)	rs3732136	32% (12/37)

nd: Frequency was not determined in the present study

Assignment to complementation group FA-L

Patient-derived primary fibroblast cultures were used to determine the constitutional FANCD2 patterns. According to the type of FA/BRCA pathway disruption, patients were classified as 'FA core', FA-D2 and a downstream group (Soulier et al. 2005). Cells of 'FA core' patients with their defects upstream of FANCD2 (and FANCI) were screened for complementation after retroviral transfer of *FANCA, -B, -C, -E, -F, -G* and *-L* cDNAs. As shown for patient 1, transfer of *FANCL* cDNA in this case led to functional correction of the MMC-sensitive cellular phenotype as evidenced by

restoration of the previously increased G2 phase cell cycle fractions to normal control levels (Fig. 1B), in contrast to transduction with *EGFP* or other *FANC* cDNAs. Concomitantly, the cells became proficient of FANCD2 monoubiquitination (see below). On the basis of such complementation results, three patients were assigned to complementation group FA-L. In case of non-mosaic patients, cultures of stimulated PBL or LCLs could be used as well for the complementation assays.

Case histories

Patient 1 was born prematurely after 35 weeks of gestation as the second child of non-consanguineous parents of German ancestry. Physical examination after birth and prior to transplantation revealed short stature, pigmentation anomalies of the skin and malformations of the skeleton, eyes and inner organs. In addition to severe growth retardation (at the 3rd percentile) and growth hormone deficiency (treated with growth hormone from her second to ninth year of life), the patient exhibited microcephaly, malposition of the thumbs, rudimentary preaxial polydactyly, cutaneous syndactyly of toes II and III, congenital hip dysplasia and Klippel-Feil deformity. Microphtalmia was present together with blepharophimosis and Sicca syndrome. Malformations of inner organs included duodenal atresia with malrotation II and crossed renal atopia combined with vesicorenal reflux causing stage IV chronic renal failure. In addition, she suffered from renal tubular acidosis type II. Statomotor development was delayed.
The clinical diagnosis of FA was considered at age 4 years following the onset of mild thrombocytopenia, neutropenia and megaloblastic anemia. Confirmation at that time was by DEB testing and cell cycle analysis of PBL (Auerbach 1993; Seyschab et al. 1995). At age 6, the patient was started on androgen therapy, which was continued until 11 years of age. 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, a gradual decrease of platelets was noted, which was followed by a decrease of neutrophil counts 2 years later, necessitating hematological stem cell transplantation (HSCT). HSCT from a fully HLA-matched unrelated stem cell donor was successfully performed using a higly reduced and modified conditioning regimen based on fludarabine, busulfan and antithymocyte globulin. Two years after successful engraftment and complete hematologic remission, the 13-year-old patient developed a squamous cell carcinoma of the tongue that was detected during a routine examination. The carcinoma was successfully removed and she is in complete remission at her present age of 16 years (Reinhard et al. 2007).

Patient 2 is the third child to second cousins of Turkish descent. He was recognized by sonography at 28 weeks of gestation with intrauterine growth retardation, ventriculomegaly, radial flexion of both hands, aplasia of the right and hypoplasia of the left radius, and aplasia of both thumbs. FA was diagnosed prenatally, the parents nonetheless decided to continue the pregnancy. The malformations, stunted growth and anomalies of skin pigmentation were confirmed after birth. Hydrocephaly required drainage by shunt installation. No hematological manifestations occurred until to the present age of 1 year.

Patients 3 is the offspring of European and native American parents. She was recognized at birth with absent thumbs and radii bilaterally, skin pigmentation anomalies and short stature. FA was confirmed by abnormal chromosome breakage rates (spontaneous 0.18, DEB-induced 10.4 breaks per cell in PBL). She also has small auditory canals and bilateral hearing loss, whose specific nature is not entirely clear. Bone marrow failure started at age 5 years and required bone marrow transplantation at 10 years of age. She is doing fine at her present age of 11.5 years.

Mutation analysis

Mutation analysis in patient 1 was performed with cDNA and gDNA from primary fibroblasts. The patient was found to be heterozygous for a 5-bp deletion in exon 7 (c.483delATCAC) and a base substitution in exon 11 (c.891C>G, I297M). The deletion causes a frameshift and premature termination of translation at amino acid position 185 (E161DfsX26). The effect of the missense

substitution was unclear initially. ESE finder software (rulai.cshl.edu/tools/ESE/) predicted the loss of a potential SC35 binding site accompanied by reinforcement of a putative SRp40 motif as a result of the substitution. In a mini gene-based splicing assay, we detected transcripts with skipping of exons 10 and 11 or 11 only. However, this observation appeared to be independent of the base substitution as it also occurred in normal controls (Fig. 1C) and was finally interpreted as alternative splicing of exons 10 and 11 or 11 only. Retroviral transfer of full length *FANCL* cDNA containing the base substitution c.891C>G failed to complement fibroblasts of patient 1, indicating the pathogenetic nature of the missense mutation (Fig.1 D).

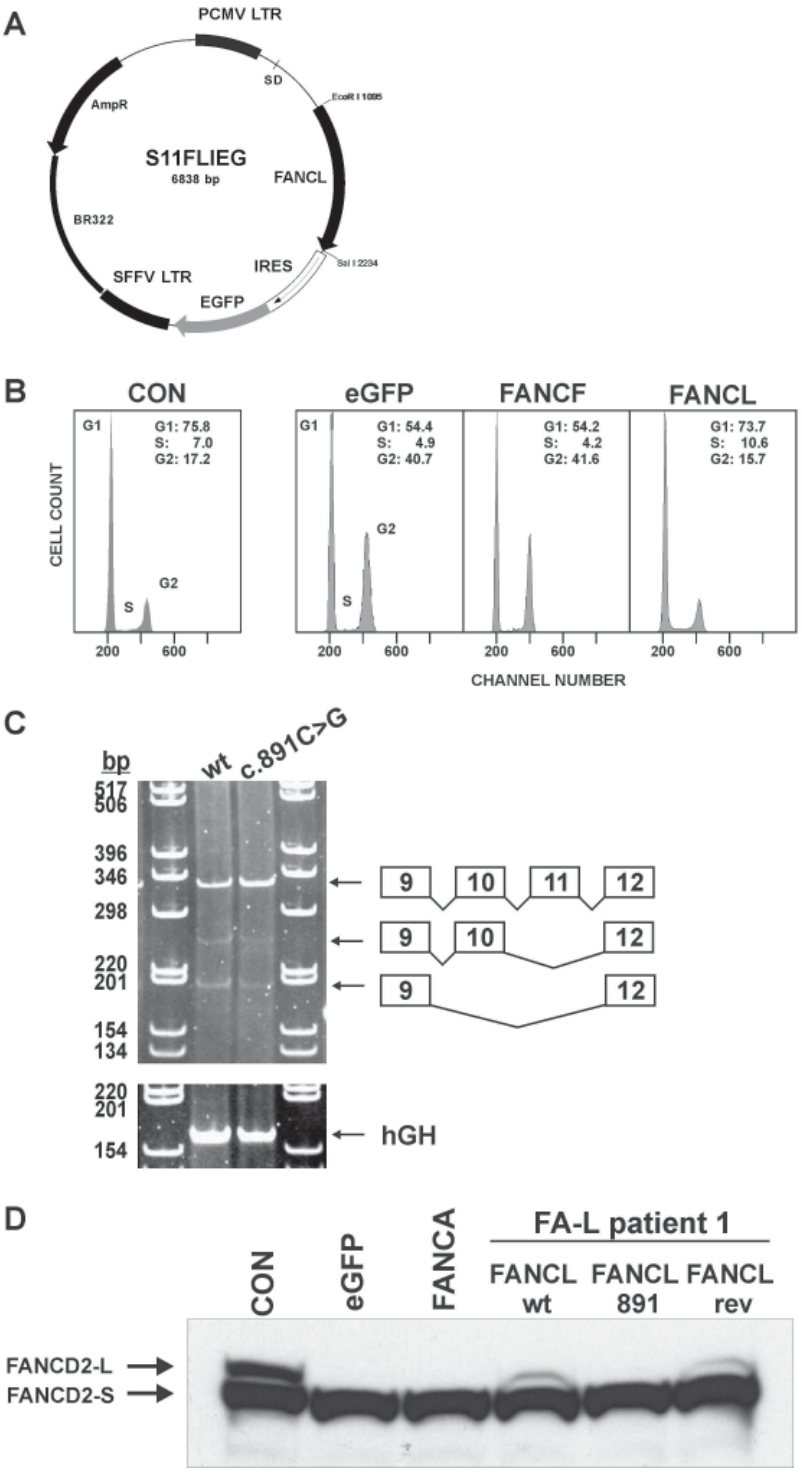


Figure 1. Assignment to complemetation group FA-L and FANCL mutations in patient 1. A) Circular map of the retroviral vector S11FLIEG that was used in complementation assays. Long terminal repeats (LTRs) and restriction sites used for construction of the vector and for site-directed mutagenesis are indicated. SD, splice donor. Bacterial resistance is *Amp*R. B) MMC sensitivity of primary fibroblasts from patient 1 results in prominent G2 phase peaks (after transduction with *eGFP* and *FANCF* vectors for negative control), but is reversed to control levels (CON) after transduction with *FANCL*-cDNA; DAPI stain. C) Polyacrylamide gel of RT-PCR products probing alternative splicing of FANCL mRNA in a mini gene-based splicing assay. The genomic region of *FANCL* exons 9 to 12 was amplified, cloned into the vector SVcrev and transfected into human cells. Isolated mRNA was reverse transcribed and amplified with vector specific primers. Both, patient 1 and control cDNA show an identical pattern of alternative splicing with skipping of exons 10 and 11 or 11 only. D) FANCD2 immunoblot with fibroblasts from FA-L patient 1. Retroviral transduction of wt*FANCL* cDNA restores FANCD2-L expression, as does *FANCL** cDNA with the somatic reversion, but *FANCL891* cDNA with the substitution c.891C>G does not, indicating that the former is a valid compensatory mutation, the latter an authentic mutation. Negative controls, *EGFP* and *FANCA* cDNA transfer.

Chromosome breakage and cell cycle analysis of PBL at 4 years of age revealed sensitivity towards MMC. In contrast, at age 10 prior to bone marrow transplantation, PBL of patient 1 proved MMC-resistant, suggesting the emergence of a mosaic status in the hematopoietic system. Two independently established LCLs likewise proved MMC-resistent as evidenced by the detection of both, the native and the monoubiquitinated FANCD2 isoform on immunoblots (Fig. 2A) and absent G2 phase arrest on cell cycle analysis (Fig. 2B) reflecting functional reversion. A de novo skipping of 10 bp preceding the 5-bp deletion was detected in PBL and lymphoblast cDNA and restored the ORF (Fig. 2C). At the genomic level, the basis of the 10-bp skipping in transcripts with the 5-bp deletion the proved to be a heterozygous base substitution c.472-2A>T (IVS6-2A>T) present in PBL, bone marrow and LCLs (at patient age 10), but not in patient´s fibroblasts (Fig. 2D). This base substitution is predicted to abrogate the splice acceptor upstream of exon 7 by disrupting the canonical AG dinucleotide. In turn, a downstream AG functions as a cryptic splice acceptor for aberrant splicing of exon 7 (Fig. 2E). This cryptic splice site is located 10 bp downstream of the normal exon boundary and 1 bp upstream of the germline deletion (c.483delATCAC). The predicted protein resulting from the combined alteration in transcripts contains a tyrosine instead of threonine at position 158, followed by a deletion of 5 amino acids (p.T158Ydel5) (Fig. 2F). This change might inflict the second WD40 repeat of FANCL, which is located between aa 147-202.

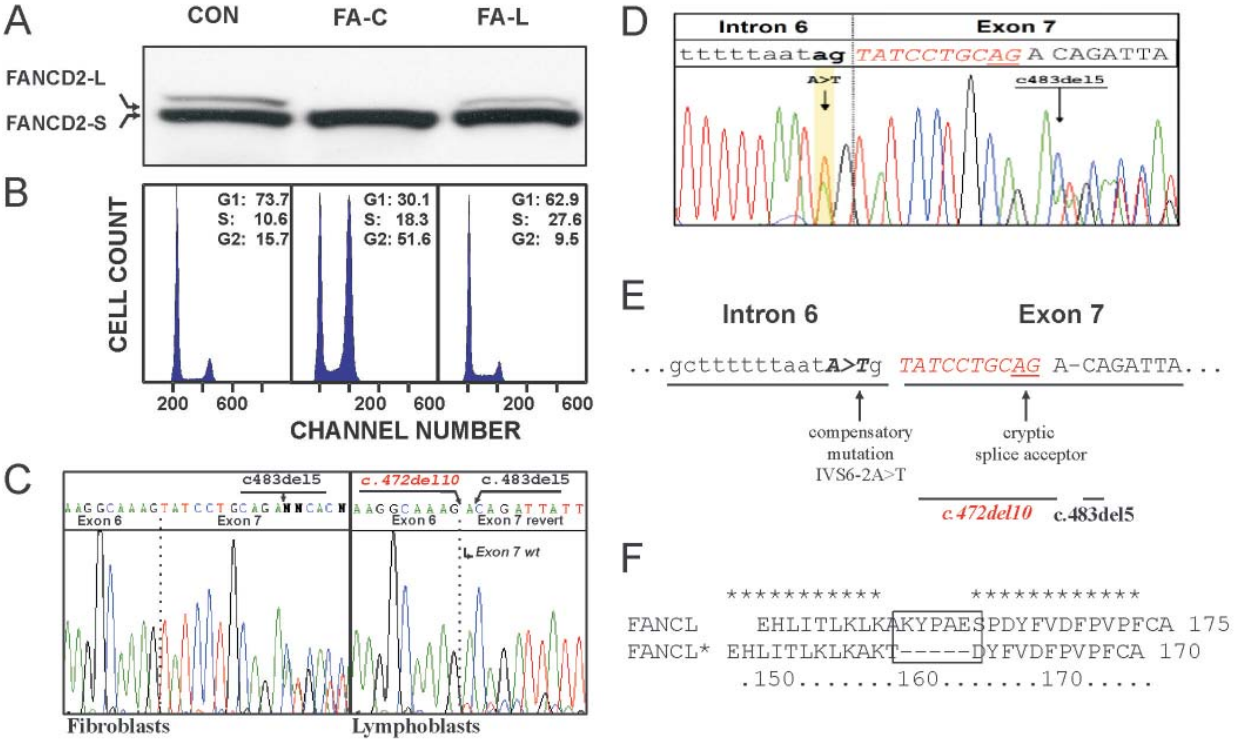


Figure 2. Molecular characterization of the somatic reversion in LCLs of patient 1. A) Revertant LCLs of FA-L patient 1 are proficient of FANCD2 monoubiquitination on immunoblots; negative control, *FANCC*-deficient cell line. B) Revertant LCLs of FA-L patient 1 lack G2-phase arrest on flow cytometric cell cycle analysis after exposure to MMC; (left to right) normal control cells, FA-C control and FA-L patient 1 LCLs; DAPI stain. C) Compared to fibroblasts, cDNA of LCLs derived from patient 1 shows the germline 5-bp deletion (c.483del5) and, in addition, somatic skipping of 10 bp (c.472del10). D) gDNA from PBL and LCLs of patient 1 shows coexistence of the germline 5-bp deletion in exon 7 and a base substition in cis, IVS6-2A>T, abrogating the normal splice acceptor upstream of exon 7. E) At the mRNA level, the base substitution IVS6-2A>T results in the usage of a cryptic splice acceptor located 10 bp downstream and skipping of the first 10 bp (red) of exon 7. F) The lack of altogether 15 bp at mRNA level (*FANCL**) restores the reading frame but leads to a chande of 1 aa (T158Y) and the deletion of 5 aa.

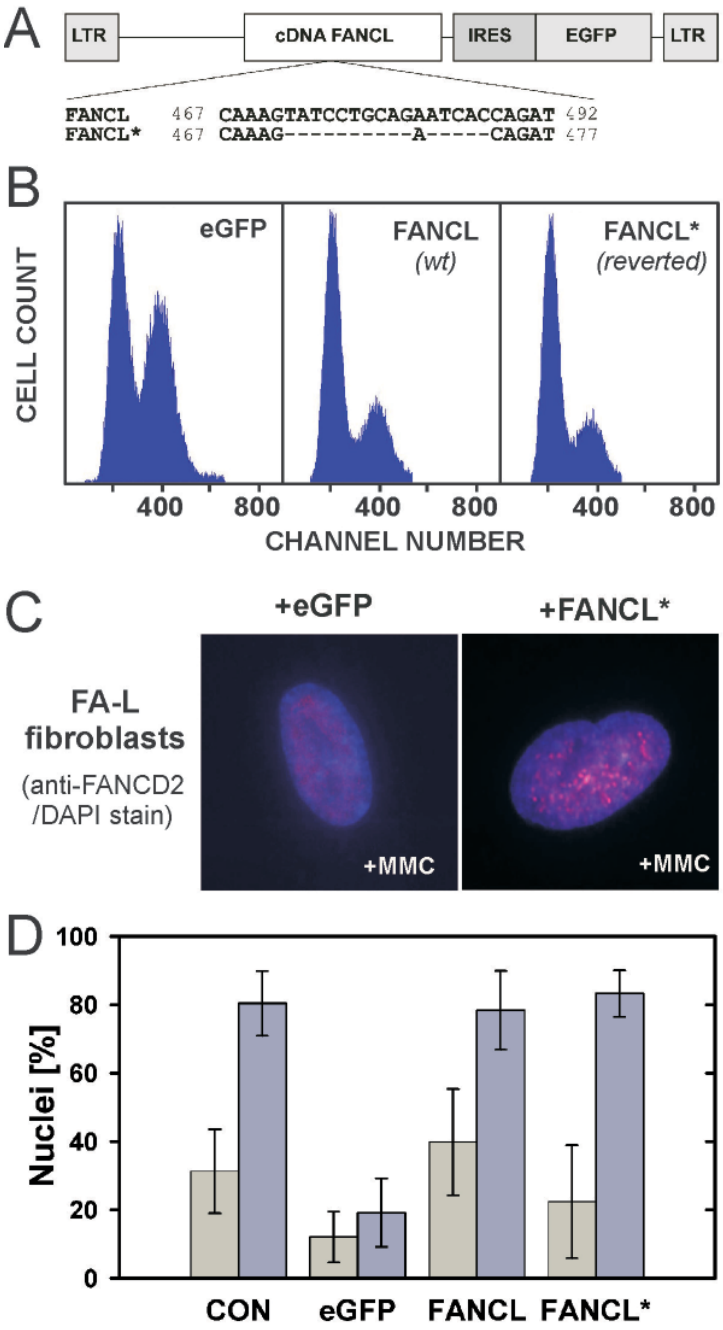


Figure 3. Evidence of the compensatory nature of the somatic reversion event in patient 1. A) Graphic presentation of a retroviral *FANCL** vector construct containing revertant cDNA from LCLs of FA-L patient 1 (with c.472del10 and c.483del5), denoted S11FL*IEG. B) Wt and revertant *FANCL* reduce G2-phase arrest of MMC-exposed fibroblasts from patient 1. Flow cytograms after transduction of fibroblasts from patient 1 with (from left to right) *EGFP* cDNA, wt *FANCL* cDNA and *FANCL** cDNA (containing the revertant sequence); Hoechst 33342 vital stain. C) Fibroblasts from patient 1 lack nuclear FANCD2 foci after exposure to MMC (left), while transduction with *FANCL** cDNA restores their formation (red, right). Nuclei were counterstained with DAPI (blue). D) Quantitative assessment of nuclear FANCD2 foci formation (mean and SD of 200 nuclei each) after transduction of fibroblasts from patient 1 with *EGFP*, wt*FANCL* and revertant *FANCL** cDNA, without (light bars) and after (dark bars) exposure toMMC. These experiments characterize IVS6-2A>T and the resulting 10-bp skipping as an authentic compensatory mutation.

In order to examine the function of the altered protein, we cloned the revertant *FANCL** cDNA into the retroviral S11 vector (Fig. 3A) and transduced patient-derived fibroblasts with this construct. As shown in Fig. 3B, *FANCL** cDNA abolished the hypersensitivity of patient's fibroblasts towards MMC, indicating complementation. Comparison between the wt and revertant *FANCL* cDNAs revealed no difference regarding complementation efficiency on cell cycle analysis. In addition, DNA damage-induced FANCD2 foci formation was restored by revertant *FANCL** cDNA (Fig. 3C). No significant difference in the number of foci per nuclei or the number of foci-containing nuclei was observed after retroviral transduction with wt*FANCL* or revertant *FANCL** cDNAs (Fig. 3D).

In case 2, lymphoblast gDNA was used for amplification and sequence analysis. Sequencing of PCR products from *FANCL* exons 6 through 14 including the adjacent intron regions revealed no mutations. Efforts to amplify exons 1 through 5 failed repeatedly; no PCR products were obtained.

A homozygous large deletion was suspected with breakpoints upstream of exon 1 and within intron 5, upstream of the binding site of the sense primer for exon 6 amplification. Using downstream walking with arbitrary forward primers in intron 5 in combination with the reverse primer for exon 6 amplification, the 3' breakpoint of the putative deletion was limited to the approximate region of IVS5-328 to IVS5-247 (c.375-328 to c.375-247). In order to determine both breakpoints exactly, we used a technique addressed as 'inverse PCR', that permits the amplification of regions of unknown sequence flanking any specified DNA segment (Fig. 4A) (Ochman et al., 1988). Restriction digests of different enzymes were ligated and thus circularized (Fig. 4B). Inverse PCR and two rounds of nested amplifications with internal primers within the known sequence revealed a PCR product exclusively with the *Not*I fraction. This was subjected to sequence analysis, which permitted identification of the breakpoints corresponding with the transition junction of sequence upstream of the *FANCL* translation start codon into intron 5 sequence (Fig. 4C). Formal description specifies the deletion EX1_5del as g.1-219_36840del37059 or c.1-219_375-248del37059. Since the 5' breakpoint of the deletion is located further upstream than all cDNA sequences available in various databases extend, it is likely that the deletion reaches into the putative *FANCL* promotor region. Therefore it is likely that neither a FANCL protein nor even a transcript are made.

In case 3, gDNA from fibroblast was used as template for amplification and exon scanning sequencing. A heterozygous 3-bp deletion in exon 12 was identified, c.1007_1009delTAT. This mutation is out of the reading frame; it deletes two original amino acids and creates a new one at the deletion union, designated as p.I336_C337delinsS. There was no second mutation detected in the exons and adjacent introns. In order to analyze the transcripts, cDNA from fibroblasts was sequenced. At the mRNA level, the small deletion appeared homozygous suggesting instability of the elusive other transcript. Therefore we designed a mismatch primer with wt*FANCL* sequence at the site of the 3-bp deletion, destined to bind and amplify preferentially the unstable transcript without the mutation at this site. Using this as antisense in combination with *FANCL* -43 (Supplementary table S3) as sense primer, we obtained two PCR products, one of which contained a 33-bp sequence from deep within intron 5. Genomic analysis showed a 5'-flanking cryptic splice acceptor. A heterozygous base substitution 3'-flanking the inserted intron sequence created a new splice donor with high complementarity for U1snRNA binding, indicated by the score of 75.10 (Splicefinder version Screen 3; http://www.splicefinder.net). This event resulted in the exonization of IVS5-2066_IVS5-2034 to create a new exon 5a. Formal description of this mutation specifies it as g.35021C>G or c.375-2066_375-2034ins33 or p.K125_L126insNYELINEKEFR.

During these studies, we recognized another site of alternative *FANCL* mRNA splicing. In intron 7, there is a direct repeat of the exon-intron junction sequence. This repeat of 15 bp length (CCTCAGGTAAATTCT) also repeats the splice donor upstream of exon 7 (c.540+15), which appears to be used. We detected minor transcripts that contain a corresponding insertion (c.540_541insGTAAATTCTCCTCAG; p.Q180_S181insVNSPQ).

The *FANCL* mutations identified in the present study and that reported by Meetei et al. (Meetei et al. 2003) are depicted in Fig. 4D.

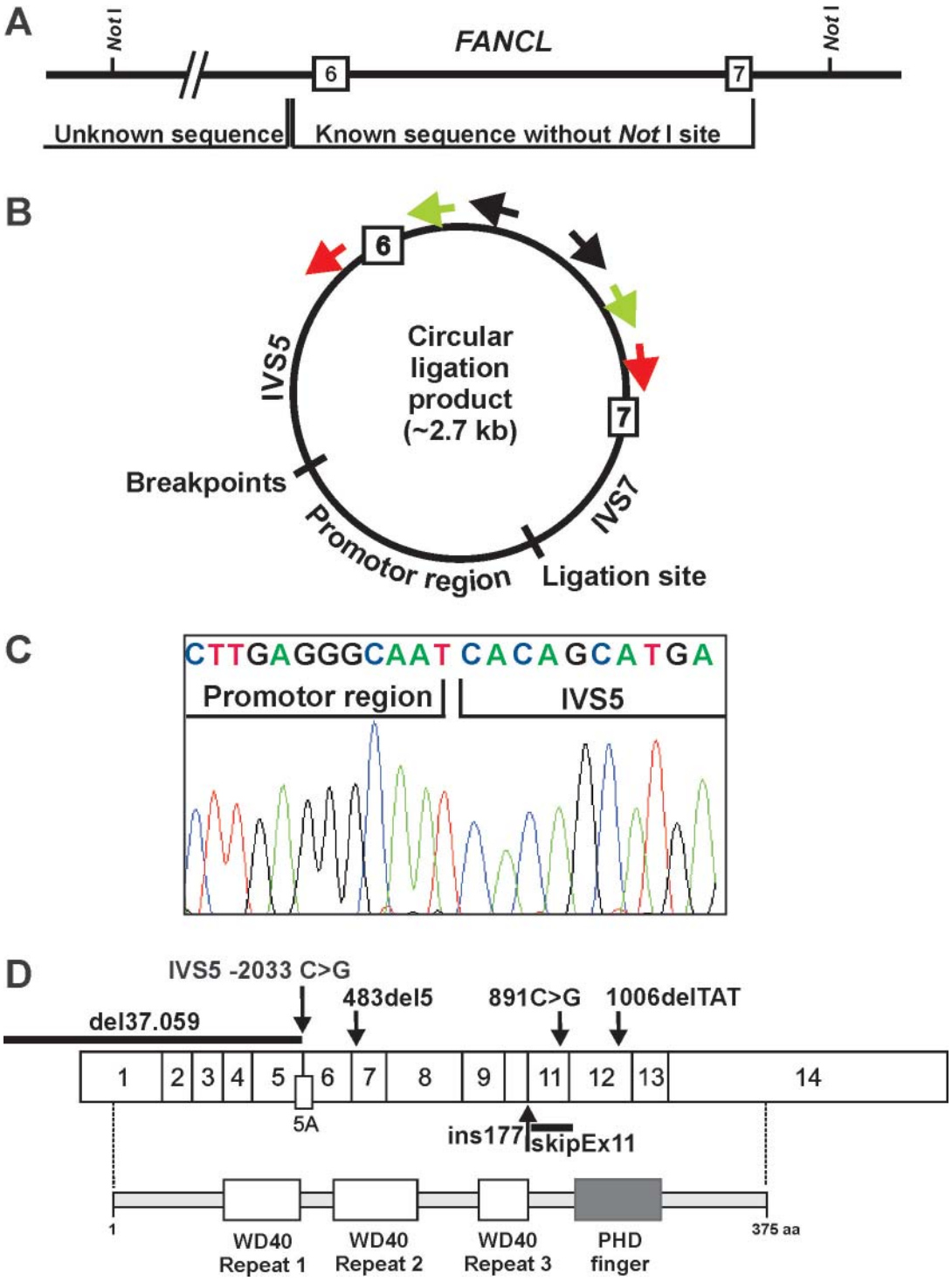


Figure 4. Mutation analyses in patient 2 and 3. A) Assay to characterize the breakpoints of a large genomic deletion of FA-L patient 2. Graphic presentation of unknown sequence containing the deletion breakpoints and an upstream *Not*I restriction site next to known sequence, a genomic fragment of *FANCL* containing exons 6 and 7, as well as a downstream *Not*I restriction site. B) Circular map of circularized *Not*I restriction products of A. Sequence elements are indicated. The arrows show primer binding sites for inverse amplification and nested PCR. C) The chromatogram of sequencing across the breakpoints in B shows the transition from putative *FANCL* promotor into IVS5 sequence, defining the deletion breakpoints. D) Positions and identity of the mutations identified in the present study are shown above a schematic presentation of *FANCL*, the mutation reported by Meetei et al. (Meetei et al. 2003) is below.

Discussion

General considerations

Three of the FANC genes represented FA subgroups with single patients when they were first reported, namely *FANCH*, *FANCL* and *FANCM* (Joenje et al. 1997; Meetei et al. 2003; Meetei et al. 2005). There are even proteins that are members of the FA core complex such as FAAP100, which does not stand for any complementation group because there is no patient with a corresponding gene defect, although the function of this protein is FA-like (Ling et al. 2007). While it may be premature to judge the success of screens for defects in recently identified genes without FA patients, the fate of the complementation groups with initially single patients is instructive. Group FA-H was withdrawn after the only patient of this group was reclassified to group FA-A (Joenje et al. 2000). Complementation group FA-M is intermediate in that it is represented by a single family for more than two years. Facts that would make this group special are not obvious to date. It is therefore to be expected that group FA-M will follow the fate of group FA-L. With the present study, the number of members of this group increases from one to four not less than 4 years after the first report of *FANCL*, which now renders FA-L a classical FA complementation group.

Genotype-phenotype correlations

With the original report it remained unclear howt the phenotypic presentation of FA-L patients is like, since there was no clinical information on the single patient who is since deceased and unavailable for study. Here we present the clinical and molecular data of three additional FA-L patients. Patient 1 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 4) 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 single 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 (Meetei et al. 2003). Leaky mutations due to aberrant splicing are known to predominate in the target of *FANCL*, the *FANCD2* gene (Kalb et al. 2006), and only very few mutations have been found in *FANCM*, another evolutionarily conserved FA gene (Meetei et al. 2005). 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) (Agoulnik et al. 2002). *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 (Lu and Bishop 2003).

Somatic reversion in patient T- and B-lymphoid cells

At age 10, examination of primary T-lymphocytes and of B-cell derived LCLs of patient 1 of the present study 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 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. To date, revertant mosaicism has been described for FA patients belonging to complementation groups A, C and D2 (Lo Ten Foe et al. 1997; Waisfisz et al. 1999; Gregory et al. 2001; Gross et al. 2002; Soulier et al. 2005, Kalb et al. 2006). Mechanisms

of reversion include intragenic crossover, gene conversion, back mutation, and compensating mutations in *cis* (Waisfisz et al. 1999; Gross et al. 2002; Mankad et al. 2006). 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 1 (Waisfisz et al. 1999). 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 the usage of an alternative splice site, located 10 base pairs downstream next to the germline deletion, 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 (Gurtan et al. 2006). 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 (Smith et al. 1999; Li and Roberts 2001). In our patient 1, the compensatory deletion evidently did not alter protein function, indicating that no structurally important domain was affected. Functionality of the reverted gene product was evidenced 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 clinical and mutational data of three additional FA-L patients. These data actually show an increasing complementation group FA-L emerging, FA-L patients present with a typical spectrum of manifestations and hematological course comparable with those of FA patients with the most common gene defects (*FANCA*, -*C* and *G*). Finally, FA-L patients reveal a spectrum of mutation and reversion similar to that reported for the commonly mutated FA genes.

References

Agoulnik AI, Lu B, Zhu Q, Truong C, Ty MT, Arango N, Chada KK, Bishop CE (2002) A novel gene, Pog, is necessary for primordial germ cell proliferation in the mouse and underlies the germ cell deficient mutation, gcd. Hum Mol Genet 11:3047-3053

Auerbach AD (1993) Fanconi anemia diagnosis and the diepoxybutane (DEB) test. Exp Hematol 21:731-733

Casado JA, Callen E, Jacome A, Rio P, Castella M, Lobitz S, Ferro T, Munoz A, Sevilla J, Cantalejo A, Cela E, Cervera J, Sanchez-Calero J, Badell I, Estella J, Dasi A, Olive T, Jose Ortega J, Rodriguez-Villa A, Tapia M, Molines A, Madero L, Segovia JC, Neveling K, Kalb R, Schindler D, Hanenberg H, Surralles J, Bueren JA (2007) A comprehensive strategy for the subtyping of patients with Fanconi anaemia: conclusions from the Spanish Fanconi Anemia Research Network. J Med Genet 44, 241-249

Chandra S, Levran O, Jurickova I, Maas C, Kapur R, Schindler D, Henry R, Milton K, Batish SD, Cancelas JA, Hanenberg H, Auerbach AD, Williams DA (2005) A rapid method for retrovirus-mediated identification of complementation groups in Fanconi anemia patients. Mol Ther 12:976-984

Digweed M, Rothe S, Demuth I, Scholz R, Schindler D, Stumm M, Grompe M, Jordan A, Sperling K (2002) Attenuation of the formation of DNA-repair foci containing RAD51 in Fanconi anaemia. Carcinogenesis 23:1121-1126

Dorsman JC, Levitus M, Rockx D, Rooimans MA, Oostra AB, Haitjema A, Bakker ST, Steltenpool J, Schuler D, Mohan S, Schindler D, Arwert F, Pals G, Mathew CG, Waisfisz Q, de Winter JP, Joenje H (2007) Identification of the Fanconi anemia complementation group I gene, FANCI. Cell Oncol 29:211-218

Freund M, Asang C, Kammler S, Konermann C, Krummheuer J, Hipp M, Meyer I, Gierling W, Theiss S, Preuss T, Schindler D, Kjems J, Schaal H (2003) A novel approach to describe a U1 snRNA binding site. Nucleic Acids Res 31:6963-6975

Frueh FW, Noyer-Weidner M (2003) The use of denaturing high-performance liquid chromatography (DHPLC) for the analysis of genetic variations: impact for dignostics and pharmacogenetics. Clin Chem Lab Med 41:452-461

Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, Grompe M, D'Andrea AD (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell 7:249-262

Godthelp BC, Wiegant WW, Waisfisz Q, Medhurst AL, Arwert F, Joenje H, Zdzienicka MZ (2006) Inducibility of nuclear Rad51 foci after DNA damage distinguishes all Fanconi anemia complementation groups from D1/BRCA2. Mutat Res 594:39-48

Gregory JJ, Jr., Wagner JE, Verlander PC, Levran O, Batish SD, Eide CR, Steffenhagen A, Hirsch B, Auerbach AD (2001) Somatic mosaicism in Fanconi anemia: evidence of genotypic reversion in lymphohematopoietic stem cells. Proc Natl Acad Sci U S A 98:2532-2537

Gross M, Hanenberg H, Lobitz S, Friedl R, Herterich S, Dietrich R, Gruhn B, Schindler D, Hoehn H (2002) Reverse mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction. Cytogenet Genome Res 98:126-135

Gurtan AM, Stuckert P, D'Andrea AD (2006) The WD40 repeats of FANCL are required for Fanconi anemia core complex assembly. J Biol Chem 281:10896-10905

Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, Williams DA. (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nat Med 2:876-882

Hanenberg H, Hashino K, Konishi H, Hock RA, Kato I, Williams DA. (1997) Optimization of fibronectin-assisted retroviral gene transfer into human CD34+ hematopoietic cells. Hum Gene Ther 8:2193-2206

Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, Leurs C, Cooper RJ, Gottsche K, Haneline L, Clapp DW, Lobitz S, Williams DA, Auerbach AD (2002) Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol 30:410-420

Heinrich MC, Hoatlin ME, Zigler AJ, Silvey KV, Bakke AC, Keeble WW, Zhi Y, Reifsteck CA, Grompe M, Brown MG, Magenis RE, Olson SB, Bagby GC (1998) DNA cross-linker-induced G2/M arrest in group C Fanconi anemia lymphoblasts reflects normal checkpoint function. Blood 91:275-287

Hildinger M, Abel KL, Ostertag W, Baum C (1999) Design of 5' untranslated sequences in retroviral vectors developed for medical use. J Virol 73:4083-4089

Huck K, Hanenberg H, Gudowius S, Fenk R, Kalb R, Neveling K, Betz B, Niederacher D, Haas R, Gobel U, Kobbe G, Schindler D (2006) Delayed diagnosis and complications of Fanconi anaemia at advanced age--a paradigm. Br J Haematol 133:188-197

Joenje H, Oostra AB, Wijker M, Di Summa FM, van Berkel CG, Ebell W, van Weel M, Pronk JC, Buchwald M, Arwert F (1997) Evidence for at least eight Fanconi anemia genes. Am J Hum Genet61:940-944

Joenje H (1997) Fanconi anemia: cytogenetic

diagnosis. Free University of Amsterdam, Amsterdam

Joenje H, Levitus M, Waisfisz Q, D'Andrea A, Garcia-Higuera I, Pearson T, van Berkel CGM, Rooimans MA, Morgan N, Mathew CG, Arwert F (2000) Complementation analysis in Fanconi anemia: assignment of the reference FA-H patient to group A. Am J Hum Genet 67:759-762

Levitus M, Rooimans MA, Steltenpool J, Cool NF, Oostra AB, Mathew CG, Hoatlin ME, Waisfisz Q, Arwert F, de Winter JP, Joenje H (2004) Heterogeneity in Fanconi anemia: evidence for 2 new genetic subtypes. Blood 103:2498-2503

Levitus M, Waisfisz Q, Godthelp BC, de Vries Y, Hussain S, Wiegant WW, Elghalbzouri-Maghrani E, Steltenpool J, Rooimans MA, Pals G, Arwert F, Mathew CG, Zdzienicka MZ, Hiom K, De Winter JP, Joenje H (2005) The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J. Nat Genet 37:934-935

Levran O, Attwooll C, Henry RT, Milton KL, Neveling K, Rio P, Batish SD, Kalb R, Velleuer E, Barral S, Ott J, Petrini J, Schindler D, Hanenberg H, Auerbach AD (2005) The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet 37:931-933

Li D, Roberts R (2001) WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases. Cell Mol Life Sci 58:2085-2097

Ling C, Ishiai M, Ali AM, Medhurst AL, Neveling K, Kalb R, Yan Z, Xue Y, Oostra AB, Auerbach AD, Hoatlin ME, Schindler D, Joenje H, de Winter JP, Takata M, Meetei AR, Wang W (2007) FAAP100 is essential for activation of the Fanconi anemia-associated DNA damage response pathway. EMBO J 26:2104-2114

Lo Ten Foe JR, Kwee ML, Rooimans MA, Oostra AB, Veerman AJ, van Weel M, Pauli RM, Shahidi NT, Dokal I, Roberts I, Altay C, Gluckman E, Gibson RA, Mathew CG, Arwert F, Joenje H (1997) Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. Eur J Hum Genet 5:137-148

Lu B, Bishop CE (2003) Late onset of spermatogenesis and gain of fertility in POG-deficient mice indicate that POG is not necessary for the proliferation of spermatogonia. Biol Reprod 69:161-168

Machida YJ, Machida Y, Chen Y, Gurtan AM, Kupfer GM, D'Andrea AD, Dutta A (2006) UBE2T is the E2 in the Fanconi anemia pathway and undergoes negative autoregulation. Mol Cell 23:589-596

Mankad A, Taniguchi T, Cox B, Akkari Y, Rathbun RK, Lucas L, Bagby G, Olson S, D'Andrea A, Grompe M (2006) Natural gene therapy in monozygotic twins with Fanconi anemia. Blood

Meetei AR, de Winter JP, Medhurst AL, Wallisch M, Waisfisz Q, van de Vrugt HJ, Oostra AB, Yan Z, Ling C, Bishop CE, Hoatlin ME, Joenje H, Wang W (2003) A novel ubiquitin ligase is deficient in Fanconi anemia. Nat Genet 35:165-170

Meetei AR, Medhurst AL, Ling C, Xue Y, Singh TR, Bier P, Steltenpool J, Stone S, Dokal I, Mathew CG, Hoatlin M, Joenje H, de Winter JP, Wang W (2005) A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. Nat Genet 37:958-963

Meetei AR, Yan Z, Wang W (2004) FANCL replaces BRCA1 as the likely ubiquitin ligase responsible for FANCD2 monoubiquitination. Cell Cycle 3:179-181

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215

Nakanishi K, Yang YG, Pierce AJ, Taniguchi T, Digweed M, D'Andrea AD, Wang ZQ, Jasin M (2005) Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci U S A 102:1110-1115

Niedzwiedz W, Mosedale G, Johnson M, Ong CY, Pace P, Patel KJ (2004) The Fanconi anaemia gene FANCC promotes homologous recombination and error-prone DNA repair. Mol Cell 15:607-620

Ochman H, Gerber AS, Hartl DL (1988) Genetic application of an inverse polymerase chain reaction. Genetics 120:621-623

Reinhard H, Peters I, Gottschling S, Ebell W, Graf N (2007) Squamous cell carcinoma of the tonguein a 13-year-old girl with Fanconi anemia. J Pediatr Hematol Oncol 29:488-491

Rosenberg PS, Socie G, Alter BP, Gluckman E (2005) Risk of head and neck squamous cell cancer and death in patients with Fanconi anemia who did and did not receive transplants. Blood 105:67-73

Seyschab H, Friedl R, Sun Y, Schindler D, Hoehn H, Hentze S, Schroeder-Kurth T (1995) Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. Blood 85:2233-2237

Sigismund S, Polo S, Di Fiore PP (2004) Signaling through monoubiquitination. Curr Top Microbiol Immunol 286:149-185

Sims AE, Spiteri E, Sims RJ 3rd, Arita AG, Lach FP, Landers T, Wurm M, Freund M, Neveling K, Hanenberg H, Auerbach AD, Huang TT (2007) FANCI is a second monoubiquitinated member of the Fanconi anemia pathway. Nat Struct Mol Biol 14:564-567

Smith TF, Gaitatzes C, Saxena K, Neer EJ (1999) The WD repeat: a common architecture for diverse functions. Trends Biochem Sci 24:181-185

Smogorzewska A, Matsuoka S, Vinciguerra P, McDonald ER 3rd, Hurov KE, Luo J, Ballif BA, Gygi SP, Hofmann K, D'Andrea AD, Elledge SJ (2007) Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. Cell 129:289-301

Soulier J, Leblanc T, Larghero J, Dastot H, Shimamura A, Guardiola P, Esperou H, Ferry C, Jubert C, Feugeas JP, Henri A, Toubert A, Socie G, Baruchel A, Sigaux F, D'Andrea AD, Gluckman E (2005) Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. Blood 105:1329-1336

Thompson LH (2005) Unraveling the Fanconi anemia-DNA repair connection. Nat Genet 37:921-922

Thompson LH, Hinz JM, Yamada NA, Jones NJ (2005) How Fanconi anemia proteins promote the four Rs: Replication, recombination, repair, and recovery. Environ Mol Mutagen 45:128-142

Waisfisz Q, Morgan NV, Savino M, de Winter JP, van Berkel CG, Hoatlin ME, Ianzano L, Gibson RA, Arwert F, Savoia A, Mathew CG, Pronk JC, Joenje H (1999) Spontaneous functional correction of homozygous fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. Nat Genet 22:379-383

Wang W (2007) Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nat Rev Genet 8, 735-748

Supplementary Tables

Supplementary Table S1. Genomic primers for *FANCL* amplification and sequencing

Designation (Exon)	Sequence (5'>3')
FANCLex1F	AGGATCTTCCCGCCAAGG
FANCLex1R	CCTAGCCCGTCACAGACTTC
FANCLex2F	GAGTTATTTTCCCTCAAATGCAA
FANCLex2R	TACTGCCTGTCCCACCAAA
FANCLex3F	TTGTCCAAATTATGTTTGTCATTC
FANCLex3R	CAACATTGTGCAAAGCAGGT
FANCLex4F	TTCACTTGGGCCTACATTTTT
FANCLex4R	AAAGAAAACATCAAATGACTGAGA
FANCLex5F	TGAAATTATGTTTTGCAATCAGG
FANCLex5R	TGACTAAAATCAGGTATAAACTGCTAA
FANCLex6F	ACCATCTCCAGTTCCCCTCT
FANCLex6R	TGAGAGCATTCACAGAGTTCATT
FANCLex7F	CCCGGCCCCTAATACATCT
FANCLex7R	CCCATGGATACTCTGGGACA
FANCLex8F	ATGTGAAGCAGGGAGAGTCG
FANCLex8R	AAGTCTGAGTCCAACTGTCATTGT
FANCLex9F	AGCCAGACCAACACCTCTTT
FANCLex9R	ATCGCGCCATTACACTACAT
FANCLex10F	CATTCTGTATCAAATGTGGCTTT
FANCLex10R	TTCTGGATCCCTGAAAGCAT
FANCLex11F	TGTGGTAGGTACCTTGGCAAT
FANCLex11R	CAGCCTCATTTTTCACTGAGAG
FANCLex12F	CCCTCCCAAATATGTACAACG
FANCLex12R	CAGGAATACTTCCTATGTTGTGTTAG
FANCLex13F	CAAAGGGAATGACATTTGGAG
FANCLex13R	ATGTTTTTGATCAGAGAATTTGAA
FANCLex14F	TTCAGAAGTGTTTTCCAAACTGT
FANCLex14R	TGAAGATGATACCAAAATTCCTTT

Supplementary Table S2. Customized, sequence-specific *FANCL* primers

Designation	Sequence (5'>3')
FANCL ex 7 for FANCL ex 7 rev (Mutation screen, patient 1)	CCTCAGTGATATTAACACACAGAAGC GTATACAGAAGAGTGTGCACAGGC
IVS6-521 IVS6-200 (Reversion, patient 1)	CCAAATCACCAAACTTGCACCTC GGCGAGGTTTCACCGTATAG
FANCLEx 9-12 F FANCLEx 9-12 R FANCLIVS9 for FANCLIVS9 rev FANCLEx10int F FANCLIVS10int3F FANCLIVS11 for FANCLIVS11int2F FANCLIVS11 rev (Alternative splicing)	TAGGTAGCCAGACCAACACCTC GATCAGGAATGGTACCGTCAAG GCTCTTGTTGCCCAGTATGTAGTG CAGTTCAGAATGCTGAGACATTGTC GCAGGAACATACATTTGTGGTAGG CATTCTTCCTGATCTTACCTTGTG AGTACAACTGTCTCTTGGAACAGGG GAACTCCAAAGTAGCTAATGAGATG TTTGATGCAGATCAGAAGTCTGTG
7F.for1 IVS5Frg11rev (Inverse nested PCR, patient 2)	CCTCAGTGATATTAACACACA GGGAACTGGAGATGGTTGAATTTG
840allel2rev (Allele-specific PCR, patient 3)	TCCTCTCAGCCACTCATATAAGCATA
840IVS5for1 840IVS5for2 840IVS5rev1 840IVS5rev2 (Exonization, patient 3)	CTCCCTCTTTATATTCATCATGGTTATG GTACAGAAAGTAGTAGGTGGTGATG CCATCTTTAATTGAATGGACGTGAAAG CCAAGAATACGCAATGCTAAAAGAC

Supplementary Table S3. *FANCL* cDNA primers

Designation	Sequence (5'>3')
FANCL -43F	CAGCAGGTCTAGAGCTTTTCTG
FANCL 293F	GACAAGAGCTGTATGCACTAC
FANCL 355F	GGAACTCTTGGTTGGGATAAAC
FANCL 671F	CAGCACGCAGAATTGCATTAGG
FANCL 745F	CCTGAGTGCTTCTTTCTTGGAG
FANCL 386R	GCATACACAAGTTTATCCCAACC
FANCL 686R	GCAATTCTGCGTGCTGTTG
FANCL 896R	TCCAGGATAGCACGAGCTG
FANCL 1321R	GACTTTGGCCTACAATTTCCCAG

2

Results

ARTICLE

Hypomorphic Mutations in the Gene Encoding a Key Fanconi Anemia Protein, FANCD2, Sustain a Significant Group of FA-D2 Patients with Severe Phenotype

Reinhard Kalb, Kornelia Neveling, Holger Hoehn, Hildegard Schneider, Yvonne Linka, Sat Dev Batish, Curtis Hunt, Marianne Berwick, Elsa Callén, Jordi Surrallés, José A. Casado, Juan Bueren, Ángeles Dasí, Jean Soulier, Eliane Gluckman, C. Michel Zwaan, Rosalina van Spaendonk, Gerard Pals, Johan P. de Winter, Hans Joenje, Markus Grompe, Arleen D. Auerbach, Helmut Hanenberg, and Detlev Schindler

FANCD2 is an evolutionarily conserved Fanconi anemia (FA) gene that plays a key role in DNA double-strand–type damage responses. Using complementation assays and immunoblotting, a consortium of American and European groups assigned 29 patients with FA from 23 families and 4 additional unrelated patients to complementation group FA-D2. This amounts to 3%–6% of FA-affected patients registered in various data sets. Malformations are frequent in FA-D2 patients, and hematological manifestations appear earlier and progress more rapidly when compared with all other patients combined (FA–non-D2) in the International Fanconi Anemia Registry. *FANCD2* is flanked by two pseudogenes. Mutation analysis revealed the expected total of 66 mutated alleles, 34 of which result in aberrant splicing patterns. Many mutations are recurrent and have ethnic associations and shared allelic haplotypes. There were no biallelic null mutations; residual FANCD2 protein of both isotypes was observed in all available patient cell lines. These analyses suggest that, unlike the knockout mouse model, total absence of FANCD2 does not exist in FA-D2 patients, because of constraints on viable combinations of *FANCD2* mutations. Although hypomorphic mutations arie involved, clinically, these patients have a relatively severe form of FA.

Fanconi anemia (FA) is a rare genome-instability disorder with the variable presence of congenital malformations, progressive bone-marrow failure (BMF), predisposition to malignancies, and cellular hypersensitivity to DNA-interstrand crosslinking agents.[1] There are at least 13 complementation groups (FA-A [MIM 607139], -B [MIM 300515], -C [MIM 227645], -D1 [MIM 600185], -D2 [MIM 227646], -E [MIM 600901], -F [MIM 603467], -G [MIM 602956], -I [MIM 609053], -J [MIM 609054], -L [MIM 608111], -M [MIM 609644], and -N [MIM 610355]), each of which is associated with biallelic or hemizygous mutations in a distinct gene.[2–4] To date, 12 of the underlying genes have been identified: *FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG/XRCC9, FANCJ/BRIP1, FANCL/PHF9, FANCM/HEF,* and *FANCN/PALB2*.[3–7] 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.[8,9] Monoubiquitination occurs in response to DNA damage and during the S phase of the cell cycle.[9,10] The monoubiquitinated FANCD2 isoform (FANCD2-L, as opposed to FANCD2-S) is targeted to nuclear foci containing proteins such as BRCA1 [MIM 113705], BRCA2, and RAD51 [MIM 179617] that are involved in DNA-damage signaling and recombinational repair.[11–14] The precise role of FANCD2 remains unknown, but FANCD2-deficient DT40 cells show defects in homologous recombination-mediated DNA double-strand break (DSB) repair, translesion synthesis, and gene conversion.[11,15,16] Therefore, FANCD2 is thought to play a central role in the maintenance of genome stability.[11,16,17]

The human and murine *Fancd2* genes show a higher degree of homology than do the corresponding *Fanca, Fancc, Fance, Fancf,* and *Fancg* genes.[18] *Fancd2*-knockout mice suffer from perinatal lethality, microphthalmia, and early epithelial cancers,[19] but it remains controversial whether the murine FA-D2 phenotype in general is more severe than the corresponding murine knockouts of the

From the Department of Human Genetics, University of Wurzburg, Wurzburg, Germany (R.K.; K.N.; H. Hoehn; D.S.); Department of Pediatric Oncology, Hematology and Immunology, University of Dusseldorf, Dusseldorf (H.S.; Y.L.; H. Hanenberg); Laboratory of Human Genetics and Hematology, Rockefeller University, New York (S.D.B.; A.D.A.); Division of Epidemiology, University of New Mexico, Albuquerque (C.H.; M.B.); Department of Genetics and Microbiology, Universitat Autónoma de Barcelona, Barcelona (E.C.; J.S.); Centre for Biomedical Research on Rare Diseases, Bellaterra, Spain (E.C.; J.S.; J.A.C.; J.B.); Hematopoiesis and Gene Therapy Division, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, Madrid (J.A.C.; J.B.); Unit of Pediatric Hematology, Hospital la Fe, Valencia (A.D.); Institut Universitaire d'Hematologie, Hôpital Saint-Louis, Paris (J.S.; E.G.); Department of Pediatric Hematology/Oncology, Erasmus Medical Center–Sophia Children's Hospital, and Dutch Childhood Oncology Group, Rotterdam (C.M.Z.); Department of Clinical Genetics and Human Genetics, Vrije Universiteit Medical Center, Amsterdam (R.v.S.; G.P.; J.P.d.W.; H.J.); Department of Medical and Molecular Genetics, Oregon Health and Science University, Portland (M.G.); and Department of Pediatrics, Indiana University School of Medicine, Indianapolis (H. Hanenberg)

Received December 21, 2006; accepted for publication February 26, 2007; electronically published April 6, 2007.

Address for correspondence and reprints: Dr. Detlev Schindler, Department of Human Genetics, University of Wurzburg, Biozentrum, Am Hubland, D-97074 Wurzburg, Germany. E-mail: schindler@biozentrum.uni-wuerzburg.de

Am. J. Hum. Genet. 2007;80:895–910. 0002-9297/2007/8005-0009$15.00
DOI: 10.1086/517616

other FA genes.[19,20] Fancd2 is required for survival after DNA damage in *Caenorhabditis elegans*.[21] Fancd2-deficient zebrafish embryos display severe developmental defects due to increased apoptosis, which underscores the importance of *Fancd2* function during vertebrate ontogenesis.[22] Finally, knock-down of *Drosophila* Fancd2 causes pupal lethality.[23] In humans, it has been estimated that complementation group FA-D2 accounts for between <1%[24] and 3%[25] of all patients with FA. The presence of *FANCD2* pseudogenes complicates mutation analysis, which may explain why there has been just one other report of a single human *FANCD2* mutation since the original description.[26,27] As a concerted effort among nine laboratories, we present a comprehensive mutation profile of the *FANCD2* gene (Ensembl Genome Browser [accession number ENSG00000144554]). We show that the FA phenotype resulting from FANCD2 deficiency is relatively severe. 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, which suggests lethality of biallelic null mutations.

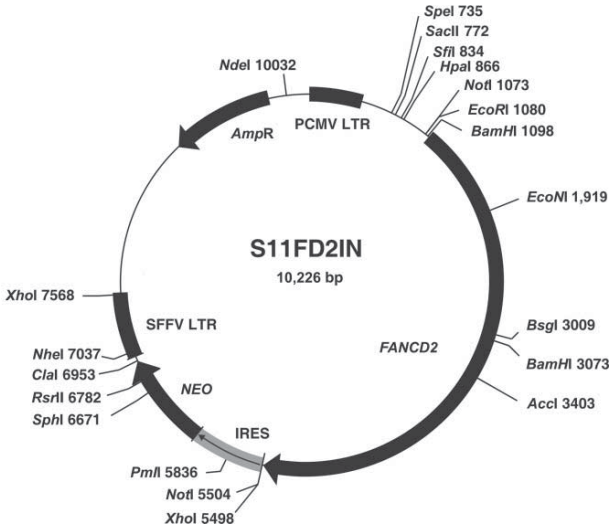


Figure 1. Circular map of vector S11FD2IN. The retroviral-expression vector S11FD2IN contains a bicistronic construct of the full-length *FANCD2* cDNA ("*FANCD2*") and the neomycin resistance gene (*NEO*). Translation of the latter is ensured by an IRES. Also shown are the long terminal repeats (LTR), the restriction sites and their positions, and the bacterial resistance (*Amp*R). Used for cloning of *FANCD2* into the target vector S11IN were the 5′ *Eco*RI and the 3′ *Sal*I (insert) and *Bam*HI (vector) sites; the latter two were destroyed by blunting.

Patients, Material, and Methods

Diagnostic Procedures

Anticoagulated 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. Clinical suspicion of FA was confirmed by the detection of cellular hypersensitivity to DNA-crosslinking agents following published procedures.[28–32] In patients with increasing and/or long-term stable blood counts, the possibility of somatic reversion leading to mosaicism of hematopoietic cells was considered, and cultured fibroblasts were used for mitomycin-C (MMC) sensitivity testing and for complementation studies.

Patient Statistics

A total of 29 fully informative FA-D2 patients (patients 1–29) were included in the present genotype-phenotype study. A fetal case (number 19) and five patients with hematopoietic mosaicism (patients 3, 14, 15, 25, and 26) were excluded from clinical follow-up studies. Four additional FA-D2 patients (patients 30–33) with incomplete clinical data were not part of the phenotype analysis, but results concerning their mutations are indicated in the text, tables, and figures.

Three end points were evaluated to determine hematologic severity: time to hematological onset, defined as "cell count of at least one lineage constantly below normal range"; period from 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 with patients in the International Fanconi Anemia Registry (IFAR), as reported elsewhere,[33] by means of log-rank test statistics. Multivariate and competing-risk analyses were not possible because of the limited number of informative patients.

Cell Culture

Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines (LCLs) were established using cyclosporin A, as described else-

Table 1. *FANCD2* cDNA Amplification Primers

PCR Fragment	Forward			Reverse			PCR Product Size (bp)
	Designation	Binding Position	Sequence (5′→3′)	Designation	Binding Position	Sequence (5′→3′)	
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	1,234
3	FA-D2, Fr.3 F	1757 to 1777	CGGCAGACAGAAGTGAATCAC	FA-D2, Fr.3 R	2979 to 2958	GTTCTTGAGAAAGGGGACTCTC	1,223
4	FA-D2, Fr.4 F	2804 to 2829	TTCTACATTGTGGACTTGTGACGAAG	FA-D2, Fr.4 R	3942 to 3922	GTCTAGGAGCGGCATACATTG	1,139
5	FA-D2, Fr.5(L) F	3761 to 3781	CAGCAGACTCGCAGCAGATTC	FA-D2, Fr.5(L) R	4700 to 4679	GACTCTGTGCTTTGGCTTTCAC	940

Table 2. *FANCD2* cDNA Sequencing Primers

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

where.[34] 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 were propagated in Earle's minimal essential medium 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 the case of fibroblasts, 5% (v/v) O_2 by replacing ambient air with nitrogen.[35] MMC treatment was for 48 h at 12 ng/ml (fibroblasts) or 15 ng/ml (LCLs) to cause cell-cycle arrest, or for 12 h at 100 ng/ml to induce monoubiquitination of the protein, FANCD2-L. In some cases, RNA stabilization was achieved by the addition of cycloheximide (CHX) to cell cultures at a final concentration of 250 μg/ml 4.5 h before RNA isolation.

Retroviral Complementation

For construction of the D2-IRES (internal ribosomal entry site)–neo retroviral expression vector S11FD2IN, the D2-IRES-puro plasmid pMMP-FANCD2[26] was cut using *Sal*I. The ends were blunted, and the fragment containing the *FANCD2* ORF was cut out with *Eco*RI and was ligated into S11IN, which was cut with *Bam*HI, was blunted, and was cut again with *Eco*RI (fig. 1). S11 vectors are based on the spleen focus–forming virus and are derived from the GR plasmid.[36] Sequencing of the retroviral plasmid S11FD2IN revealed three reported polymorphisms in the *FANCD2* ORF—c.1122A→G, c.1509C→T, and c.2141C→T[26]—and another silent base substitution, c.3978C→T. Stable retroviral packaging cells were generated by infection of PG13 cells and selection in G418 (Sigma), as described elsewhere.[37] In addition, the cDNAs encoding enhanced green fluorescent protein (*GFP*) and *FANCA* cDNAs were separately cloned into the vector S11IN for transduction of the cells under study, with GFP serving to monitor complete selection and FANCA serving as negative complementation control.

Retroviral transduction of cultured cells followed published protocols.[38,39] Selection of transduced cells was in G418 (Sigma) at a final concentration of 0.8–1.2 mg/ml for ~10 d. Transduced cells were analyzed for their sensitivity to MMC, with use of flow cytometry, to assess survival rates and cell-cycle arrest.[39,40]

Immunoblotting

FANCD2 immunoblotting was performed as described elsewhere,[9] with minor modifications. Detection was by the chemiluminescence technique with use of standard enhanced chemoluminescence reagent (Amersham) or SuperSignalWestFemto (Pierce).

Mutation and Haplotype Characterization

Primers used for cDNA amplification are shown in table 1, and those additionally used for cDNA sequencing are shown in table 2 (GenBank accession numbers NM_033084 and AF340183).

A total of seven large amplicons (superamplicons) were generated with primers that are unique to certain regions of the functional *FANCD2* gene. These primers and the sizes of the superamplicons I–VII are shown in table 3. The superamplicons served as templates in place of genomic DNA. They were used to amplify the genomic sequence before sequencing; an exception to this was superamplicon V, which was used for direct sequencing. Genomic primers for the amplification of all *FANCD2* exons and adjacent intron regions and their sizes are displayed in table 4. Additional genomic mutation-specific primers are shown in table 5.

For haplotyping, four microsatellite markers flanking *FANCD2* on chromosome 3 were studied: *D3S1597,* a dinucleotide repeat at 9.340 Mb; *D3S1038,* a dinucleotide repeat at 10.488 Mb; *D3S3611,* another dinucleotide repeat at 10.529 Mb; and *D3S1675,* a tetranucleotide repeat at 10.643 Mb. Primers used for microsatellite amplifications are specified in table 6. Two of the forward primers were 5′-Cy5-; the other two 5′-Cy5.5- primers were labeled for multiplex fragment analysis with DNA Sizing standards on a CEQ 8000 Genetic Analysis System (both Beckman Coulter).

Results

Assignment to and Frequency of Group FA-D2

Figure 2 demonstrates our strategy for the assignment of cultured FA cells to group FA-D2. MMC sensitivity was demonstrated by cell-cycle analysis that showed G2-phase arrest of the tested LCLs (fig. 2*B,* lane 2).[30–32] The apparent absence of FANCD2 bands on standard-exposure immunoblots suggested their belonging to complementation group FA-D2 (fig. 2*A,* lane 2).[41] Transduction of putative FA-D2 LCLs with *FANCD2* cDNA with use of the retroviral vector S11FD2IN restored FANCD2 expression and function, as reflected by the emergence of both FANCD2 isoforms (FANCD2-S and -L) (fig. 2*A,* lane 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 (fig. 2*A,* lane 1, and fig. 2*B,*

Table 3. *FANCD2* Superamplicon Primers

The table is available in its entirety in the online edition of *The American Journal of Human Genetics.*

Table 4. *FANCD2* Exon Primers

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

lanes 1 and 3). Transduction of D2 LCLs with *GFP* or *FANCA* resulted in neither the restoration of either FANCD2 isoform nor a normalization of G2 phase arrest, as exemplified for *FANCA* with use of S11FAIN, as shown in figure 2*A* and 2*B,* lanes 4. In cases with suspected hematopoietic mosaicism, cultured fibroblasts were assayed using a corresponding strategy (fig. 2*C* and 2*D*). As shown in table 7, only a minority of patients were assigned to group FA-D2 by primary mutation analysis. This group includes four affected siblings of four different index patients and an unrelated deceased patient with only DNA available.

In the North American IFAR collection, of 630 classified patients with FA, 7 (patients 19–25) were fully informative clinically and were included in the present cohort; another (patient 32) is among the four additional patients. Results of immunoblotting data alone suggested that 10 more IFAR patients belonged to the FA-D2 group, for a maximum estimate of 18 FA-D2 subjects in the IFAR group of 630. Among the patients referred to the two German laboratories, 15 of 243 patients with FA were assigned to complementation group FA-D2. The latter figures suggest that the proportion of patients who meet criteria for complementation group D2 may be higher than reported elsewhere.[2,24,25]

Clinical Data for FA-D2 Patients

Including, where appropriate, information from a prenatal case (number 19), phenotypic details in the present cohort of 29 FA-D2 patients with adequate clinical information are shown in tables 8 and 9. With the exception of two families (those of patients 19 and 22), there was no general tendency for increased rates of spontaneous abortions in this study. Among the 28 fully informative FA-D2 patients, there was only a single malignancy observed (acute myelogenous leukemia [AML] in patient 1), and there was no apparent overrepresentation of malignancies in the parents or grandparents of the FA-D2 patients in our cohort.

The median age at diagnosis of these FA-D2 patients was 4 years and 5 mo ($n = 29$). When the fetal case (number 19) and five mosaic patients (patients 3, 14, 15, 25, and 26) were excluded, the median age at transfusion dependency was 10 years and 10 mo ($n = 23$). Figure 3 compares the progressive hematological course and the outcome of our group of FA-D2 patients with that reported elsewhere for 754 North American IFAR patients.[42] BMF in our D2 group ($n = 23$) occurred at an earlier age (median for FA-D2 patients was 2.4 years vs. 6 years and 7 mo for IFAR; $P = .001$) (fig. 3*A*), and the period from BMF to HSCT was shorter (median age at HSCT for FA-D2 patients [$n = 9$] was 5 years and 6 mo vs. 11 years and 4 mo for IFAR [$n = 218$]; $P < .08$) (fig. 3*B*). Age at HSCT for our FA-D2 patients was earlier than in the IFAR patients of combined

Table 5. *FANCD2* Mutation-Specific Primers

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

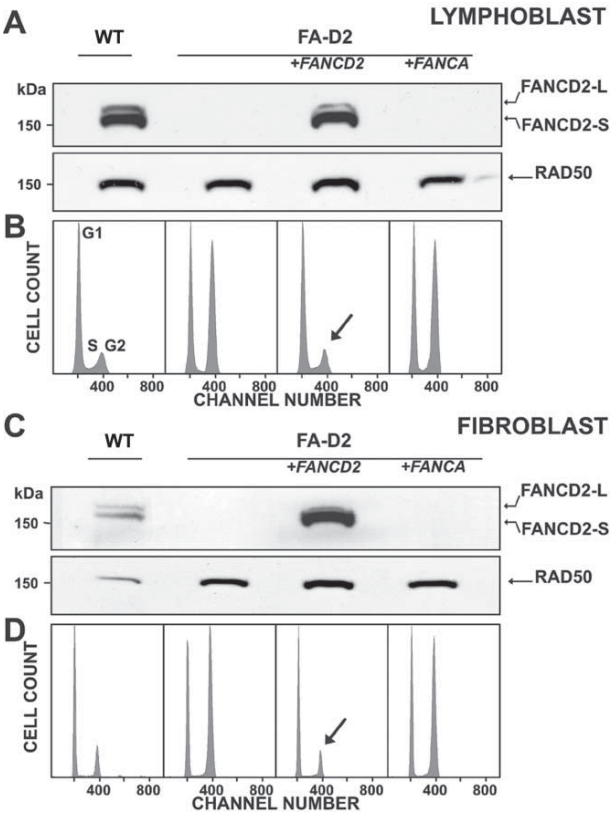


Figure 2. Delineation of FA-D2. *A,* Assignment to group FA-D2 on the basis of the absence of either FANCD2 band on immunoblots after exposure of the patients' cells to MMC, here shown for an LCL from patient 6 (*lane 2*). Transduction with *FANCD2* cDNA with use of S11FD2IN restores both isoforms of FANCD2—S and L (*lane 3*)—similar to a nontransduced normal control (*lane 1*). Transduction with *FANCA* cDNA in the same vector fails to show such restoration (*lane 4*). *B,* Assignment to group FA-D2 on the basis of cell-cycle analysis. After exposure to MMC, the LCL of the same patient shows pronounced G2-phase arrest (56.6%) (*lane 2*; Hoechst 33342 staining). Transduction with *FANCD2* cDNA by use of S11FD2IN reduces the G2 phase to normal (14.9%) (*lane 3, arrow*), similar to the nontransduced 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*). Panels C and D are analogous to panels A and B and show complementation with cultured fibroblasts from patient 10; staining in panel D was with 4′,6-diamidino-2-phenylindole (DAPI). G2-phase proportions in panel D are 20.3% (*lane 1,* control), 61.3% (*lane 2,* nontransduced FA), 19.9% (*lane 3, FANCD2*-transduced FA), and 58.5% (*lane 4, FANCA*-transduced FA). RAD50 [MIM 604040] was used as the loading control in panels A and C. WT = wild type.

Table 6. Microsatellite Primers

STR	Genomic Position (Mb)	Primer Sequence (5′→3′) Sense	Antisense
D3S1597	9.34	AGTACAAATACACACAAATGTCTC	CAATTCGCAAATCGTTCATTGCT
D3S1038	10.49	AAAGGGGTTCAGGAAACCTG	CCCTCCAGTAAGAGGCTTCCTAG
D3S3611	10.53	GCTACCTCTGCTGAGCATATTC	CACATAGCAAGACTGTTGGGGGC
D3S1675	10.64	GGATAGATGGATGAATGGATGGC	CCTCTCTAACTACCAATTCATCCA

groups (median age for FA-D2 patients was 10 years and 11 mo vs. 27 years and 11 mo for IFAR; $P < .01$) (fig. 3*C*). Of 23 FA-D2 patients, 9 patients of our cohort received HSCT. Kaplan-Meier estimates (fig. 3*D*) suggest that our FA-D2 patients ($n = 23$) may have a shorter life span than the IFAR patients overall, since their survival curve falls below that of the IFAR patients after age 9 years; however, the difference of median survival (11 years and 4 mo for FA-D2 patients vs. 24 years and 3 mo for IFAR) was not significant, because the number of nonmosaic FA-D2 patients (two) of our cohort who reached adulthood was too low for statistical support.

FANCD2 *and the* FANCD2 *Pseudogenes*

BLAT searches (Human BLAT Search) identified two pseudogene regions: *FANCD2-P1* spanning 16 kb, located ~24 kb upstream of *FANCD2,* and *FANCD2-P2* spanning 31.9 kb, located ~1.76 Mb downstream of *FANCD2* (fig. 4*A*). *P1* and *P2* are in the same orientation as the functional gene. They are characterized by high sequence homology with certain *FANCD2* exons and have retained ordered succession of their exon/intron equivalents, compared with the functional gene. On the other hand, the exon replicas of *FANCD2-P1* and *FANCD2-P2* have acquired numerous deletions and insertions. Striking sequence similarity of the D2 pseudogenes extends into some *FANCD2* introns, particularly in the regions of IVS21-IVS26. Thus, *P1* and *P2* reveal recognizable patterns of conserved gene structure (fig. 4*B*). *FANCD2-P1* is roughly a copy of the front portion of *FANCD2,* including, with intermittent gaps, the region of exons 1–18 (homology with *FANCD2* exons 1 and 12–16 and the 3′ portion of exon 18). The region upstream of *FANCD2-P1* shares homology with the putative *FANCD2* promoter predicted within the CpG-rich region of ~800 bp upstream of the start codon of the functional gene.[60] The corresponding region upstream of *P1* is interrupted by an *Alu*Y element (RepeatMasker). *FANCD2-P2* is an approximate match of the middle portion of *FANCD2*. Including gaps, it spans the region of exons 12–28 (homology with *FANCD2* exons 12–14 and 17–28).

Mutations in FANCD2

Unique amplification of the functional *FANCD2* gene was achieved using primers that exclude pseudogene sequences. In *FANCD2* regions sharing extensive homology with *FANCD2-P1* and *-P2,* seven superamplicons (fig. 4*C*) were used for genomic mutation screens. Studies at the RNA level were implemented to guide the genomic analyses. All identified mutations and their predicted consequences at the protein level are compiled in table 10. The distribution of the mutations among the individual patients is shown in table 7.

Mutations Affecting Pre-mRNA Splicing

In peripheral-blood lymphocytes (PBLs), LCLs, and cultured fibroblasts from unaffected controls, two species of *FANCD2* cDNAs were consistently detected by sequence analysis of the regions corresponding to exon 22 (fig. 5*A* and 5*B*) and exons 15–17 (data not shown). This observation suggests low-level skipping of these exons, consistent with FANCD2 RNA being subject to alternative splicing. mRNA stabilization via CHX treatments of cultured cells resulted in a relative increase of the alternatively spliced mRNA species (fig. 5*A* and 5*C*), implying instability of the alternatively spliced FANCD2 mRNAs. On genomic sequencing, alternative splicing of exon 22 was not associated with any mutation or variant in exon 22 or the adjacent intronic regions in 25 controls.

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 (fig. 5*A* and 5*D*). Patients 9 and 10, with balanced levels of exon 22 skipping and retention, were compound heterozygous carriers of the mutation c.1948-16T→G. A different base substitution preceding exon 22, c.1948-6C→A, was present on one allele of the compound-heterozygous patients 2, 8, 14, 15, and 20, likewise resulting in similar levels of exon 22 skipping and retention. Both mutations—c.1948-16T→G and 1948-6C→A—are predicted to disrupt the splice-acceptor recognition in intron 21 suggested by impaired scores of the 3′ splice site relative to the wild type (see, e.g., table 11). Both result in skipping of exon 22.

Three apparently unrelated patients (patients 6, 12, and 30) showed balanced levels of skipping and retention of exon 5 due to heterozygous insertional mutagenesis by an *Alu* element between positions c.274–57 and c.274–56 into an AT-rich target sequence in IVS4. Integration site,

Table 7. Laboratory Diagnostic Data for the Cohort of 29 FA-D2 Patients

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 8. Clinical Phenotype of the Cohort of FA-D2 Patients

Malformation	No. of Affected/ Total[a]	Frequency (%)
Symptom:		
Microcephaly	25/28	89
(Intrauterine) growth retardation	25/29	86
Anomalies of skin pigmentation	21/28	75
Radial-ray defects	21/29	72
Microphthalmia	17/28	61
Renal anomalies	10/28	36
Malformations of the external ear	9/28	32
Brain anomalies	9/29	31
Including hydrocephalus	5/29	17
Psychomotor retardation[b]	8/28	29
Hypogonadism/genital anomalies	7/28	25
Hip dysplasia/dislocation	6/28	21
Heart anomalies	4/28	14
Malformations of the gastrointestinal tract	4/28	14
Distinct syndromic association:		
Michelin-tire baby syndrome (MIM 156610)	2/28	7
VACTERL-like association (MIM 192350)	1/28	4
Holoprosencephaly (MIM 236100)	1/28	4
Kartagener syndrome (MIM 244400)	1/28	4

[a] A prenatal case (number 19) was partially informative. Pertinent information was used where applicable.
[b] Including attention-deficit/hyperactivity disorder.

type, length, and orientation of the *Alu* and the duplicated *FANCD2* intron sequence were identical in all three patients. We did not detect any such *Alu* insertions in 300 unaffected control alleles.

Aberrant splicing of exons 4, 5, 10, 13, 15–17, 28, and 37 was observed in other patients also (analysis of the regulatory splec sequences by ESEfinder and Rescue-ESE). Patients 28 and 29 showed skipping of exon 4 caused by 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 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 reported elsewhere.[26] 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 second-to-the-last 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 the exclusion of the corresponding sequence from the mature mRNA. A 2-bp deletion in exon 16 (c.1321_1322delAG) in patient 18 causes skipping of exons 15–17. In that case, aberrant splicing occurs in the same position as low-grade alternative splicing in normal controls, but at heterozygous levels. Patients 10 and 22 showed inclusion of a 27-bp sequence of intron 28 into mRNA because of a splice-donor mutation (c.2715+1G→A) and the use of a cryptic splice-donor downstream. Patient 11 had a base substitution in exon 37 (c.3707G→A), reported elsewhere,[26] that abrogates the normal splice acceptor 25 bp upstream and activates a cryptic site 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 of the 24 nt in between. With the exception of those of patient 1, all of these splicing aberrations were due to heterozygous mutations. Patient 1 showed homozygous exonization of an IVS9 fragment because of a mutation in intron 9 (c.696-121C→G), which activates cryptic splice sites. Predicted scores and consequences of some of these splice mutations are computed in tables 11 and 12. Apart from 1321_1322delAG, which causes skipping of exons 15–17, all mutations affecting splicing in the patients in this study result in frameshifts and subsequent premature termination of translation. More than half—that is, 30 of 58 mutated alleles of the 29 clinically informative FA-D2 patients, or 34 of all 66 alleles—were splicing mutations, which makes that the most prevalent type of mutation.

Other Mutations

There were five different heterozygous nonsense mutations in nine patients from six families (c.757C→T in siblings 23 and 24, c.1092G→A in patient 7, c.2404C→T in patient 21, c.2775_2776CC→TT in siblings 14 and 15, and

Table 9. Clinical Diagnostic Data for the Cohort of 29 FA-D2 Patients

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 10. Identified *FANCD2* Mutations and Their Effects

Location and Patient No(s).	Mutation[a]		Consequence[a]
	Genomic DNA	RNA	Protein
Exon 2:			
32	c.2T→C	r.2T→C	Failure of normal translation initiation
Intron 3:			
28 and 29	c.206-2A→T (*IVS3-2* A→T)	r.206_273del68 (exon 4 skipping)	p.A69DfsX7
Intron 4:			
6, 12, and 30	c.274-57_-56insinv*Alu*Yb8nt36_319 +dup c.274-69_−57[b]	r.274_377del104 (exon 5 skipping)	p.I92YfsX7
Exon 5:			
26 and 27	c.376A→G	r.376A→G+r.377_378ins13 (aberrant splicing)	p.S126RfsX12
Exon 9:			
19	c.692T→G	r.692T→G	p.L231R
Intron 9:			
1	c.696-121C→G (*IVS9-12* 1C→G)	r.695+1619_696-126ins34 (exonization)	p.S232insQNNFX
18	c.696-2A→T (*IVS9-2* A→T)	r.696_783del88 (exon 10 skipping)	p.S232RfsX6
Exon 10:			
23 and 24	c.757C→T	r.757C→T	p.R253X
31 and 33	c.782A→T	r.696_783del88 (exon 10 skipping)	p.S232RfsX6
Exon 11:			
9	c.810_812delGTC	r.810_812delGTC	p.S271del
Exon 12:			
7	c.904C→T	r.904C→T	p.R302W
Intron 12:			
8	c.990-1G→A (*IVS12-1* G→A)	r.990del8 (aberrant splicing)	p.S330RfsX16
Exon 13:			
7	c.1092G→A	r.1092G→A	p.W364X
Intron 14:			
33	g.13377_17458dup4082 (duplication, including exons 11–14)	r.784_1134dup (duplication of 351 nt in-frame)	p.262_378dup (duplication of 117 aa)
Exon 16:			
18	c.1321_1322delAG	r.1135_1545del411 (exon 15–17 skipping)	p.V379_K515del
23 and 24	c.1367T→G	r.1367T→G	p.L456R
31	c.1370T→C	r.1370T→C	p.L457P
Exon 17:			
28, 29	g.22875_23333del459 (c.1414-71_c.1545+256del459)	r.1414_1545del132	p.E472_K515del
Intron 21:			
3, 4, 5, 9, 10, 13, and 25	c.1948-16T→G (*IVS21-16* T→G)	r.1948_2021del74 (exon 22 skipping)	p.E650X
2, 8, 14, 15, and 20	c.1948-6C→A (*IVS21-6* C→A)	r.1948_2021del74 (exon 22 skipping)	p.E650X
Exon 26:			
21	c.2404C→T	r.2404C→T	p.Q802X
16, 17, 19, 21, 22, and 30	c.2444G→A	r.2444G→A	p.R815Q
Exon 28:			
20	c.2660delA	r.2660delA	p.E888RfsX16
Intron 28:			
10 and 22	c.2715+1G→A (*IVS28*+1G→A)	r.2715_2716ins27 (aberrant splicing)	p.E906LfsX4
Exon 29:			
14 and 15	c. 2775_2776CC→TT	r. 2775_2776CC→TT	p.R926X
11	c.2835dupC	r.2835dupC	p.D947RfsX3
Exon 34:			
12	c.3453_3456delCAAA	r.3453_3456delCAAA	p.N1151KfsX46
Exon 36:			
2	c.3599delT	r.3599delT	p.I1200KfsX12
Exon 37:			
32	c.3706C→A	r.3684_3707del24 (aberrant splicing)	p.R1228S_F1235del
11	c.3707G→A	r.3684_3727del44 (aberrant splicing)	p.H1229EfsX7
Exon 38:			
6, 26, and 27	c.3803G→A	r.3803G→A	p.W1268X

[a] Nomenclature is according to the Human Genome Variation Society.

[b] This *Alu* was identical to the evolutionary young subfamily Yb8.[43,44] It was lacking its annotated nucleotides 1–35, had integrated in reverse orientation (with its poly-A tail toward 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 both sides. Altogether, the insertion length was 298 bp.

c.3803G→A in patient 6 and siblings 26 and 27) (tables 7 and 10). In addition, we detected five different missense mutations in 11 patients from nine families (c.692T→G in patient 19; c.904C→T in patient 7, identical to a mutation reported elsewhere[26]; c.1367T→G in siblings 23 and 24; c.1370T→C in patient 31; and c.2444G→A in siblings 16 and 17 and patients 19, 21, 22, and 30). These amino acid substitutions were classified as missense mutations because of their absence from unaffected controls, their absence from FA-D2 patients of our cohort with other biallelic mutations, and their occurrence at evolutionarily conserved residues (ClustalW; ExPASy). Missense mutations were either compound heterozygous in combination with other types of *FANCD2* mutations or were homo-

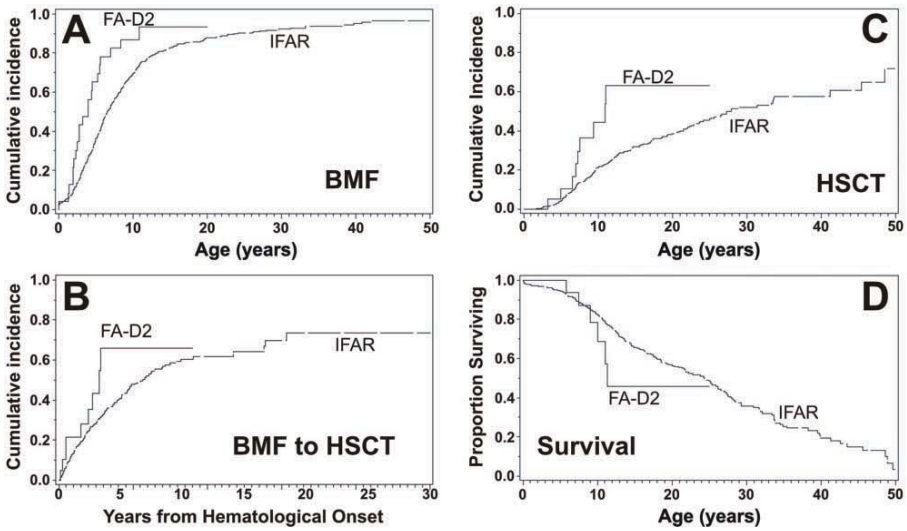


Figure 3. Clinical course of 23 fully informative, nonmosaic FA-D2 patients in this study. *A,* The cumulative incidence of BMF of the FA-D2 patients in the present study (FA-D2) precedes that of all patients with FA in the IFAR[42] ($P = .001$). *B,* The period from BMF to HSCT, which was shorter in the patients of the present study than in those of the IFAR[42] (trending, $P < .08$). *C,* Cumulative incidence of HSCT of the FA-D2 patients in our study, which likewise antedates that of all patients in the IFAR[42] ($P < .01$). *D,* Kaplan-Meier curves of survival, which suggest higher death rates of the FA-D2 patients than of all patients in the IFAR aged >10 years.

zygous in consanguineous families. Three unrelated patients had small deletions (c.2660delA in patient 20, c.3453_3456delCAAA in patient 12, and c.3599delT in patient 2) resulting in frameshifts. Another small deletion was in frame and affected a single codon (c.810_812delGTC in patient 9). There was only a single small frameshift duplication (c.2835dupC in patient 11). A large genomic deletion (g.22875_23333del459) spanning the entire exon 17 (similar to a mutation reported elsewhere without defined breakpoints[26]) and the 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. A large genomic duplication in patient 33 included exons 11–14 and resulted in the insertion of 132 aa. Both gross gene rearrangements retained the reading frame. In all of our patients, nonsense mutations, deletions, and insertions affected exclusively single alleles in combination with splice or missense mutations. A unique case was a compound heterozygous start codon mutation (c.2T→C) in patient 32.

Figure 6 illustrates the distribution of *FANCD2* mutations that were identified in this study, including those of four FA-D2 patients who were reported elsewhere.[26,27]

Ethnic Associations and Shared Alleles

Relatively severe birth defects and early hematological onset were observed in three patients (4, 5, and 13) who were homozygous for the splice mutation c.1948-16T→G with exon 22 skipping. These three patients and two other homozygotes with reverse mosaicism in the hematopoietic system (patients 3 and 25) were all from four consanguineous Turkish families. Of two other FA-D2 patients who were compound heterozygotes for this mutation, one was also of Turkish origin; the other came from the eastern Czech Republic. The splice mutation c.1948-6C→A, likewise leading to exon 22 skipping, was detected in five patients (2, 8, 14, 15, and 20), including two sisters (patients 14 and 15). These patients came from three families in northern Germany and an American family of German ancestry (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, from a consanguineous Spanish family (patients 16 and 17), with the homozygous missense substitution c.2444G→A. 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 ancestry (patient 22), and another had Spanish and Portuguese ancestry (patient 30). An allele with the insertion of an *Alu*Yb8 element was found in compound heterozygous patients of German (patient 6), Danish (patient 12), and Spanish/Portuguese (patient 30) descent. No other mutations occurred in more than two families.

On haplotype analysis using markers flanking *FANCD2,* all patients homozygous for the intron 21 mutation detected in the Turkish population were homozygous for markers *D3S1597, D3S1938, D3S3611,* and *D3S1675.* The resulting haplotype was shared, in a heterozygous state, with the nonconsanguineous compound heterozygous

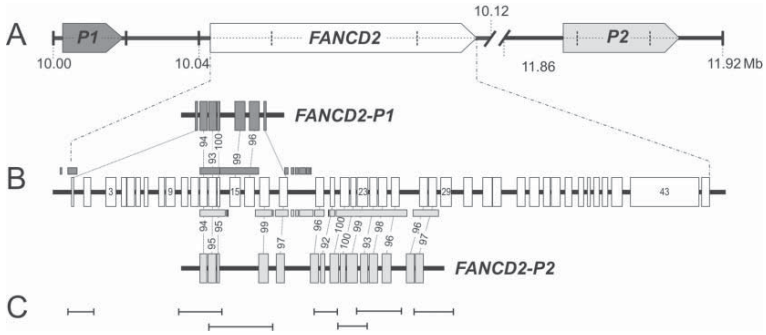


Figure 4. Topography of *FANCD2,* its pseudogenes, and the superamplicons. *A,* The two pseudogenes—*FANCD2-P1* and *FANCD2-P2*—located upstream and downstream, respectively, of the functional *FANCD2* gene. All three have the same orientation. The scale denotes Mb on chromosome 3. *B, FANCD2* exons and their pseudogene equivalents, connected by dashed lines, indicating percentages of nucleotide identity. Homology also extends into many introns nearby, as indicated by the boxes beyond and below the active gene. *C,* Graphic presentation of the positions and sizes of 7 superamplicons relative to the active gene shown in panel B. These amplicons represent *FANCD2* exon-exon or exon-intron regions. Unique primer-binding sites ensure specific amplification.

Turkish patient (patient 10). The Czech patient (patient 9) with the same 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) on at least one side of their mutated *FANCD2* gene. This finding suggests that c.1948-6C→A is an old mutation, with 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 (patient 21), a patient with Sicilian ancestry (patient 22), and a patient of Spanish/Portuguese descent (patient 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). Sequence analysis of the parents indicated that both substitutions were on the same allele. A single patient (patient 19) with the mutation c.2444G→A shared neither the haplotype nor the polymorphism c.2702G→T. Apart from c.2702G→T, which was also observed without association with the mutation c.2444G→A, the only new *FANCD2* polymorphisms detected in our study were c.3978C→T (synonymous base substitution, exon 41) and c.4478A→G (3′ UTR, according to GenBank accession number NM_033084, or intron 43, according to accession number AF340183). All others have been reported elsewhere.[26] Despite clear ethnic association of the patients with the insertion of an *Alu*Yb8 element in intron 4, 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 <10% of complete *Alu*Yb8 elements in the human genome (Human BLAT Search) further suggests that the *Alu* insertion goes back to a single event and is an ancient rather than a recurrent mutation.

Reverse Mosaicism

Among the 28 fully informative FA-D2 patients in this study (excluding fetal case number 19), five (patients 3, 14, 15, 25, and 26) developed reverse mosaicism in the hematopoietic system. Mosaic patients were recognized by the fact that they had levels of both FANCD2-S and -L in protein from LCLs that were comparable to those of unaffected controls (fig. 7*A*). They also had low chromosome breakage rates in blood and blood-derived LCLs (table 7), and they had lost the typical G2-phase arrest of their lymphocytes after exposure to MMC (fig. 7*C*). Nonetheless, these patients displayed the characteristic clinical FA phenotype. Their cultured fibroblasts failed to show either FANCD2 isoform on standard immunoblots (fig. 7*B*). They were sensitive to MMC, as indicated by elevated chromosome breakage and G2-phase arrest (fig. 7*C*). Molecular studies confirmed these findings. Two patients with heterozygous base substitutions in the coding sequence, resulting in a nonsense (patient 14) and a splice (patient 26) mutation, showed reversion to the respective wild-type bases in primary blood cells and LCLs. The mechanism of these reversions is not clear and could involve back mutation, recombination with loss of heterozygosity, or recombination with gene conversion. Intragenic mitotic crossover is the likely but not proven mechanism of mosaicism in the sibling of patient 14 (patient 15) who had retained her dinucleotide substitution in her peripheral-

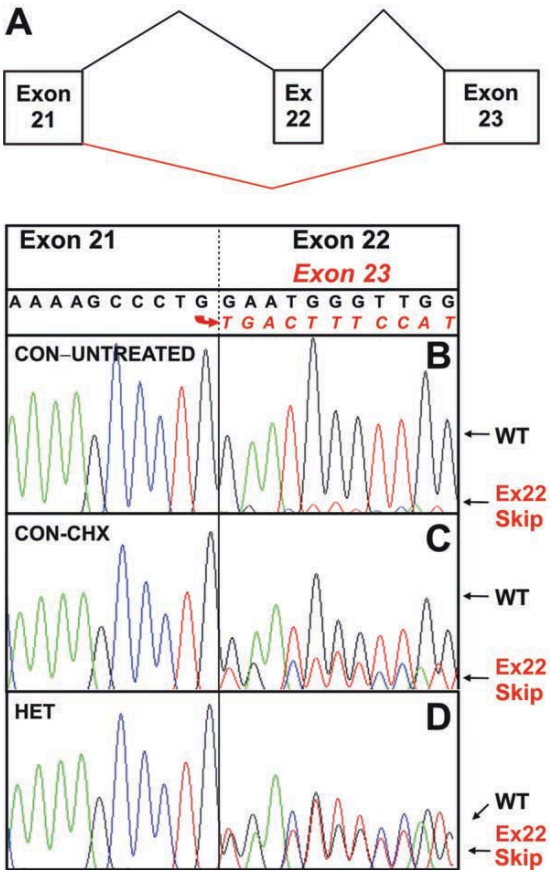


Figure 5. Exon 22 splicing. *A,* Schematic depiction of the splicing patterns resulting from exon 22 retention or skipping. *B,* cDNA sequencing in an LCL from a normal control (CON), showing predominance of exon 22 sequence following that of exon 21 but also low levels of underlying sequence readable as exon 23. *C,* Treatment of the same LCL from a normal control with CHX for 4 h before cDNA synthesis, which increases the relative level of sequence with exon 22 skipping. *D,* cDNA sequencing in an LCL from a compound heterozygote (HET) for splice-acceptor mutation in intron 21, c.1948-16T→G (patient 9), which shows comparable levels of inclusion and exclusion of exon 22 sequence following that of exon 21. Ex = exon; WT = wild type.

blood cells. Two patients (3 and 25) with the c.1948-16T→G splice mutation had different second-site mutations nearby. The compensatory mutation of patient 3 was c.1954G→A (p.V652I), detected in blood, bone marrow, and an LCL. The compensatory mutation of patient 25 was c.1953G→T (p.W651C), detected in blood. c.1954G→A restored exon 22 retention correctly. c.1953G→T cDNA was not available. Clinically, three of five mosaic patients (3, 14, and 15) in the present cohort experienced a mild or protracted hematological course. The other two of five patients (patients 25 and 26) had no apparent benefit from their mosaicism; one of them required relatively early HSCT, and the other died of intracranial hemorrhage (table 9). The rate of 17% mosaic FA-D2 patients in our study is within the 15%[46] to 20%[47] or 25%[48] range reported for other complementation groups. With a rate comparable to *FANCA, FANCD2* appears to be another FA gene particularly prone to reverse mosaicism.

Residual FANCD2 Protein

cDNA sequencing of LCLs of patients 3, 4, 5, and 13 showed nearly complete exon 22 skipping. However, we consistently observed a small amount of correctly spliced mRNA retaining exon 22 (fig. 8*A*; compare with fig. 5*A*). Genomic sequencing identified homozygosity of these patients for the common underlying mutation, the base substitution c.1948-16T→G in IVS21. Homozygosity for this mutation was also observed in the deceased patient 25 with no cDNA available. All of these patients were products of consanguineous matings. In all LCLs that were homozygous for the above splicing mutation, we were able to demonstrate minute amounts of FANCD2 protein. A more surprising finding, however, was the presence of residual FANCD2 protein in PBLs and LCLs of every tested FA-D2 patient. Detection of residual protein required overexposure of FANCD2 immunoblots (fig. 8*B*). Unlike standard exposures that revealed no FANCD2 bands in most of the FA-D2 cell lines (see, e.g., fig. 2), 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 lines, all 21 LCLs available from our 29 fully informative FA-D2 patients had minute but unequivocal amounts of residual protein (table 7). This was also true for CD3/CD28/IL-2–stimulated PBL cultures from patient 13. Patients 4, 17, and 19 with no available LCLs remained untested. In contrast, our mosaic patients displayed levels of FANCD2 protein in the control range. They had normal chromosome-breakage rates and

Table 11. ***FANCD2*** **3′ Splice-Acceptor Calculations**

Exon/Intron and Designation	Sequence	MaxEntScan Score[a]
4:		
Consensus	ctcttctttttctgcatagCTG	9.12
c.206-2A→T	ctcttctttttctgcat**t**gCTG	.76
10:		
Consensus	tctttttctaccattcacagTGA	7.39
c.696-2A→T	tctttttctaccattcac**t**gTGA	−.97
13:		
Consensus	ttcctctctgctacttgtagTTC	6.19
c.990-1G→A	ttcctctctgctacttgta**t**TTC	−2.56
22:		
Consensus	tgtttgtttgcttcctgaagGAA	6.43
c.1948-16T→G	tgtt**g**gtttgcttcctgaagGAA	5.58
c.1948-6C→A	tgtttgtttgcttc**a**tgaagGAA	4.51
37:		
Consensus	ACTTTTGTTGTTTTCTTCCGTGT	2.10
c.3706C→A	ACTTTTGTTGTTTTCTTC**A**GTGT	10.14

[a] MaxEntScan::score3ss for human splice sites.

Table 12. ***FANCD2*** **Splice-Donor Calculations**

Exon/Intron and Designation	Sequence	Score (Splicefinder)	Difference	Result
5:				
Consensus	CAGgtgtggag	LC4 12||3|2|		
c.376A→G	C**G**Ggtgtggag	LC2 2|8||3|2	Large	Malfunction
Cryptic splice donor	GAGgcatggaa[a]	HC1 |12|3|2|		Gain of function
9:				
Consensus	acggtaactta	LC4 ||12|2||		
c.696-121C→G	ACGgtaa**g**tta	HC3 ||17||	Large	Gain of function
10:				
Consensus	AAGgtagaaaa	LC4 |12|||2|		
c.782A→T	A**T**Ggtagaaaa	LC3 ||10|||2|	Small	Malfunction
28:				
Consensus	AAGgtattgga	LC4 |12|||||		
c.2715+1G→A	AAG**a**tattgga	No score	Large	Malfunction
Cryptic splice donor	AAGgtttgtga[a]	LC4 |10||5||		Gain of function

[a] Mutation-activated cryptic splice donor; calculation available only for the consensus dinucleotide gt.

lacked G2-phase arrest, whereas the nonmosaic patients had high chromosome breakage and G2 arrest. We therefore consider it unlikely that undetected mosaicism accounts for the presence of residual protein in the remainder of our patients. In support of this conclusion, we were able to detect residual protein, including both -S and -L bands, in fibroblasts from patients 3, 14, and 26 when the blot of figure 7*B* was overexposed (data not shown). Densitometry suggested reductions of residual FANCD2 protein on the order of 1 of 100 to 1 of 1,000 relative to wild type, with the degree of expression differing greatly among individual LCLs (fig. 8*B*). FA-D2 LCLs with the highest

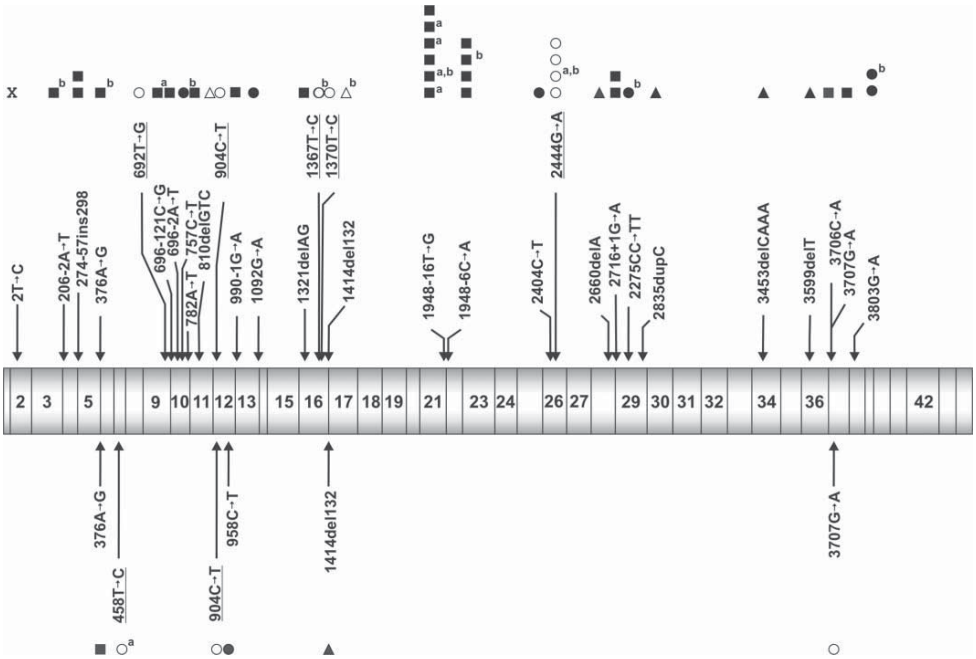


Figure 6. Positions and identity of mutations detected in *FANCD2*. Mutations identified in the present study are shown above, mutations reported elsewhere[24,45] are indicated below the schematic display of *FANCD2* cDNA. Blackened squares (■) represent mutations resulting in aberrant splicing patterns, blackened circles (•) represent nonsense mutations, unblackened circles (○) represent missense mutations, blackened triangles (▲) represent frameshift deletions or duplications, and unblackened triangles (Δ) represent in-frame deletions or duplications. Missense mutations are depicted above or below the other mutations and are underlined. Superscript a at the right upper corner of a symbol denotes homozygous occurrence (2 alleles); superscript b denotes an affected sibling (relationship bias). Mutation 3707G→A was originally reported as a missense mutation,[26] whereas we characterized it as a splicing mutation.

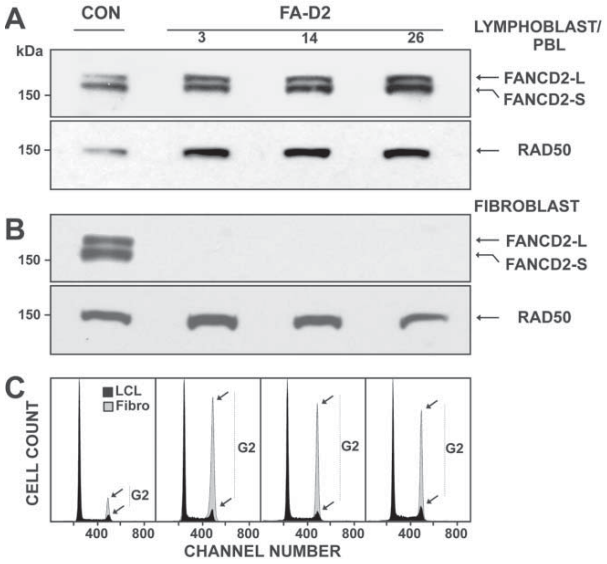


Figure 7. Reverse mosaicism. Blood-derived cells from FA-D2 patients with reverse mosaicism of the hematopoietic system (patients 3 and 26, LCLs; patient 14, stimulated PBL; *panel A, lanes 2, 3,* and *4*) reveal both FANCD2 bands at levels similar to a random normal control (*lane 1*) after exposure to MMC. In contrast, neither FANCD2 band was present in fibroblasts from the same patients, but only in the control (*B*). RAD50 was used as loading control in panels A and B. The LCLs and PBL used in panel A fail to show G2-phase arrest on flow cytometric cell cycle distributions in response to MMC (*panel C*) (*black histograms* indicate DAPI stain; control (CON), 8.0% G2; patient 3, 8.8% G2; patient 14, 8.8% G2; patient 26, 10.4% G2), whereas the corresponding cultured FA-D2 fibroblasts retain high G2-phase accumulations, which is again in contrast to the non-FA control (*superimposed gray histograms*; CON, 22.6% G2; patient 3, 53.2% G2; patient 14, 56.0% G2; patient 26, 54.8% G2). PBLs in panel A were stimulated with anti-CD3, anti-CD28, and Il-2 and, in panel B, with PHA.

levels of residual FANCD2 were used to examine its characteristics on overexposed blots. The intensity of the FANCD2-L band increased as a function of the concentration of the DNA crosslinking agent (fig. 8*C*) 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, apparently influenced by the high proportion of patients from specific ethnic groups in the present study. [2,24,25] The relatively large proportion of FA-D2 patients with Turkish ancestry in the present study 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 with the European FA population. The proportion of FA-C patients in the IFAR is 15%,[49] compared with only 10% in the European data set.[2] This is due to the relatively high frequency of Ashkenazi Jewish patients with FA in the IFAR who have the prevalent *FANCC* mutation c.456+4A→G (formerly referred to as "IVS4+4A→G"),[33] comprising 7.5% of all IFAR patients and 50% of these FA-C patients. On the basis of our present data, we estimate that ~6% of patients with FA belong to complementation group D2. This estimate is supported by recent studies with figures of 4 of 53 (~7.5%),[46] 3 of 73 (~4.1%),[50] and 5%.[49]

The FA-D2 patients in our cohort displayed anomalies and malformations typical of FA such that there were no exceptional clinical features that had not been observed elsewhere.[51] However, it is remarkable that not a single FA-D2 patient lacked phenotypical manifestations, whereas the proportion of patients with FA without anomalies and malformations is generally estimated to be as high as 30%.[24] Growth retardation was present in 86% of the present cohort, substantially higher than the 58%[52] and 63%[24] reported. Microcephaly was present in 89% of the FA-D2 cases; in contrast, Faivre et al.[52] found anomalies of the head in only 56%. Anomalies of skin pigmentation were present in 75% of our FA-D2 cohort, compared with 71% and 64% reported elsewhere.[24,52] Of our FA-D2 patients, 72% had radial-ray defects, in contrast to only 47%[52] or 49.1%[51] of all patients with FA. Of the patients in the present study, 61% had microphthalmia, whereas 38% has been the reported percentage in other patients with FA.[24] As with these rather common phenotypic alterations, FA-D2 patients also showed 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% such individuals in other studies.[24,52] A third of our FA-D2 patients had anomalies of the brain, whereas other studies report such alterations on the order of 4.5%,[52] 7.7%,[51] and 8%[24] of their patients with FA. Of our FA-D2 patients with brain anomalies, 17% had hydrocephalus, in contrast to 4.6% reported elsewhere.[51] Since several laboratories contributed to the present study and since all of our FA-D2 patients came from groups of patients with previously unassigned FA, it is unlikely that our rates reflect major biases. However, a more severe D2 phenotype has also been observed in *Drosophila,* comparing *Fancd2* and *Fancl* knock-down.[23] 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 time the patients were aged 2 years. The median age at diagnosis in our cohort was 4.5 years, which is considerably younger than for other patients with FA, for whom the diagnosis is made in only 30% of patients before onset of hematological manifestations at the median age of 7.6 years.[53] In addition to an earlier median

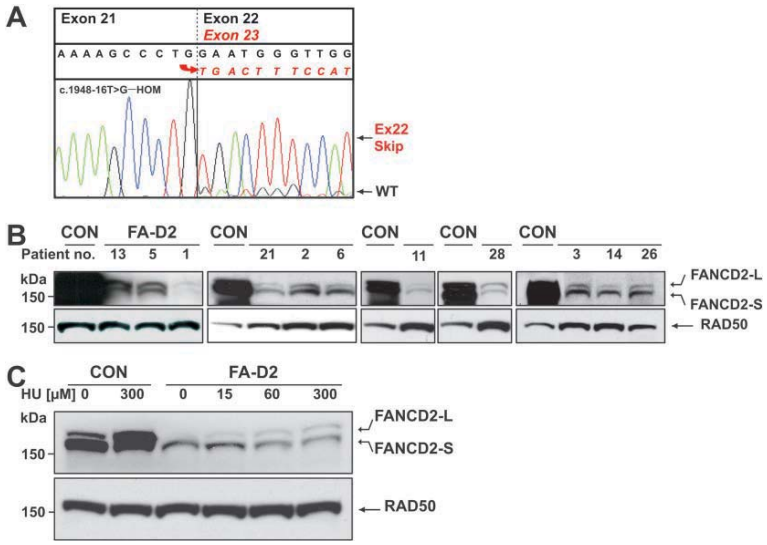


Figure 8. Residual FANCD2 protein. *A,* Exon 23 sequence following that of exon 21 (exon 22 exclusion, aberrant splicing), which prevails in cDNA from homozygotes for the splice-acceptor mutation in intron 21, c.1948-16T→G, but, at low level, underlying sequence is readable as exon 22 (exon inclusion). Depicted are results from patient 5. *B,* Blood-derived cells from nonmosaic FA-D2 patients (exemplified 13, 5, 1, 21, 2, 6, 11, and 28) show faint but conspicuous FANCD2 bands of both species in response to MMC exposure exclusively on overexposed immunoblots, as indicated by the very intense FANCD2 signals of the normal controls (CON) (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. *C,* 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-dependent response. Normal control LCLs are distinctive by their prominent FANCD2 signals.

age of hematological onset (i.e., BMF) in our patients with FA, there was a shorter median period between BMF and HSCT, earlier HSCT, and a tendency toward shorter median survival than of all FA-affected patients listed in the IFAR.[42] Because of 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 rather frequent therapeutic option in FA-D2 patients. In theory, however, deficient ATM [MIM 607585]/ATR [MIM 601215]–dependent phosphorylation of FANCD2[45,54,55] could involve additional toxicity of conditioning regimens. Collectively, our data suggest that FA-D2 patients represent a group with frequent but typical congenital anomalies and malformations and with relatively early hematological manifestations, compared with most other FA complementation groups.

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. In our cohort, LCLs and PBLs from 21 fully informative, nonmosaic study FA-D2 patients showed traces of residual FANCD2 protein. Importantly, the residual protein always consisted of both FANCD2 isoforms, and the typical time- and dose-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.[10,56] 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. These differences render any quantitative mutation-specific comparisons of residual FANCD2 protein levels close to impossible. The existence of residual protein has been described elsewhere for other FA-D2 patients,[9,26,27,46] but our study confirms residual protein as a consistent and, in all likelihood, essential feature of cells of FA-D2 patients. Somatic reversion as a cause of residual protein levels could be excluded in many of the patients in this study, because the diagnosis of FA in these patients was based on hypersensitivity to cross-linking agents of their cells in vitro. It could be argued that these LCLs might include very small, undetected amounts of reverted cells and thus might actually represent low-proportion mosaics. However, reverse mosaicism has been widely observed only at certain rates (<25%) and not at the frequency at which we detected residual FANCD2 protein. Moreover, reverted cells, once present, tend to outgrow nonreverted FA cells rather rapidly, at least in LCLs, which never occurred in our long-term studies. Finally, detection of residual protein with both FANCD2-S and -L bands in some of our FA-D2 fibroblast

lines without the possibility of mosaicism strongly argues in favor of residual protein and against somatic reversion. Of note, the vast majority of our FA-D2 patients are homozygous or compound heterozygous for at least a missense mutation or a nucleotide substitution affecting splicing without altering the canonical AG and GT dinucleotides of splice sites. Missense mutations might lead only to a partial degradation of FANCD2, whereas the other type of mutations causing exon skipping or aberrant splicing might compete against but not abrogate the original splice site, guaranteeing a residual amount of FANCD2.

FANCD2 is targeted to chromatin after DNA damage-dependent monoubiquitination. In this process, FANCE binds to FANCD2, providing the critical bridge linking FANCD2 to FANCC and the rest of the FA core complex.[57] The binding site of FANCD2 for FANCE has not been determined. Our data suggest that this binding site, in addition to K561 and apart from an intact FA nuclear core complex, must be retained by mutated residual FANCD2 protein to become monoubiquitinated.

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 merely 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 the work of Garcia-Blanco et al.[58]). The tight regulation of FANCD2 expression and activation and the presence of low-abundant wild-type 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 the work of Mansfield et al.[59]).

Acknowledgments

We thank Richard Friedl (Wurzburg), for expert flow cytometry; Birgit Gottwald (Wurzburg), for dedicated cell culture work; Daniela Endt (Wurzburg), for sequencing; Dr. Sabine Herterich (Wurzburg), for microsatellite analyses; and Kerstin Goettsche and Silke Furlan (Dusseldorf), for assistance with retroviral vectors. We are grateful to Dr. Heidemarie Neitzel (Berlin), for providing patient DNAs, and to Ralf Dietrich (Unna), for facilitating personal contacts with FA-affected families and for arranging for insight into their medical histories. The GR plasmid for construction of diagnostic retroviral vectors was kindly provided by Dr. Christopher Baum (Hamburg). We thank Dr. Birgit Pils (Oxford, United Kingdom), for help with database searches; Dr. Heiner Schaal (Dusseldorf), Dr. Adrian Krainer (Cold Spring Harbor, NY), and Dr. Chris Smith (Cambridge, United Kingdom), for advice with the characterization of some of the splice-site mutations. Dr. Markus Schmugge (Zurich) and Dr. Eva Seemanova (Prague) are gratefully acknowledged for providing clinical information, as are Drs. John Wagner (Minneapolis), David Williams (Cincinnati), Farid Boulad (New York), and many other physicians who provided clinical data for the IFAR. We are deeply obliged to all of the participating patients and families and to the many clinicians who contributed to the present work through their patient care. This work was supported in part by grants from the Deutsche Fanconi-Anämie-Hilfe (to H. Hoehn and D.S.), the Schroeder-Kurth Fund (to R.K. and D.S.), and the Senator Kurt und Inge Schuster Foundation (to R.K.) and by National Institutes of Health grants R37 HL32987 (to A.D.A.) and R01 CA82678 (to M.B. and A.D.A.). The work of J.S. was funded by European Union grant FI6R-CT-2003-508842; Spanish Ministries of Science grant SAF2006-03340; Spanish Ministries of Health grants PI051205, G03/073, and PI061099; and the La Caixa Foundation Oncology Programme. The Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas has been supported by Spanish Ministry of Health grant G03/073; VI Framework Program of the E.U. grant CONSERT, Ref. 005242; and Spanish Interministerial Commission for Science and Technology grant SAF 2005-00058). J.P.d.W., H.J., and H. Hanenberg were supported by the Fanconi Anemia Research Fund; J.P.d.W. and H.J. were supported by the Dutch Cancer Society.

Web Resources

Accession numbers and URLs for data presented herein are as follows:

ClustalW, ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX (for the polypeptide sequences compared using the Windows interface ClustalX [v. 1.81, for the multiple sequence-alignment program])

CpG Island Explorer, http://bioinfo.hku.hk/cpgieintro.html (for promoter analyses [v. 1.9, at the settings GC 60%, CpG O/E ratio 0.7, and minimum length 500 nt)

Ensembl Genome Browser, http://www.ensembl.org/ (for *FANCD2* genomic sequences [accession number ENSG00000144554] and *Fancd2* sequence information for other species)

ESEfinder, http://rulai.cshl.edu/tools/ESE/ (for analysis of regulatory splice sequences)

ExPASy, http://www.expasy.org/sprot/ (for the Swiss-Prot Fancd2 protein sequences of different species, including *Homo sapiens*)

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for FA-D2 [accession number NM_033084, 43 exons]) and FANCD2 [accession number AF340183, 44 exons], used as the human *FANCD2* cDNA reference)

Human BLAT Search, http://genome.ucsc.edu/cgi-bin/hgBlat (for genomic *FANCD2* sequences)

Human Genome Variation Society, http://hgvs.org/ (for the mutation nomenclature)

MaxEntScan, http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html (for estimation of deduced splice-acceptor function, with use of a maximum entropy model)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, -N, BRCA1, RAD51, RAD50, ATM/ATR, RAD50, Michelin-tire baby syndrome, VACTERL-like association, holoprosencephaly, and Kartagener syndrome)

RepeatMasker, http://www.repeatmasker.org/ (for analysis of repetitive elements)

Rescue-ESE (http://genes.mit.edu/burgelab/rescue-ese (for analysis of regulatory splice sequences)

Splicefinder, http://www.uni-duesseldorf.de/rna/ (for predicted splice-donor performance)

References

1. Joenje H, Patel KJ (2001) The emerging genetic and molecular basis of Fanconi anaemia. Nat Rev Genet 2:446–457
2. Levitus M, Rooimans MA, Steltenpool J, Cool NF, Oostra AB, Mathew CG, Hoatlin ME, Waisfisz Q, Arwert F, de Winter JP, et al (2004) Heterogeneity in Fanconi anemia: evidence for 2 new genetic subtypes. Blood 103:2498–2503
3. Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, Kalb R, Neveling K, Kelly P, Seal S, Freund M, et al (2007) Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet 39:162–164
4. Xia B, Dorsman JC, Ameziane N, de Vries Y, Rooimans MA, Sheng Q, Pals G, Errami A, Gluckman E, Llera J, et al (2007) Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. Nat Genet 39:159–161
5. Levran O, Attwooll C, Henry RT, Milton KL, Neveling K, Rio P, Batish SD, Kalb R, Velleuer E, Barral S, et al (2005) The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet 37:931–933
6. Meetei AR, Medhurst AL, Ling C, Xue Y, Singh TR, Bier P, Steltenpool J, Stone S, Dokal I, Mathew CG, et al (2005) A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. Nat Genet 37:958–963
7. Levitus M, Waisfisz Q, Godthelp BC, de Vries Y, Hussain S, Wiegant WW, Elghalbzouri-Maghrani E, Steltenpool J, Rooimans MA, Pals G, et al (2005) The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J. Nat Genet 37:934–935
8. Meetei AR, Sechi S, Wallisch M, Yang D, Young MK, Joenje H, Hoatlin ME, Wang W (2003) A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. Mol Cell Biol 23:3417–3426
9. Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, Grompe M, D'Andrea AD (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell 7:249–262
10. Taniguchi T, Garcia-Higuera I, Andreassen PR, Gregory RC, Grompe M, D'Andrea AD (2002) S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. Blood 100:2414–2420
11. Thompson LH, Hinz JM, Yamada NA, Jones NJ (2005) How Fanconi anemia proteins promote the four Rs: replication, recombination, repair, and recovery. Environ Mol Mutagen 45:128–142
12. Nakanishi K, Yang YG, Pierce AJ, Taniguchi T, Digweed M, D'Andrea AD, Wang ZQ, Jasin M (2005) Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci USA 102:1110–1115
13. Niedzwiedz W, Mosedale G, Johnson M, Ong CY, Pace P, Patel KJ (2004) The Fanconi anaemia gene FANCC promotes homologous recombination and error-prone DNA repair. Mol Cell 15:607–620
14. Digweed M, Rothe S, Demuth I, Scholz R, Schindler D, Stumm M, Grompe M, Jordan A, Sperling K (2002) Attenuation of the formation of DNA-repair foci containing RAD51 in Fanconi anaemia. Carcinogenesis 23:1121–1126
15. Yamamoto K, Hirano S, Ishiai M, Morishima K, Kitao H, Namikoshi K, Kimura M, Matsushita N, Arakawa H, Buerstedde JM, et al (2005) Fanconi anemia protein FANCD2 promotes immunoglobulin gene conversion and DNA repair through a mechanism related to homologous recombination. Mol Cell Biol 25:34–43
16. Mirchandani KD, D'Andrea AD (2006) The Fanconi anemia/BRCA pathway: a coordinator of cross-link repair. Exp Cell Res 312:2647–2653
17. Levitus M, Joenje H, de Winter JP (2006) The Fanconi anemia pathway of genomic maintenance. Cell Oncol 28:3–29
18. Blom E, van de Vrugt HJ, de Winter JP, Arwert F, Joenje H (2002) Evolutionary clues to the molecular function of Fanconi anemia genes. Acta Haematol 108:231–236
19. Houghtaling S, Timmers C, Noll M, Finegold MJ, Jones SN, Meyn MS, Grompe M (2003) Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. Genes Dev 17:2021–2035
20. Carreau M (2004) Not-so-novel phenotypes in the Fanconi anemia group D2 mouse model. Blood 103:2430
21. Dequen F, St-Laurent JF, Gagnon SN, Carreau M, Desnoyers S (2005) The *Caenorhabditis elegans* FancD2 ortholog is required for survival following DNA damage. Comp Biochem Physiol B Biochem Mol Biol 141:453–460
22. Liu TX, Howlett NG, Deng M, Langenau DM, Hsu K, Rhodes J, Kanki JP, D'Andrea AD, Look AT (2003) Knockdown of zebrafish Fancd2 causes developmental abnormalities via p53-dependent apoptosis. Dev Cell 5:903–914
23. Marek LR, Bale AE (2006) *Drosophila* homologs of FANCD2 and FANCL function in DNA repair. DNA Repair (Amst) 5:1317–1326
24. Tischkowitz M, Dokal I (2004) Fanconi anaemia and leukaemia—clinical and molecular aspects. Br J Haematol 126:176–191
25. Taniguchi T, D'Andrea AD (2006) The molecular pathogenesis of Fanconi anemia: recent progress. Blood 107:4223–4233
26. Timmers C, Taniguchi T, Hejna J, Reifsteck C, Lucas L, Bruun D, Thayer M, Cox B, Olson S, D'Andrea AD, et al (2001) Positional cloning of a novel Fanconi anemia gene, *FANCD2*. Mol Cell 7:241–248
27. Borriello A, Locasciulli A, Bianco AM, Criscuolo M, Conti V, Grammatico P, Cappellacci S, Zatterale A, Morgese F, Cucciolla V, et al (2007) A novel Leu153Ser mutation of the Fanconi anemia *FANCD2* gene is associated with severe chemotherapy toxicity in a pediatric T-cell acute lymphoblastic leukemia. Leukemia 21:72–78
28. Joenje H (1997) Fanconi anemia: cytogenetic diagnosis. Free University of Amsterdam, Amsterdam
29. Auerbach AD (2003) Diagnosis of Fanconi anemia by diepoxybutane analysis. In: Current protocols in human genetics, supplement 37. John Wiley, New York and London, pp 8.7.1–8.7.15
30. Berger R, Le Coniat M, Gendron MC (1993) Fanconi anemia: chromosome breakage and cell cycle studies. Cancer Genet Cytogenet 69:13–16
31. Seyschab H, Friedl R, Sun Y, Schindler D, Hoehn H, Hentze S, Schroeder-Kurth T (1995) Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. Blood 85:2233–2237
32. Heinrich MC, Hoatlin ME, Zigler AJ, Silvey KV, Bakke AC, Keeble WW, Zhi Y, Reifsteck CA, Grompe M, Brown MG, et al (1998) DNA cross-linker-induced G2/M arrest in group C

Fanconi anemia lymphoblasts reflects normal checkpoint function. Blood 91:275–287
33. Kutler DI, Auerbach AD (2004) Fanconi anemia in Ashkenazi Jews. Fam Cancer 3:241–248
34. Neitzel H (1986) A routine method for the establishment of permanent growing lymphoblastoid cell lines. Hum Genet 73:320–326
35. Schindler D, Hoehn H (1988) Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. Am J Hum Genet 43:429–435
36. Hildinger M, Abel KL, Ostertag W, Baum C (1999) Design of 5′ untranslated sequences in retroviral vectors developed for medical use. J Virol 73:4083–4089
37. Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, Williams DA (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nat Med 2:876–882
38. Hanenberg H, Hashino K, Konishi H, Hock RA, Kato I, Williams DA (1997) Optimization of fibronectin-assisted retroviral gene transfer into human $CD34^+$ hematopoietic cells. Hum Gene Ther 8:2193–2206
39. Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, Leurs C, Cooper RJ, Gottsche K, Haneline L, Clapp DW, et al (2002) Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol 30:410–420
40. Chandra S, Levran O, Jurickova I, Maas C, Kapur R, Schindler D, Henry R, Milton K, Batish SD, Cancelas JA, et al (2005) A rapid method for retrovirus-mediated identification of complementation groups in Fanconi anemia patients. Mol Ther 12:976–984
41. Shimamura A, de Oca RM, Svenson JL, Haining N, Moreau LA, Nathan DG, D'Andrea AD (2002) A novel diagnostic screen for defects in the Fanconi anemia pathway. Blood 100:4649–4654
42. Kutler DI, Singh B, Satagopan J, Batish SD, Berwick M, Giampietro PF, Hanenberg H, Auerbach AD (2003) A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood 101:1249–1256
43. Carter AB, Salem AH, Hedges DJ, Keegan CN, Kimball B, Walker JA, Watkins WS, Jorde LB, Batzer MA (2004) Genome-wide analysis of the human Alu Yb-lineage. Hum Genomics 1:167–178
44. Roy-Engel AM, Carroll ML, Vogel E, Garber RK, Nguyen SV, Salem AH, Batzer MA, Deininger PL (2001) Alu insertion polymorphisms for the study of human genomic diversity. Genetics 159:279–290
45. Ho GP, Margossian S, Taniguchi T, D'Andrea AD (2006) Phosphorylation of FANCD2 on two novel sites is required for mitomycin C resistance. Mol Cell Biol 26:7005–7015
46. Soulier J, Leblanc T, Larghero J, Dastot H, Shimamura A, Guardiola P, Esperou H, Ferry C, Jubert C, Feugeas JP, et al (2005) Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. Blood 105:1329–1336
47. Callen E, Casado JA, Tischkowitz MD, Bueren JA, Creus A, Marcos R, Dasi A, Estella JM, Munoz A, Ortega JJ, et al (2005) A common founder mutation in FANCA underlies the world's highest prevalence of Fanconi anemia in Gypsy families from Spain. Blood 105:1946–1949
48. Lo Ten Foe JR, Kwee ML, Rooimans MA, Oostra AB, Veerman AJ, van Weel M, Pauli RM, Shahidi NT, Dokal I, Roberts I, et al (1997) Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. Eur J Hum Genet 5:137–148
49. Kennedy RD, D'Andrea AD (2005) The Fanconi anemia/BRCA pathway: new faces in the crowd. Genes Dev 19:2925–2940
50. Casado JA, Callen E, Jacome A, Rio P, Castella M, Lobitz S, Ferro T, Munoz A, Sevilla J, Cantalejo A, et al (2006) A comprehensive strategy for the subtyping of Fanconi anemia patients: conclusions from the Spanish Fanconi Anemia research network. J Med Genet (electronically published November 14, 2006; accessed March 27, 2007)
51. Auerbach AD, Buchwald M, Joenje H (2001) Fanconi anemia, 8th ed. Vol 1. McGraw-Hill, New York, pp 753–768
52. Faivre L, Guardiola P, Lewis C, Dokal I, Ebell W, Zatterale A, Altay C, Poole J, Stones D, Kwee ML, et al (2000) Association of complementation group and mutation type with clinical outcome in fanconi anemia. Blood 96:4064–4070
53. Huret JL (2002) Fanconi anaemia. In: Atlas Genet Cytogentic Oncol Haematol (http://atlasgeneticsoncology.org/kprones/FA10001.html) (accessed March 27, 2007)
54. Andreassen PR, D'Andrea AD, Taniguchi T (2004) ATR couples FANCD2 monoubiquitination to the DNA-damage response. Genes Dev 18:1958–1963
55. Pichierri P, Rosselli F (2004) The DNA crosslink-induced S-phase checkpoint depends on ATR-CHK1 and ATR-NBS1-FANCD2 pathways. EMBO J 23:1178–1187
56. Holzel M, van Diest PJ, Bier P, Wallisch M, Hoatlin ME, Joenje H, de Winter JP (2003) FANCD2 protein is expressed in proliferating cells of human tissues that are cancer-prone in Fanconi anaemia. J Pathol 201:198–203
57. Pace P, Johnson M, Tan WM, Mosedale G, Sng C, Hoatlin M, de Winter J, Joenje H, Gergely F, Patel KJ (2002) FANCE: the link between Fanconi anaemia complex assembly and activity. EMBO J 21:3414–3423
58. Garcia-Blanco MA, Baraniak AP, Lasda EL (2004) Alternative splicing in disease and therapy. Nat Biotechnol 22:535–546
59. Mansfield SG, Chao H, Walsh CE (2004) RNA repair using spliceosome-mediated RNA *trans*-splicing. Trends Mol Med 10:263–268
60. Wang Y, Leung FC (2004) An evaluation of new criteria for CpG islands in the human genome as gene markers. Bioinformatics 20:1170–1177

Table 3.

FANCD2 Superamplicon Primers

Superamplicon	Containing Exon(s)	Forward		Reverse		PCR Product Size (bp)
		Designation	Sequence ($5'\rightarrow3'$)	Designation	Sequence ($5'\rightarrow3'$)	
I	1 and 2	hFANCD2_exon1_F	TATGCCCGGCTAGCACAGAA	hFANCD2_super_1_2_R	GGCCCACAGTTTCCGTTTCT	4,346
II	3	hFANCD2_super_3_3_F	GTGTCACGTGTCTGTAATCTC	hFANCD2_super_3_3_R	CTGGGACTACAGACACGTTTT	2,323
III	7, 8, and 9	hFANCD2_super_7_14_F	TGGGTTTGGTAGGGTAATGTC	hFANCD2_exon9_R	TACTCATGAAGGGGGGTATCA	4,595
IV	10, 11, 12, 13, and 14	hFANCD2_exon10_F	GCCCAGCTCTGTTCAAACCA	hFANCD2_super_7_14_R	TTAAGACCCAGCGAGGTATTC	5,635
V	13, 14, 15, 16, and 17	FA-D2, sup13-I17 F	CATGGCAGGAACTCCGATCTTG	FA-D2, sup13-I17 F	CTCCCTTAAAAGCTCAAAGCTCAAGTTC	8,858
VI	19 and 20	hFANCD2_super_19_22_F	ACGTAATCACCCCTGTAATCC	hFANCD2_exon20_R	TGACAGAGCGAGACTCTCTAA	2,749
VII	21, 22, and 23	FA-D2, 21_23, F	GCTTCTAGTCACTGTCAGTTCACCAG	FA-D2, 21_23, R	ACGTTGGCCAGAAAGTAATCTCAG	2,518
VIII	23, 24, 25, and 26	hFANCD2_super_23_29_F	GGCCTTGTGCTAAGTGCTTTT	hFANCD2_exon26_R	TCAGGGATATTGGCCTGAGAT	3,252
IX	27, 28, and 29	hFANCD2_exon27_F	GCATTCAGCCATGCTTGGTAA	hFANCD2_super_23_29_R	CACTGCAAACTGCTCACTCAA	3,371
X	30	hFANCD2_super_30_32_F	CCAAAGTACTGGGAGTTTGAG	hFANCD2_exon30_R	TACCCAGTGACCCAAACACAA	2,186
XVI	31 and 32	hFANCD2_exon31_F	CCATTGCGAACCCTTAGTTTC	hFANCD2_super_30_32_R	ACCCTGGTGGACATACCTTTT	299
XII	33 and 34	hFANCD2_super_33_36_F	GAGCAATTTAGCCTGTGGTTTT	hFANCD2_exon34_R	TATAGCAAGAGGGCCTATCCA	3,457
XIII	35 and 36	hFANCD2_exon35_F	TTAGACCGGGAACGTCTTAGT	hFANCD2_super_33_36_R	TCTGGGCAACAGAACAAGCAA	2,040
XIV	43a	hFANCD2_super_43_44_F	AGGGTCCTGAGACTATATACC	hFANCD2_exon43a_R	AGCATGATCTCGGCTCACCA	2,040
XV	44	hFANCD2_exon44_F	CACCCAGAGCAGTAACCTAAA	hFANCD2_super_43_44_R	ACCATCTGGCCGACATGGTA	464

Table 4.

CITED IN TEXT | |

FANCD2 Exon Primers

Exon	Forward		Reverse		PCR Product Size (bp)
	Designation	Sequence (5′→3′)	Designation	Sequence (5′→3′)	
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

Table 5. CITED IN TEXT | |

FANCD2 Mutation-Specific Primers

Designation	Sequence (5′→3′)	PCR/Sequencing
FA-D2, exon4-6 F	GAAGGAAAACTATGGTAGGAAACTGGTG	PCR/Sequencing
FA-D2, exon 6 R	CAGATGTATTAGGCTAATAAGCACAG	PCR/Sequencing
FA-D2, exon4-i6 R	CCAGAAGCAGTTTGATGAGACTCTTAG	PCR/Sequencing
FA-D2, exon i4F	GCTTTCCAAAAGAAGCTCTTTCAGAC	PCR/Sequencing
FA-D2, exon4-IVS F	GGAGACACCCTTCCTATCCCAAAG	PCR/Sequencing
FA-D2, exon 5F	GAGTGGGCTAGAATGATTTTTAACAGC	PCR/Sequencing
FA-D2, exon 5 R	CTCTGAGGAAAACAGAGCTTACAC	PCR/Sequencing
D2_AluYb9 F	GCAATCTCGGCTCACTGCAAGCTC	PCR/Sequencing
D2_IVS4/AluYb9, R	GCTGTTAAAAATCATTCTACTTTGGGAGG	PCR/Sequencing
FA-D2, ex 10 F	GACTTGACCCAAACTTCCTATTGAA*	PCR/Sequencing
FA-D2, ex 14 F	TCGTGTTTCGCTGATGTGT	PCR/Sequencing
FA-D2, ex 11 R	CCGGATTAGTCAGTATTCTCAGTTAG	PCR/Sequencing
IVS14+2411 R	CGAGACCATCCTGACTAACACG	PCR/Sequencing
IVS14+2512 R	GATACCCCTTAAGAATACAGAGC	PCR/Sequencing
FANCD2_16S	AGAGCTAGGGAGGAGAAGTCTGA	PCR
FANCD2_18A	GAGCTGAGATCGTGCCAACT	PCR
FANCD2_17S	TGGTCAAGTTACACTGGCATATT	Sequencing
FANCD2_17A	CCATCCTTCAGCAATCACTC	Sequencing
D2_P2_21_23 F	GTTTTCTGATACTTGGAAACTACTGGCTTG	PCR/Sequencing
D2_P1_21_23 R	GACACAGAGGTAGCAAAGGATGTTC	PCR/Sequencing
FA-D2, ex21_23, int1	CTATGATGAATTTGCCAACCTGATCC	Sequencing
FA-D2, ex21_23, int2	GAGGGCTCCTTCACTTAATAACAATC	Sequencing
FA-D2, ex21_23, int3	GTATTGTTTACCTGCTGGCTGGTTG	Sequencing
FA-D2_sup_exon26 II F uniq	TAGGGTCACAAGCCTAATCTCCTTT	PCR
FA-D2_sup_exon26 II R uniq	GGCCATGATGAATAATCTTTCTTTTGTTTG	PCR

Table 7.

Laboratory Diagnostic Data for the Cohort of 29 FA-D2 Patients

Patient Number	Kindred Sibling	Cell Type of Laboratory Diagnosis	G2-Phase Arrest (%), G2/GF		Breaks/Cell		Technique of Complementation Group Assignment	*FANCD2* Mutation		Somatic Mosaicism
			Spontaneous	MMC/DEB	Spontaneous	MMC/DEB		Allele 1	Allele 2	
1	1/I	Lymphocyte	65.7	ND	.07	4.5 (MMC)/6.6 (MMC)	IB of LCL	c.696-121C→G (exonization) *p.S232insQNNFX*	c.696-121C→G (exonization) *p.S232insQNNFX*	None (residual protein)
2	2/I	Lymphocyte	54.3	70.1 (MMC)	.09	1.4 (MMC)/1.5 (DEB)	IB and RC of LCL	c.1948-6C→A (exon 22 skipping) *p.E650X*	c.3599delT *p.I1200KfsX12*	None (residual protein)
3	3/I	Lymphocyte (before mosaicism)	38.6 (before mosaicism)	46.6 (MMC) (before mosaicism)	.04	.06 (MMC)	RC of fibroblasts	c.1948-16T→G (exon 22 skipping) *p.E650X*	c.1948-16T→G (exon 22 skipping) *p.E650X*	1954G→A (exon 22), V652I, reconstitutes exon 22 recognition (blood, BM, LCL)
4	4/I	Lymphocyte	45.7	63.6 (MMC)	ND	ND	RC of fibroblasts	c.1948-16T→G (exon 22 skipping) *p.E650X*	c.1948-16T→G (exon 22 skipping) *p.E650X*	None (no LCL)
5	4/II	Lymphocyte	44.5	58.9 (MMC)	ND	ND	RC of fibroblasts	c.1948-16T→G (exon 22 skipping) *p.E650X*	c.1948-16T→G (exon 22 skipping) *p.E650X*	None (residual protein)
6	5/I	Lymphocyte	34.5	64.7 (MMC)	.05	4.7 (MMC)/ 5.6 (DEB)	IB and RC of LCL	c.274-57_-56insinvAluYb8nt36_319 +dup c.274–69_–57 (exon 5 skipping) *p.I92YfsX7*	c.3803G→A *p.W1268X*	None (residual protein)
7	6/I	Lymphocyte	34.8	51.5 (MMC)	ND	ND	IB of LCL	c.904C→T *p.R302W*	c.1092G→A *p.W364X*	None (residual protein)
8	7/I	Lymphocyte	45.6	58.4 (MMC)	.06	1.3 (MMC)	IB of LCL	c.990-1G→A (aberrant splicing) *p.S330RfsX16*	c.1948-6C→A (exon 22 skipping) *p.E650X*	None (residual protein)
9	8/I	Lymphocyte	65.3	70.9 (MMC)	.12	ND	IB of LCL	c.810_812delGTC *p.S271del*	c.1948-16T→G (exon 22 skipping) *p.E650X*	None (residual protein)
10	9/I	Lymphocyte	38.4	58.4 (MMC)	ND	ND	RC of fibroblasts	c.1948-16T→G (exon 22 skipping) *p.E650X*	c.2715+1G→A (aberrant splicing) *p.E906LfsX4*	None (residual protein)

11	10/I	Lymphocyte	55.4	65.8 (MMC)	? (Wien)	? (Wien)	IB of LCL	c.3707G→A (aberrant splicing) *p.H1229EfsX7*	c.2835dupC *p.D947RfsX3*	None (residual protein)
12	11/I	Lymphocyte	40.2	61.1 (MMC)	ND	ND	IB of LCL	c.274-57_–56insinvAlu Yb8nt36_319 +dup c.274–69_–57 *p.I92YfsX7*	c.3453_3456delCAAA *p.N1151KfsX46*	None (residual protein)
13	12/I	Lymphocyte	27.6	57.8	.11	1.08 (MMC)/2.9 (DEB)	IB of LCL	c.1948-16T→G (exon 22 skipping) *p.E650X*	c.1948-16T→G (exon 22 skipping) *p.E650X*	None (residual protein in T cells and LCL)
14	13/I	Lymphocyte fibroblast	20.9	32.1 (MMC)	ND	ND	Mutation analysis (by sibling)	c.1948-6C→A (exon 22 skipping) *p.E650X*	2775_2776CC→TT *p.R926X*	2775_2776 CC (blood, LCL)
15	13/II	Lymphocyte fibroblast	25.0	35.4 (MMC) 69.2 (MMC)	0	.11 (MMC)/.02 (DEB)	RC of fibroblasts	c.1948-6C→A (exon 22 skipping) *p.E650X*	2775_2776CC→TT *p.R926X*	Recombination
16	14/I	Lymphocyte	ND	ND	.04	1.78 (DEB)	IB of LCL	c.2444G→A *p.R815Q*	c.2444G→A *p.R815Q*	None (residual protein)
17	14/II	Lymphocyte	ND	ND	.12	3.1 (DEB)	Mutation analysis (by sibling)	c.2444G→A *p.R815Q*	c.2444G→A *p.R815Q*	None (no LCL)
18	15/I	Lymphocyte	ND	ND	.12	1.5 (DEB)	IB of LCL	c.696-2A→T (exon 10 skipping) *p.S232RfsX6*	c.1321_1322delAG (aberrant splicing) *p.V379_K515del*	None (residual protein)
19	16/I	Fetal blood	ND	ND	ND	3.7 (DEB)	RC of fetal fibroblasts	c.692T→Gpat *p.L231R*	c.2444G→Amat *p.R815Q*	Not done
20	17/I	Lymphocyte	ND	ND	.02	8.4 (DEB)	IB and RC of LCL	c.1948–6C→Amat, (exon 22 skipping) *p.E650X*	2660delApat *p.E888RfsX16*	None (residual protein)
21	18/I	Lymphocyte	ND	ND	.02	5.4 (DEB)10.3 (DEB)	IB of LCL	c.2404C→T *p.Q802X*	c.2444G→A *p.R815Q*	None (residual protein)
22	19/I	Lymphocyte	ND	ND	.04	3.7 (DEB)	RC of fetal fibroblasts from 880/2 (early spontaneous abortion)	c.2444G→Apat *p.R815Q*	c.2715+1G→Amat (aberrant splicing) *p.E906LfsX4*	None (residual protein)
23	20/I	Lymphocyte	ND	ND	.08	7.4 (DEB)	IB of LCL	c.757C→T *p.R253X*	c.1367T→G *p.L456R*	None (residual protein)
24	20/II	Lymphocyte	ND	ND	.20	8.9 (DEB)	IB of LCL	c.757C→T *p.R253X*	c.1367T→G *p.L456R*	None (residual protein)

25	21	Lymphocyte	ND	ND	Data missing	Data missing	Mutation analysis	c.1948–16T→G (exon 22 skipping) *p.E650X*	c.1948–16T→G (exon 22 skipping) *p.E650X*	1953G→T (W651C) (blood, LCL)
26	22/I	Fibroblast	22.2 (fibroblast)	54 (fibroblast, 300 nM ~ 100 ng/ml MMC)	.04	.16 (300 nM ~ 100 ng/ml MMC)	Mutation analysis in fibroblasts (by sibling)	c.376A→G (aberrant splicing) *p.S126RfsX12*	c.3803G→A *p.W1268X*	376A (blood)
27	22/II	Lymphocyte	ND	ND	.12	>10 (MMC)	IB and IP of LCL	c.376A→G (aberrant splicing) *p.S126RfsX12*	c.3803G→A *p.W1268X*	None (residual protein)
28	23/I	Lymphocyte	ND	ND	.10	6.0 (MMC)	IB, IP and RC of LCL	c.206–2A→T (exon 4 skipping) *p.A69DfsX7*	g.22875_23333del459 (c.1414–71_c.1545+256del459) *p.E472_K515del*	None (residual protein)
29	23/II	Lymphocyte	ND	ND	.12	8.1 (MMC)	Mutation analysis (by sibling)	c.206-2A→T (exon 4 skipping) *p.A69DfsX7*	g.22875_23333del459 (c.1414–71_c.1545+256del459) *p.E472_K515del*	None (residual protein)

NOTE.—DEB=diepoxybutane; RC=retroviral complementation; IB=immunoblotting; IP=immunoprecipitation; ND=not determined; G2=G2 phase fraction of the cell cycle; GF=growth fraction; G2/GF=ratio G2 phase fraction over GF.

Table 9.

CITED IN TEXT | |

Clinical Diagnostic Data for the Cohort of 29 FA-D2 Patients

Patient Number	Kindred/Sibling	Consanguinity	Sex	Ethnicity/Nationality	Age at Diagnosis	Clinical Presentation	Hematologic Manifestation(s)	Survival at Last Follow-Up	Family History
1	1/I	Unknown	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), and aplasia of the corpus callosum	BMF as of age 2 years and 4 mo, transfusions from age 3 years and 2 mo, AML at age 7.0 years	Deceased at age 7 years and 6 mo (AML, pneumonia)	No SABs; no known cancer
2	2/I	Absent	F	While German	5 Years and 7 mo	GR, pigmentation anomalies, microcephaly, microphtalmia, low-set thumbs, duplicate kidney (R), dysplastic hips	BMF as of age 5 years and 7 mo, cortisol from age 8 years, transfusions from age 8 years and 4 mo, androgen from age 9 years and 2 mo	Deceased at age 11 years and 4 mo (subarachnoidic hemorrhage)	1 SAB; MGM: cervical cancer at age 40 years
3	3/I	Cousins of 1st°	M	White Turk	1 Years and 11 mo	IUGR, pigmentation anomalies, microcephaly, hypoplastic thumbs (L>R), syndactyly II/III toes, hypogenitalism, and glomerulosclerosis	Stable partial mosaicism, BMF as of age 11 years, cortisol and androgen from age 12 years, transfusions from age 18 years and 9 mo,	Deceased at age 20 years and 7 mo (viral encephalitis after BMT)	No SABs, no known cancer

							and BMT at age 19 years and 7 mo		
4	4/I	Cousins of 2nd°	F	White Turk	5 years and 10 mo	IUGR, pigmentation anomalies, microcephaly, microphtalmia, hypoplastic thumb (R), hydocephalus internus, hypoplastic corpus callosum, mental retardation, and hyperactivity/attention-deficit disorder	BMF as of age 2 years and 6 mo, transfusions from age 2 years and 6 mo, subdural hemorrhage at age 6 years, and BMT at 7 years	8 years and 3 mo	No SABs; no known cancer
5	4/II	Cousins of 2nd°	M	White Turk	4 Years and 5 mo	IUGR, microcephaly, microphtalmia, strabismus, mental retardation, and hyperactivity/attention-deficit disorder	BMF as of age 3 years and 3 mo, transfusions from age 3 years and 3 mo, and oxymetholon from age 5 years and 9 mo	6 Years and 11 mo	No SABs; no known cancer
6	5/I	Absent	M	White German	2 Years and 6 mo	GR, microcephaly, microphtalmia, absent antihelix (R), radial-ray hypoplasia, preaxial hexadactyly (R), duplicate pelvic kidney (R), maldescensus of the testes, micropenis, dysplastic hips, hypoplastic corpus callosum, misshaped brain ventricles, and psychomotor retardation	BMF as of age 2 years and 9 mo, and BMT at age 3 years and 3 mo.	4 Years and 4 mo	No SABs; PGM cancer at age 70 years; otherwise, no cancer history
7	6/I	Absent	F	White Italian	2 Years	IUGR, pigmentation anomalies, microcephaly, microphtalmia, absent thumbs, short radii, absent antihelix (R), and closed	BMF as of age 4.5 Years	12 Years	No SABs; no cancer history

						auditory canals			
8	7/I	Absent	M	White German	3 Years and 9 mo	Pigmentation anomalies, microcephaly, "flat" auricles-absent antihelix?, ptosis, short thumbs, and hyperactivity/attention-deficit disorder	BMF as of age 4 years	4 Years and 4 mo	No SABs; no cancer history
9	8/I	Absent	F	White Czech	2 Years and 11 mo	IUGR, microcephaly, brain atrophy, patent ductus arteriosus, esophagus atresia, tracheoesophageal fistula (IIIb), hypoplastic kidneys, polycystic ovary (L), triphangeal digitalized thumbs, pedes equinovari, and rib anomaly (VACTERL-like association)	BMF as of age 2 years and 10 mo and transfusions from age 2 years and 11 mo	Deceased at age 5 years and 10 mo (hemorrhage)	1 SAB (first trimester); no cancer in the family
10	9/I	Absent	F	White Turk	7 mo	IUGR, pigmentation anomalies, microcephaly, hydrocephalus internus, absent corpus callosum, microphtalmia, small mouth, low-set ears, hypoplastic thumbs, unilateral triphalangeal (R), pelvic kidney (L), hip luxation, and psychomotor retardation	BMF as of age 2 years	2 years and 3 mo	1 SAB (first trimester); 1 pregnancy terminated because of hydrocephalus and renal agenesis; PGF bronchus cancer
11	10/I	Absent	F	White Austrian	10 Years and 10 mo	IUGR, pigmentation anomalies, microcephaly, hypoplastic thumbs, and ectopic kidney (R)	BMF as of age 10 years and 10 mo, transfusions from age 10 years and 10 mo, MDS (RAEB-t) with del(7)(q32) at age 10 years and 10 mo,	11 Years and 11 mo	No SABs; no cancer history

							BMT at age 11 years and 1 mo		
12	11/I	Absent	M	White Dane	3 Mo	IUGR, atresia of the duodenum, microcephaly, dilated lateral ventricles and stenosis of the aquaeduct (hydrocephalus), hypoplasia of the corpus callosum, microphtalmia, closed auditory channels, hypoplastic thumbs, and micropenis	BMF as of age 2 wk	5 Mo	No SABs; PGM and MPGM breast cancer, MGGF prostate cancer
13	12/I	Cousins of 1st°	M	White Turk	5 Years and 5 mo	IUGR, pigmentation anomalies, microcephaly, microphalmia, psychomotor retardation, and Michelin-tire baby syndrome	BMF as of age 1 years and 5 mo	5 Years and 8 mo	No SABs; no cancer history
14	13/I	Absent	F	White German	34 Years and 2 mo	IUGR, microcephaly, and mild radial-ray hypoplasia	None	34 Years	No SABs; no cancer history
15	13/II	Absent	F	White German	21 Years and 11 mo	IUGR, microcephaly, radial -ray hypoplasia, dysplasia of mandibula, anomalies of the teeth, dysplasia of hip (R), and mental retardation	Transfusions from age 17 years and 6 mo, MDS (RARS-RAEB) at age 17 years and 6 mo	Deceased at age 23 years and 5 mo (pneumonia, invasive aspergillosis, and hemorrhage)	No SABs; no cancer history
16	14/I	Cousins of 3°	M	White Spanish	6 Years	Patent ductus arteriosus, pigmentation anomalies, bifid thumb (R), and hypogonadism	BMF as of age 7 years (very mild hypoplasia of the myeloid series)	25 Years	No SABs; MGF lung, and PGF stomach cancer
17	14/II	Cousins of 3°	M	White Spanish	8 Mo	Pigmentation anomalies, microphtalmia, hypoplastic thumb (R), absent os	Blood cell counts at low-range	20 Years	No SABs; MGF lung, and PGF

						metacarpale I (L), and glandular hypospadia	normal levels		stomach cancer
18	15/I	Absent	F	White Spanish	5 Years and 3 mo	IUGR, pigmentation anomalies, microcephaly, microphtalmia, hypotelorism, and annular pancreas	BMF as of age 5 years and 3 mo, androgen, G-SCF, EPO and transfusions from age 5 years and 3 mo, BMT at age 10 years and 11 mo	Deceased at age 11 years and 1 mo (graft failure/did not take)	No SABs; MMGM colon cancer
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), and lateral cerebral ventricular dilation (hydrocephalus)	NA	NA, 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 and basal cell cancer
20	17/I	Absent	M	White maternal German, paternal Dutch	4 Years and 5 mo	IUGR, pigmentation anomalies, microcephaly, and microphtalmia	BMF as of age 2 years, BMT at age 5 years	9 Years (4 years post BMT)	1 SAB, M 3× basal cell, MMMGM melanoma, MMGF breast, PGM bowel cancer
21	18/I	Absent	M	White Hispanic	7 Mo	IUGR, café-au-lait spots,	None yet	10 Years and 3	No SAB; one

				(Mexican)		microcephaly, microphtalmia, hearing loss (auditory canals? sensory hearing impairment?), absent thumbs and radii, intestinal atresia, renal defects, genital abnormalities (undescended testes), and learning disabilities		mo	cancer
22	19/I	Absent	M	White 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), and hypoplastic thumb with ponce flottant (L)	BMF as of age 1 year and 4 mo	2 Years and 6 mo	5 Miscarriages with one positive for FA; PGM breast, MPGM cervix and lung cancer, 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), and horseshoe kidney	None	1 Years and 4 mo	GM: 2 miscarriages; cancer only in great-GP generation
24	20/II	Absent	F	Maternal African American/Caucasian, paternal African American	4 Years and 6 mo	IUGR, café-au-lait spots, microcephaly, and microphtalmia	BMF starting from age 4 years and 6 mo	5 Years and 9 mo	GM: 2 miscarriages; cancer only in great-GP generation
25	21/I	Cousins of 1st°	M	White Turk	5 Years and 5 mo	IUGR, pigmentation anomalies, microcephaly, microphtalmia, and pelvic kidney	BMF starting from age 4 years and oxymetholone and prednisone from age 5 years and 5 mo	Deceased at age 9 years (intracranial hemorrhage)	No SAB; no cancer history

26	22/I	Absent	F	White Dutch	5 Years	IUGR (asymmetrical); pigmentation anomalies; microcephaly; ventriculomegaly (hydrocephalus) and multiple developmental anomalies of the brain, possibly holoprosencephaly; hypotelorism; microphtalmia; narrow auditory canals; hypoplastic os metacarpale I; renal aplasia (R); dysplasia of the hip (L); and growth-hormone deficiency	BMF as of age 5 years, transfusions from age 6 years and 9 mo, and GCSF and BMT at age 7 years and 10 mo	9 Years	No SAB; no cancer history
27	22/II	Absent	M	White Dutch	3 Years	IUGR, pigmentation anomalies, microcephaly, hypoplastic corpus callosum; hypertelorism, blepharophimosis, and preaxial hexadactyly (L)	BMF as of age 2 years and 1 mo, transfusions from age 5 years and 8 mo; BMT at age 7 years and 7 mo	7 Years and 9 mo	No SAB; no cancer history
28	23/I	Absent	M	White Dutch	8 Years and 6 mo	IUGR, pigmentation anomalies, microcephaly, Kartagener syndrome with situs inversus, and mild mental retardation	BMF as of age 8 years and 5 mo, no transfusions, BMT at age 9 years and 5 mo	Deceased at age 10 years and 1 mo (gastrointestinal hemorrhage due to necrotizing enterocolitis post BMT)	1 SAB; no cancer history
29	23/II	Absent	M	White Dutch	5 Years and 8 mo	IUGR, pigmentation anomalies, microcephaly, and microphtalmia	BMF as of age 5 years and 8 mo and BMT at age 6 years and 7 mo	7 Years and 10 mo	1 SAB; no cancer history

NOTE.—L=left; R=right; GR=growth retardation; IUGR=intrauterine growth retardation; BMT=bone-marrow transplantation; MDS=myelodysplastic syndrome;

SAB=spontaneous abortion; MGM=maternal grandmother; PGF=paternal grandfather; MPGM=maternal and paternal grandmother; MGGF=maternal greatgrandfather; MGF=maternal grandfather; MMGM=mother of the maternal grandmother; MMGF=mother of the maternal grandfather; MMMGM=mother of the maternal greatgrandmother; GP=grandparent.

ERRATA

In the May 2007 issue of the *Journal,* in the article entitled "Hypomorphic Mutations in the Gene Encoding a Key Fanconi Anemia Protein, FANCD2, Sustain a Significant Group of FA-D2 Patients with Severe Phenotype" by Kalb et al. (80:895–910), there were errors in the text and in table 3.

On page 897, in the "Retroviral Complementation" section, the text should read "In addition, cDNAs of the enhanced green fluorescent protein (*GFP*) and *FANCA* genes were separately cloned into the vector S11IN…." On page 900, in the "Mutations Affecting Pre-mRNA Splicing" section, "splec" should be "splice." On page 905, in the "Residual FANCD2 Protein" section, "(data not shown)" should be "(fig. 8*B,* right panel)." Therefore, in figure 8, the description of panel B should be "*B*, Blood-derived cells from nonmosaic FA-D2 patients (exemplified 13, 5, 1, 21, 2, 6, 11, and 28) and fibroblasts from mosaic FA-D2 patients (exemplified 3, 14, and 26) show faint but conspicuous FANCD2 bands of both species in response to MMC exposure exclusively on overexposed immunoblots, as indicated by the very intense FANCD2 signals of the normal control LCLs (CON) (patient 13, stimulated PBL; patients 5, 1, 21, 2, 6, 11, and 28, LCLs; patients 3, 14, 26, cultured fibroblasts; loading control, RAD50)." In the figure 6 legend, two reference numbers were incorrect. The sentence should be "Mutations identified in the present study are shown above, mutations reported elsewhere[26,27] are indicated below the schematic display of FANCD2 cDNA."

The revised table 3 contains seven superamplicons that were actually used in the study. Their numbering corresponds to their order shown in figure 4*C*. The table has been modified accordingly.

The authors and the *Journal* regret these errors.

Table 3. *FANCD2* Superamplicon Primers

		Forward		Reverse		PCR Product
Superamplicon	Containing Exons	Designation	Sequence (5′→3′)	Designation	Sequence (5′→3′)	Size (bp)
I	1 and 2	hFANCD2_exon1_F	TATGCCCGGCTAGCACAGAA	hFANCD2_super_7_15_R	GGCCCACAGTTTCCGTTTCT	4,346
II	10, 11, 12, 13, 14, and 15	hFANCD2_exon10_F	GCCCAGCTCTGTTCAAACCA	hFANCD2_super_7_15_R	TTAAGACCCAGCGAGGTATTC	5,635
III	13, 14, 15, 16, and 17	hFANCD2_super_13_17_F	CATGGCAGGAACTCCGATCTTG	hFANCD2_super_13_17_R	CTCCCTTAAAAGCTCAAAGCTCAAGTTC	8,858
IV	19 and 20	hFANCD2_super_19_22_F	ACGTAATCACCCCTGTAATCC	hFANCD2_exon20_R	TGACAGAGCGAGACTCTCTAA	2,749
V	21, 22, and 23	hFANCD2_21_23_F	GCTTCTAGTCACTGTCAGTTCACCAG	hFANCD2_21_23_R	ACGTTGGCCAGAAAGTAATCTCAG	2,518
VI	22, 23, 24, 25, and 26	hFANCD2_super_22_29_F	GGCCTTGTGCTAAGTGCTTTT	hFANCD2_exon26_R	TCAGGGATATTGGCCTGAGAT	3,252
VII	27, 28, and 29	hFANCD2_exon27_F	GCATTCAGCCATGCTTGGTAA	hFANCD2_super_22_29_R	CACTGCAAACTGCTCACTCAA	3,371

2

Results

BRIEF COMMUNICATIONS

nature genetics

The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia

Orna Levran[1], Claire Attwooll[2], Rashida T Henry[1], Kelly L Milton[1], Kornelia Neveling[3], Paula Rio[4,6], Sat Dev Batish[1], Reinhard Kalb[3], Eunike Velleuer[4], Sandra Barral[5], Jurg Ott[5], John Petrini[2], Detlev Schindler[3,7], Helmut Hanenberg[4,7] & Arleen D Auerbach[1,7]

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** online). 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 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** online). 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

[1]Laboratory for Human Genetics & Hematology, The Rockefeller University, New York, New York, USA. [2]Memorial Sloan-Kettering Cancer Center, New York, New York, USA. [3]Institute of Human Genetics, University of Wuerzburg, Germany. [4]Department of Pediatric Oncology, Hematology and Immunology, Children's Hospital, Heinrich Heine University, Duesseldorf, Germany. [5]Laboratory of Statistical Genetics, The Rockefeller University, New York, New York, USA. [6]Present address: CIEMAT/Marcelino Botin Foundation, Madrid, Spain. [7]These authors contributed equally to this work. Correspondence should be addressed to A.D.A. (auerbac@rockefeller.edu).

Published online 21 August 2005; doi:10.1038/ng1624

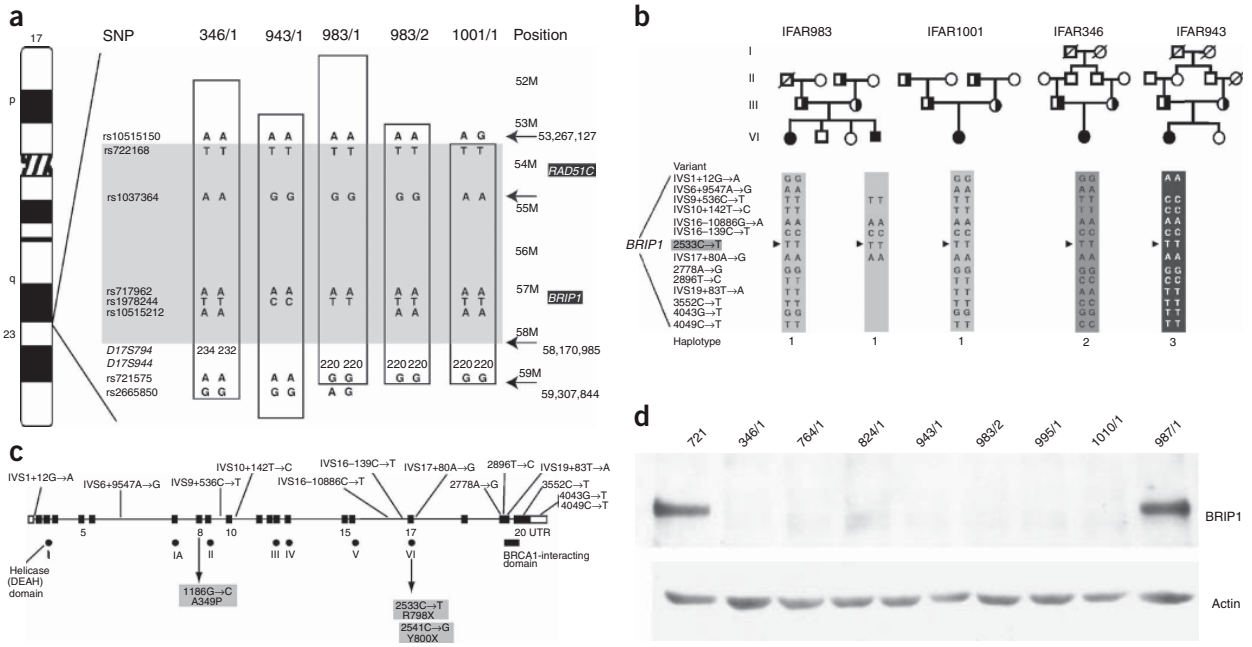


Figure 1 *BRIP1* is defective in Fanconi anemia. (**a**) The 5-Mb region of shared homozygosity among the five individuals is highlighted. The map position and the candidate genes (*RAD51C* and *BRIP1)* are indicated on the right. The open boxes indicate the full region of homozygosity for each individual. (**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.

(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).

Because we identified mutations in *BRIP1*, we analyzed the BRIP1 protein by immunoblotting (**Supplementary Methods** online). 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.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS
We thank the affected individuals and their families for providing tissue samples for these studies; the many physicians who referred their patients

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	80037A→T	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} diepoxybutane (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.

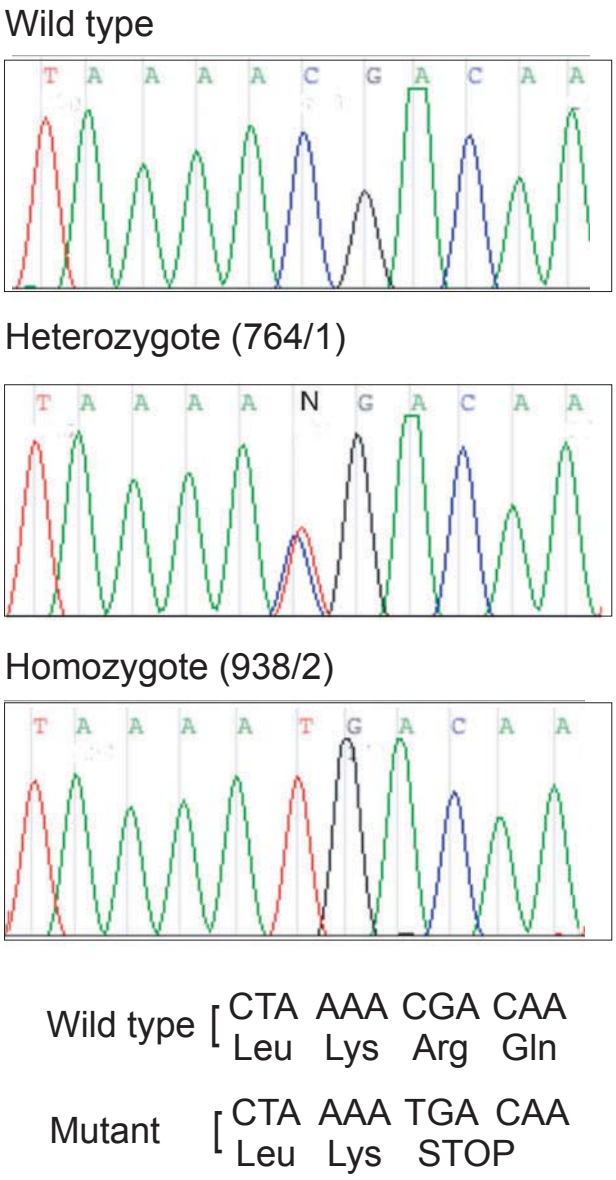
to the IFAR and helped in sending the tissue samples for their contributions; A. Dawson, B. Chodirker and G. Graham for providing samples from the Inuit families, which were instrumental to the success of the mapping experiments; S. Cantor for the monopool BRIP1 antibody; Myriad Genetic Laboratories 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.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 23 May; accepted 1 July 2005
Published online at http://www.nature.com/naturegenetics/

1. Kutler, D.I. *et al. Blood* **101**, 1249–1256 (2003).
2. Levitus, M. *et al. Blood* **103**, 2498–2503 (2004).
3. Garcia-Higuera, I. *et al. Mol. Cell* **7**, 249–262 (2001).
4. Meetei, A.R. *et al. Nat. Genet.* **35**, 165–170 (2003).
5. Meetei, A.R. *et al. Nat. Genet.* **36**, 1219–1224 (2004).
6. Howlett, N.G. *et al. Science* **297**, 606–609 (2002).
7. Gschwend, M. *et al. Am. J. Hum. Genet.* **59**, 377–384 (1996).
8. Liu, Y., Masson, J.-Y., Shah, R., O'Regan, P. & West, S.C. *Science* **303**, 243–246 (2004).
9. Cantor, S.B. *et al. Cell* **105**, 149–160 (2001).
10. Rutter, J.L. *et al. Hum. Mutat.* **22**, 121–128 (2003).
11. Cantor, S. *et al. Proc. Natl. Acad. Sci. USA* **101**, 2357–2362 (2004).
12. Moynahan, M.E., Chiu, J.W., Koller, B.H. & Jasin, M. *Mol. Cell* **4**, 511–518 (1999).
13. Chen, J.J., Silver, D., Cantor, S., Livingston, D.M. & Scully, R. *Cancer Res.* **59**, 1752s–1756s (1999).



Supplementary Figure 1. 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.

The BRCA1 interacting helicase BACH1/BRIP1 is deficient in Fanconi anemia

Levran et al.

Nature Genetics

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 NetAffx™ Analysis Center (Affymetrix, Inc. Santa Clara, CA). SNPs with ambiguous positions were excluded.

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/cgi-bin/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, 150mM 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

1. Auerbach, A.D., Rogatko, A., & Schroeder-Kurth, T.M. *Blood.* **73**, 391-396 (1989).
2. Hanenberg, H. *et al*. *Exp Hematol*. **30**, 410-420 (2002).
3. Garcia-Higuera, I. *et al*. *Mol. Cell* **7**, 249-262 (2001).
4. Levran, O. *et al*. *Hum. Mutat.* **25**,142-149 (2005).
5. Rutter, J. L. *et al*. *Hum*. *Mutat*. **22**,121-128 (2003).
6. Carney, J.P. *et al*. *Cell* **93**, 477–486 (1998).

2

Results

BRIEF COMMUNICATIONS

nature genetics

Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer

Sarah Reid[1], Detlev Schindler[2], Helmut Hanenberg[3,4], Karen Barker[1], Sandra Hanks[1], Reinhard Kalb[2], Kornelia Neveling[2], Patrick Kelly[1], Sheila Seal[1], Marcel Freund[3], Melanie Wurm[3], Sat Dev Batish[5,6], Francis P Lach[5], Sevgi Yetgin[7], Heidemarie Neitzel[8], Hany Ariffin[9], Marc Tischkowitz[10,11], Christopher G Mathew[12], Arleen D Auerbach[5] & Nazneen Rahman[1]

PALB2 was recently identified as a nuclear binding partner of BRCA2. Biallelic *BRCA2* mutations cause Fanconi anemia subtype FA-D1 and predispose to childhood malignancies. We identified pathogenic mutations in *PALB2* (also known as *FANCN*) in seven families affected with Fanconi anemia and cancer in early childhood, demonstrating 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 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[1]. The syndrome is genetically heterogeneous with 12 complementation groups currently recognized, 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)[2,3].

BRCA2 is a DNA repair protein with a key role in the repair of DNA double-strand breaks by homologous recombination[4]. *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[5]. Subsequently, biallelic *BRCA2* mutations were found to cause a rare subtype of Fanconi anemia, FA-D1 (ref. 6). The phenotype of biallelic *BRCA2* mutations differs from other Fanconi anemia subtypes, most notably with respect to the high risks of childhood solid tumors, particularly Wilms tumor and medulloblastoma, which occur very rarely in other Fanconi anemia subtypes[6–10].

Although Fanconi anemia and childhood embryonal tumors are attributable to *BRCA2* in many individuals, we identified individuals with this phenotype who lacked *BRCA2* mutations. 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 (ref. 11). PALB2 colocalizes with BRCA2, promoting its localization 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[11]. 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 (**Supplementary Methods** and **Supplementary Table 1** online). We identified pathogenic mutations in seven families (**Fig. 1a** and **Table 1**). In four affected individuals (GESH, IFAR-847, LNEY and IFAR-849), we identified biallelic mutations that resulted in premature protein truncation. 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, IFAR-007 and ICR-60, but their parents all carried truncating *PALB2* mutations. We also sequenced *PALB2* in 352 control chromosomes (176 normal individuals) and did not identify any truncating mutations, providing further evidence that such mutations are pathogenic in the individuals with Fanconi anemia.

We saw one mutation, 3549C→G (leading to amino acid change Y1183X), in two separate families. We also identified a different mutation in a third family at the same nucleotide, 3549C→A, which also results in Y1183X. 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[12]. However, there was no detectable PALB2 protein in lymphoblastoid cells from individuals IFAR-847 and IFAR-849, who both carry

[1]Section of Cancer Genetics, Institute of Cancer Research, Sutton, Surrey SM2 5NG, UK. [2]Department of Human Genetics, University of Wuerzburg, D-97074 Wuerzburg, Germany. [3]Department of Pediatric Oncology, Hematology and Immunology, Heinrich Heine University, D-40225 Duesseldorf, Germany. [4]Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA. [5]Laboratory of Human Genetics and Hematology, The Rockefeller University, 1230 York Ave., New York, New York 10021, USA. [6]Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, New York, New York 10021, USA. [7]Hacettepe University, Ihsan Dooramacy Children Hospital, Division of Pediatric Hematology, 06100 Ankara, Turkey. [8]Department of Human Genetics Charité-Universitaetsmedizin, D-13353 Berlin, Germany. [9]Paediatric Haematology-Oncology Unit, University of Malaya Medical Centre, Kuala Lumpur, Malaysia. [10]Cancer Prevention Center, The Sir Mortimer B. Davis Jewish General Hospital, Montreal, Quebec H3T 1E2, Canada. [11]Program in Cancer Genetics, Departments of Oncology and Human Genetics, McGill University, Montreal, Quebec H3A 1B1, Canada. [12]Kings College London School of Medicine, Department of Medical and Molecular Genetics, Guy's Hospital, London SE1 9RT, UK. Correspondence should be addressed to N.R. (nazneen.rahman@icr.ac.uk).

Received 14 August; accepted 27 November; published online 31 December 2006; doi:10.1038/ng1947

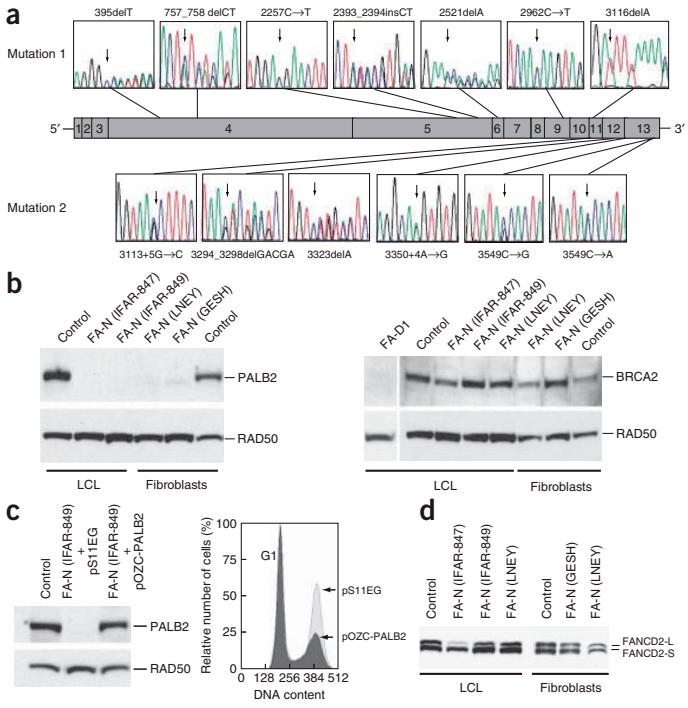


Figure 1 Evidence that PALB2 deficiency causes FA-N. (**a**) Mutation analysis. Schematic representation of *PALB2* showing the position of mutations identified in FA-N cases. Further details of the mutations and the individuals in which they were identified are given in **Table 1**. The 3549C→G mutation (leading to amino acid change Y1183X) was identified in two separate families. We obtained informed consent from all families and the research was approved by the London Multicentre Research Ethics Committee (05/MRE02/17). (**b**) PALB2 and BRCA2 immunoblotting. PALB2 protein is absent in cells from FA-N cases IFAR-847 (lymphoblastoid cell line (LCL)), IFAR-849 (LCL), LNEY (fibroblast) and GESH (fibroblast) and is present in control cells (left). BRCA2 is present in FA-N and control cells and is absent in FA-D1 cells (right). RAD50 was used as a loading control. (**c**) Complementation of the cellular phenotype. Transduction of cells from IFAR-849 with the vector pOZC-PALB2 restores PALB2 expression to a similar level as in a normal control, whereas the mock vector pS11EG (expressing GFP) does not (left). Overlay of two cell cycle distributions represented by histograms acquired with flow cytometry after exposure of an LCL from IFAR-849 to 15 ng/ml MMC for 48 h (right). The light gray histogram shows noncomplementation when cells were transduced with a mock vector expressing GFP (pS11EG) (G1 phase, 42%; S, 15%; G2, 43%; G2 phase arrest is typical of FA). The darker histogram demonstrates complementation after transduction of isogenic cells with the PALB2-expressing vector pOZC-PALB2 (G1 phase, 57%; S, 17%; G2, 26%; G2 phase is restored). This effect has been reported in complemented FA cell lines from other subtypes[15]. (**d**) FANCD2 monoubiquitination. There is normal FANCD2 monoubiquitination in FA-N cells, similar to control cells.

Y1183X, indicating that the mutation results in a null allele and confirming its pathogenicity (**Fig. 1b**).

We performed protein blot analysis on lymphoblastoid cells or fibroblasts from four individuals with biallelic *PALB2* mutations. Using an antibody that recognizes the N terminus of PALB2, we found absence of PALB2 protein in each case (**Fig. 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 (**Fig. 1c** and **Supplementary Methods**). 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 (FANCD2-L) is translocated to DNA repair foci, where it colocalizes with various proteins involved in the DNA damage response, including BRCA2 (refs. 2,3). Given the close functional relationship between BRCA2 and PALB2, one would predict that PALB2 acts downstream of FANCD2. We confirmed this in different cell types from individuals IFAR-847, IFAR-849, LNEY and GESH, which show normal monoubiquitination of FANCD2 (**Fig. 1d**).

The phenotype of FA-N is in many ways typical of Fanconi anemia and includes growth retardation and variable congenital malformations (**Table 1**). 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, markedly reduced lymphocyte survival and increased chromosome breakage on exposure to MMC (**Supplementary Fig. 1** online). There was also no formation of nuclear RAD51 foci in PALB2-deficient fibroblasts after ionizing irradiation. Again, this is similar to cells with biallelic *BRCA2* mutations and differs from other Fanconi anemia subtypes[13] (**Supplementary Fig. 1**).

All seven individuals with FA-N developed cancers 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 and early mortality associated with biallelic *PALB2* mutations is thus very similar to that associated with biallelic *BRCA2* mutations[10]. The reasons for the association between childhood solid tumors and deficiency of BRCA2 or PALB2 are unclear but are probably related to functions not shared by other Fanconi anemia 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[5]. 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 note in this regard are the cancer histories in the seven FA-N families we have identified. The mother of IFAR-007 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 individuals are known to have developed cancer, although most are still under 50 years of age. However, the maternal grandmother of ICR-60 and a maternal great-grandmother of GESH developed breast cancer at 52 years and 20 years of age, respectively. Mutational analyses of *PALB2* in individuals with adult-onset cancer, particularly familial

Table 1 *PALB2* mutations and clinical features of FA-N

		Mutation 1		Mutation 2		Clinical features	
ID	Origin	Nucleotide change	Effect	Nucleotide change	Effect	Cancer (age at diagnosis)	Other features
LOAO[a]	Albanian[b], Moroccan[c]	395delT	V132fs	3113+5G→C	r.2835_3113del279/A946fs	Medulloblastoma (3.5 yrs)	Growth retardation, radial ray hypoplasia, absent right kidney
GESH	German	757_758delCT	L253fs	3294_3298 delGACGA	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], North American[c]	2257C→T	R753X	3549C→A	Y1183X	Wilms tumor (1 yr)	Severe IUGR, postnatal growth retardation, microcephaly, microphthalmia, skin hyperpigmentation
LNEY	German	2393_2394insCT	T799fs	3350+4A→G	r.3350insGCAG/F1118fs	Medulloblastoma (4 yrs)	Growth retardation, microcephaly, microphthalmia, bifurcated anus
IFAR-007[a,d]	North American[b], African ancestry[c]	2521delA	T841fs	3323delA	Y1108fs	Wilms tumor (1.5 yrs), medulloblastoma (1.5 yrs)	Growth retardation, microcephaly, skin hyper- and hypopigmentation, horseshoe kidney, gonadal dysgenesis
ICR-60[a]	British	2962C→T	Q988X	3549C→G	Y1183X	Medulloblastoma (2.3 yrs)	Growth retardation, microcephaly, hypoplastic thumb
IFAR-849	North American	3116delA	N1039fs	3549C→G	Y1183X	Neuroblastoma (0.7 yrs), AML (2 yrs)	Growth retardation, microcephaly, VSD, ASD, thumb and radial anomalies, skin hyperpigmention

AML, acute myelogenous leukemia; IUGR, intrauterine growth retardation; ASD, atrial septal defect, VSD, ventricular septal defect. North American individuals are of European ancestry.
[a]No samples were available from the affected individuals; therefore, the mutations were identified in parental samples. [b]Origin relates to mutation 1. [c]Origin relates to mutation 2. [d]This individual's clinical details have been published previously[14].

breast cancer, will clarify the role of monoallelic *PALB2* mutations in cancer susceptibility.

Mutations in *BRCA2* and *PALB2* together account for almost all the individuals we studied having both Fanconi anemia and childhood solid tumors. However, there are rare individuals with Fanconi anemia 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 unrecognized subtypes of Fanconi anemia.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We would like to thank all the families with Fanconi anemia for cooperating in this study and the many physicians that have referred families for participation in the research. In particular, we thank the doctors and counselors who provided samples and information from the FA-N cases, including R. Beier, T. Brenn, R.C. Lewandowski, M. Hamre, R.E. Harris, A. Carlson, V.M. Der Kaloustian, N. Wong and M. Buyukpamukcu. We thank B. Xia and D. Livingston for the PALB2 antibody and the pOZC-PALB2 vector; K. Spanova and S. Fielding for technical assistance with PALB2 sequencing; R. Friedl, B. Gottwald and S. Furlan for the flow cytometry analyses and handling of patients' cells and M. Stratton, A. Futreal and R. Scott for comments on the manuscript. We acknowledge use of controls from the British 1958 Birth Cohort DNA collection, funded by the Medical Research Council grant G0000934 and the Wellcome Trust grant 068545/Z/02. This work forms part of the UK Childrens' Cancer and Leukaemia Group's FACT (Factors Associated with Childhood Tumours) Study. The work was supported in part by the following: R37HL32987 and R01CA82678 from the US National Institutes of Health (A.D.A.); the Daniel Ayling Fanconi Anaemia Trust (C.G.M.); the Deutsche Fanconi-Anamie-Hilfe e V., Schroeder-Kurth-Fond and the Fanconi Anemia Research Fund (D.S., H.H.); the Deutsche Forschungsgemeinschaft and Elterninitiative Kinderkrebsklinik Duesseldorf e. V. (H.H.); the Jewish General Hospital Weekend to End Breast Cancer, Rethink Breast Cancer, Canada and Canadian Foundation for Innovation (M.T.); the Breast Cancer Campaign (P.K.) and, primarily, the Institute of Cancer Research and Cancer Research UK (S.R., S.H., K.B., S.S., N.R.).

AUTHOR CONTRIBUTIONS

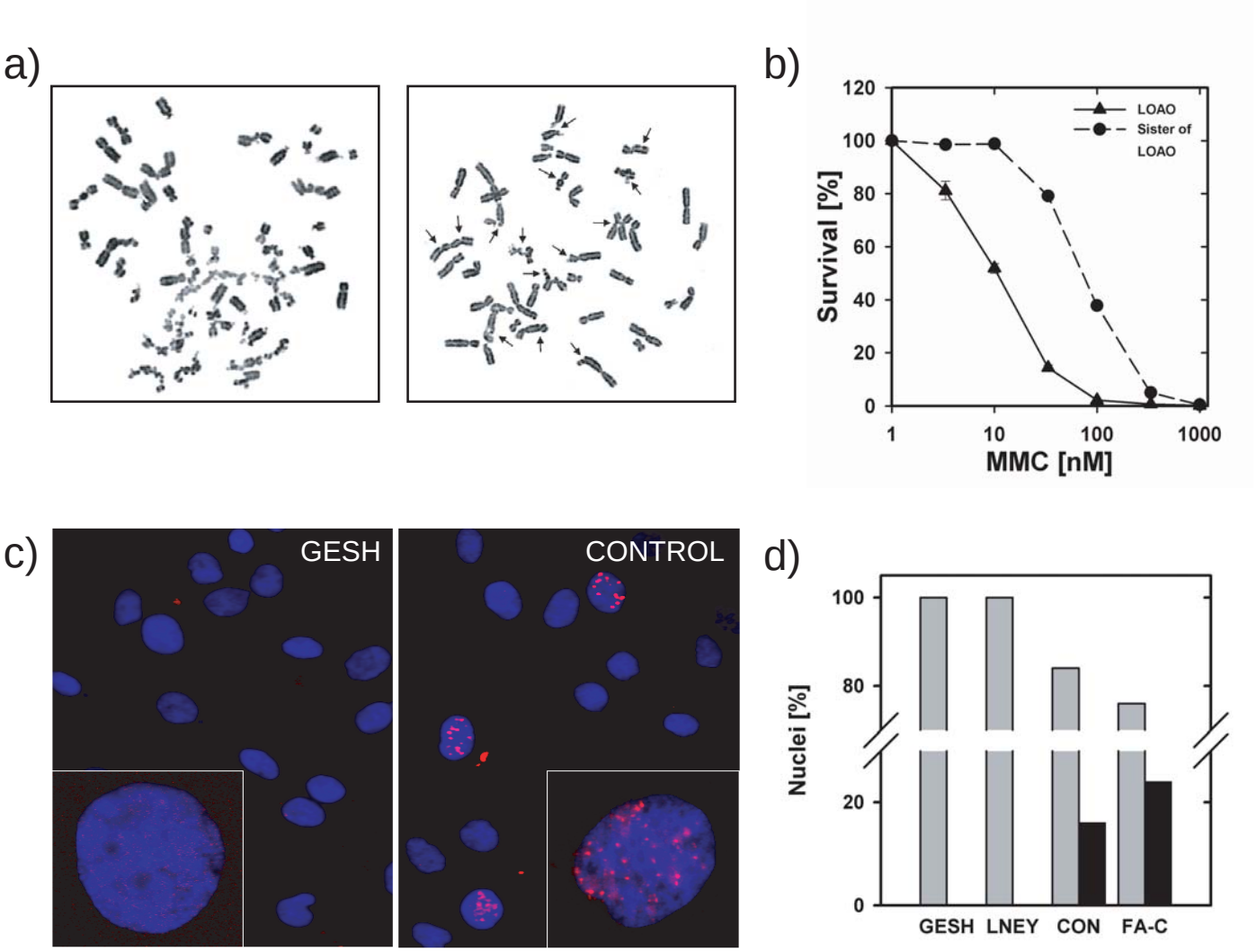
The study was designed by N.R. The mutational and PALB2 protein blot analyses were performed by S.S., K.B., S.H., P.K. and S.S. under the direction of N.R. The cellular, protein blot and complementation analyses and investigation of effects of splicing mutations were performed by D.S., H.H., R.K., K.N., M.F., M.W. and H.N. The phenotypic assessment, sample collection and characterization with respect to Fanconi anemia subgroups was performed by D.S., H.H., S.D.B., F.P.L., S.Y., H.A., M.T., C.G.M. and A.D.A. The manuscript was written by N.R with contributions from the other authors.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at http://www.nature.com/naturegenetics
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Kutler, D.I. *et al. Blood* **101**, 1249–1256 (2003).
2. Niedernhofer, L.J., Lalai, A.S. & Hoeijmakers, J.H.J. *Cell* **123**, 1191–1198 (2005).
3. Taniguchi, T. & D'Andrea, A.D. *Blood* **107**, 4223–4233 (2006).
4. Shivji, M.K. & Venkitaraman, A.R. *DNA Repair (Amst.)* **3**, 835–843 (2004).
5. The Breast Cancer Linkage Consortium. *J. Natl. Cancer Inst.* **91**, 1310–1316 (1999).
6. Howlett, N.G. *et al. Science* **297**, 606–609 (2002).
7. Offit, K. *et al. J. Natl. Cancer Inst.* **95**, 1548–1551 (2003).
8. Hirsch, B. *et al. Blood* **103**, 2554–2559 (2004).
9. Wagner, J.E. *et al. Blood* **103**, 3226–3229 (2004).
10. Reid, S. *et al. J. Med. Genet.* **42**, 147–151 (2005).
11. Xia, B. *et al. Mol. Cell* **22**, 719–729 (2006).
12. Lejeune, F. & Maquat, L.E. *Curr. Opin. Cell Biol.* **17**, 309–315 (2005).
13. Godthelp, B.C. *et al. Mutat. Res.* **594**, 39–48 (2006).
14. de Chadarevian, J.P., Vekemans, M. & Bernstein, M. *Arch. Pathol. Lab. Med.* **109**, 367–369 (1985).
15. Chandra, S. *et al. Mol. Ther.* **12**, 976–984 (2005).



Supplementary Figure 1. Cellular phenotype of PALB2 deficiency. **a)** Severe chromosomal damage in a metaphase from a fibroblast culture of GESH, treated with 50ng/ml MMC for 24 hours (left panel). Breakage rates (breaks per cell) were: GESH, spontaneous 1.58, with 10ng/ml MMC 5.75, with 50ng/ml MMC >10 (23/25 multiaberrant metaphases); FA means, spontaneous 0.26, 10ng/ml 0.72, 50ng/mls 1.92; normal control means, spontaneous 0.02, 10ng/ml 0.06, 50ng/ml 0.18. The right panel shows 14 chromatid and chromosome breaks, indicated by arrows, in a lymphocyte metaphase from a blood culture from the same patient, after exposure to 100ng/ml MMC for 24 hours. **b)** Dose-response curves demonstrate diminished survival of primary T cells from patient LOAO as opposed to his unaffected sibling (cells are not available from LOAO to perform this experiment in a complemented cell line). **c)** No cells with >5 RAD51 nuclear foci were detectable in fibroblasts from GESH after induction by irradiation with 8 Gy, whereas a control showed a proportion of cells with >5 RAD51 signals (red) per nucleus (blue). **d)** The proportion of nuclei with >5 RAD51 foci (black columns) was ~0% in fibroblasts from GESH and LNEY compared to 16% in control cells and 24% in FA-C cells (grey columns, proportion of nuclei with <5 foci).

Supplementary Table 1
Primers and PCR conditions for *PALB2* coding exons

Exon	Primer Sequence 5'-3'		Size(bp)	PCR
	Forward	Reverse		
1	GGATTTAATTGGCCGGAGTT	GACACAAAGCCAGGCCTAAA	309	68-50
2-3	ACCTTTCCACTTGCCCAGTA	GGGAAAAAGAACAATAGCCAAA	400	68-50
4A	GCCTGAATGAAATGTCACTGATT	GGTGATCTAGCAGGATTTTTGC	495	68-50
4B	CCCTAGTGGTGAGCAAAAGC	TTCAAGGTGCTGACTACTACCG	388	68-50
4C	ACCAACTGCCCAACCAGA	TGGTTTTCATTTGCTGGTAAG	357	68-50
4D	AAGTAAAAGTGGCCAACTGC	TTTTTCTTGACATCCAAATGACTC	387	68-50
4E	GCAGAAAAACATTCTTGCACA	AAGGAAGTGCCAGGCAAATA	600	68-50
5A	GATTGTCTGTTTTGTTGGGTTT	GGTCCTCTTCTAAGTCCTCCATT	395	68-50
5B	AAAGAGGGAAGCTGTATTTTTCC	CTGCCTGAACTGTCGAATTG	398	68-50
5C	CACCTGCTTTCCCCATCTTA	GGCATTTCATTCCTTCAGAGA	389	68-50
6	AGTGGGTAATGCAGGCAGAC	TGACTGAATTCTTTTCAGTTCATT	213	68-50
7	TGCTTTGCATAAAACAGCACT	TGGTAAGCTGCCCATCTACA	293	68-50
8	TGGAAAATCTGGATTAAACAAAAA	TGCACTTAAAACCAGCTGACA	221	68-50
9	ATTAAAAGGTTACTCCTCACATCAC	CCCAACTTTCTCTGAAACCTGT	287	68-50
10	CCTAGAGACTGCTTTAGTGCAAA	TTCACAACAACCCTGTAAAATTAG	250	68-50
11	TTTTCTGAATACTGGTTTGTTGGA	CGGGGAAGGTTTGTTCATTA	244	68-50
12	TGCCAGATCTTTATTTTTCCTGA	TGTGTTTGCACAGTGCCTTT	281	68-50
13	TGGTTTTGGGAACATGGTTT	TTAAGTGTCATTCAGATATTCTCCTTT	400	68-50

SUPPLEMENTARY 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[1,2], 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[3], and western blot analysis for FANCD1 (ref. 4), FANCD2 (ref. 5) and FANCJ[6]. *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). 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.

Analysis of splice site mutations

To isolate RNA we used the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) from which cDNA was prepared using oligo(dT)20 primers and

SuperScriptII (Invitrogen). cDNA was amplified with primers GAAATCAGAGAGATCAGGAATTTAforward/TAGGGTTAATCACAATGAGCT GAAACreverse to show exon skipping of 9 and 10 in LOV (father of LOAO) and CAGTCTGTCACAAAGCCTATTCTGforward/GAGTCATCCCTGTGCCAAAGrev erse to show the insertion of 4bps in LNEY. Amplified DNA was sequenced as detailed above.

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 (gift of B Xia). 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[5].

Transduction and complementation

For complementation studies, the γ-retroviral vectors pOZC-PALB2 (ref 7) 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 ng/ml (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 (Supplementary Fig 1b). Transduction was with FANC cDNAs and GFP cDNA as a

control, separately cloned into a S11-type γ-retroviral vector as described[3]. 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. All slides were evaluated independently by several investigators.

References

1. Auerbach, A.D., Rogatko, A., & Schroeder-Kurth, T. M. International Fanconi Anemia Registry: relation of clinical symptoms to diepoxybutane sensitivity. *Blood* **73**, 391-396 (1989).
2. Seyschab, H. *et al.* Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. *Blood* **85**, 2233-2237 (1995).
3. Hanenberg, H. *et al.* Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. *Exp. Hematol.* **30**, 410-20 (2002).
4. Popp, H. *et al.* Screening Fanconi anemia lymphoid cell lines of non-A, C, D2, E, F, G subtypes for defects in BRCA2/FANCD1. *Cytogenet. Genome Res.* **103**, 54-47 (2003).
5. Garcia-Higuera, I. *et. al.* Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol. Cell* **7**, 249-262 (2001).

6. Levran, O. *et al.* The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. *Nat. Genet.* **37**, 931-933 (2005).

7. Xia, B. *et al*. Control of BRCA2 Cellular and Clinical Functions by a Nuclear Partner, PALB2. *Molecular Cell* **22**, 719-729 (2006).

2

Results

BRIEF COMMUNICATIONS

FANCI is a second monoubiquitinated member of the Fanconi anemia pathway

Ashley E Sims[1,6], Elizabeth Spiteri[2,6], Robert J Sims III[1], Adriana G Arita[1], Francis P Lach[2], Thomas Landers[2], Melanie Wurm[3], Marcel Freund[3], Kornelia Neveling[4], Helmut Hanenberg[3,5], Arleen D Auerbach[2] & Tony T Huang[1]

Activation of the Fanconi anemia (FA) DNA damage–response pathway results in the monoubiquitination of FANCD2, which is regulated by the nuclear FA core ubiquitin ligase complex. A FANCD2 protein sequence–based homology search facilitated the discovery of FANCI, a second monoubiquitinated component of the FA pathway. Biallelic mutations in the gene coding for this protein were found in cells from four FA patients, including an FA-I reference cell line.

FA is a rare chromosome-instability syndrome associated with developmental abnormalities, bone-marrow failure, predisposition to cancer and cellular hypersensitivity to DNA cross-linking agents[1,2]. The disorder is genetically heterogeneous, with 13 complementation groups currently defined, 12 of which are associated to distinct genes. Eight FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM) form a core complex with catalytic ubiquitin E3 ligase activity required for the activation of FANCD2 by monoubiquitination[2–4]. Monoubiquitinated FANCD2 is targeted to BRCA1-containing DNA damage and repair sites in chromatin (nuclear foci) to assist in DNA repair, along with three other downstream FA members, FANCJ (also called BRIP1)[5], FANCD1 (BRCA2)[6], and FANCN (PALB2)[7,8]. It is assumed that FANCD2 monoubiquitination is the key trigger activating the FA pathway. Negative regulation of the FA pathway can be achieved, in part, through FANCD2 deubiquitination by USP1 (refs. 9,10). It is unknown whether the FA core complex controls the monoubiquitination of other protein substrates to aid in DNA cross-link repair and genomic maintenance[11]. Experiments in chicken DT40 cells have hinted at a FANCD2-independent

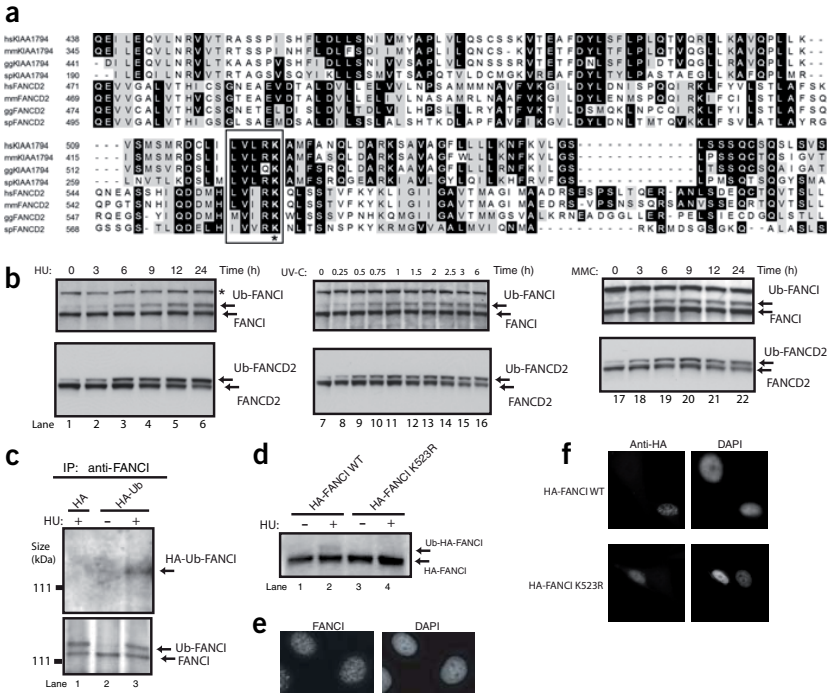


Figure 1 FANCI monoubiquitination is site-specific and DNA damage dependent. (**a**) CLUSTALW (http://align.genome.jp/) alignment of the conserved region of FANCD2 and KIAA1794 (FANCI) identified by the LVI/LRK motif (boxed). Black and gray boxes, identical and similar amino acids, respectively; asterisk, conserved monoubiquitination site; hs, *Homo sapiens*; mm, *Mus musculus*; gg, *Gallus gallus*; sp, *Strongylocentrotus purpuratus*. Amino acid positions are indicated. (**b**) Time course of FANCD2 and FANCI monoubiquitination (Ub) induced by hydroxyurea (HU), UV-C or MMC and analyzed by western blotting. (**c**) HeLa cells were transiently transfected with an HA vector or HA-ubiquitin construct and treated as indicated. Cell extracts were immunoprecipitated with anti-FANCI and probed with anti-HA (upper gel) or anti-FANCI (lower gel). (**d**) HeLa cells were transfected with HA-tagged wild-type (WT) FANCI or K523R mutant and treated as indicated. Cell extracts were immunoprecipitated using anti-FANCI and probed with anti-HA. (**e**) FANCI localization to DNA damage nuclear foci was examined by indirect immunofluorescence using antibodies against either FANCI or HA and DAPI counter-staining. Representative image of HU-treated HeLa cells stained for endogenous FANCI, HA-FANCI WT or K523R mutant is shown. (**f**) Cells were transfected with indicated constructs and analyzed as in **e**.

[1]Department of Biochemistry, New York University School of Medicine, New York, New York 10016, USA. [2]Laboratory of Human Genetics and Hematology, The Rockefeller University, New York, New York 10021, USA. [3]Department of Pediatric Hematology, Oncology and Clinical Immunology, Children's Hospital, Heinrich Heine University, D-40225 Duesseldorf, Germany. [4]Department of Human Genetics, University of Wuerzburg, D-97074 Wuerzberg, Germany. [5]Department of Pediatrics, Wells Center for Pediatric Research, Riley Hospital for Children, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA. [6]These authors contributed equally to this work. Correspondence should be addressed to T.T.H. (tony.huang@med.nyu.edu).

Received 12 March 2007; accepted 20 April 2007; published online 25 April 2007; doi:10.1038/nsmb1252

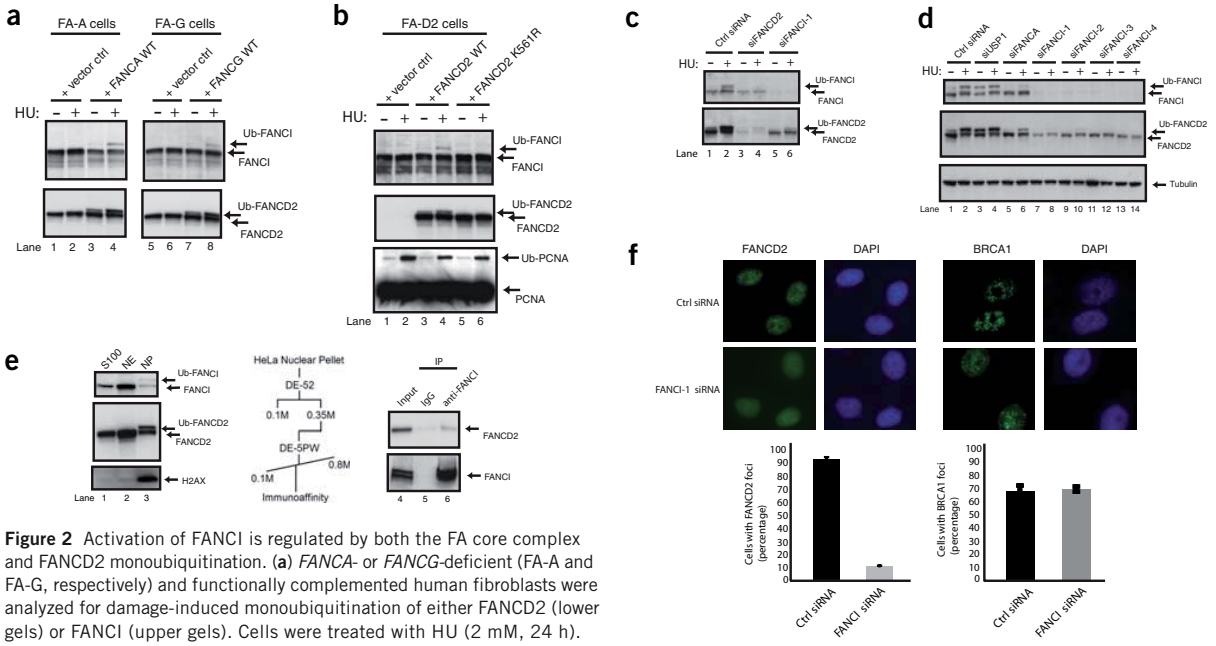


Figure 2 Activation of FANCI is regulated by both the FA core complex and FANCD2 monoubiquitination. (**a**) *FANCA*- or *FANCG*-deficient (FA-A and FA-G, respectively) and functionally complemented human fibroblasts were analyzed for damage-induced monoubiquitination of either FANCD2 (lower gels) or FANCI (upper gels). Cells were treated with HU (2 mM, 24 h). (**b**) *FANCD2*-deficient (FA-D2) human fibroblast cell line (PD20) stably expressing vector control, wild-type FANCD2 or the FANCD2 K561R mutant[14] was examined for monoubiquitination of FANCI (as in **a**). (**c**) HeLa cells were transfected with siRNAs targeting the indicated genes, treated with HU (2 mM, 24 h) and probed for FANCI (upper gel) or FANCD2 (lower gel). (**d**) HeLa cells were transfected with indicated siRNAs and treated with HU (2 mM, 18 h). Monoubiquitination of both FANCI and FANCD2 was analyzed by western blotting. (**e**) HeLa cells were fractionated into cytoplasm (S100), nuclear extract (NE) and nuclear pellet (NP). NP was further biochemically fractionated, immunoprecipitated using anti-FANCI or control IgG, and western blotted with anti-FANCI (lower gel) or anti-FANCD2 (upper gel). (**f**) HeLa cells were treated with siRNA against *FANCI* or Ctrl sequence and stimulated with HU (18 h) before paraformaldehyde fixation. FANCD2 and BRCA1 proteins were detected by indirect immunofluorescence using antibodies against FANCD2 and BRCA1. Nuclear foci were quantified (error bars show s.d. from three independent experiments).

function of the FA core complex[12]. The only FA complementation group that has not been assigned to a gene is FA-I or FANCI. Cells of FA-I patients presumably have an intact FA core complex but are known to be defective in FANCD2 monoubiquitination[13].

To identify novel substrates of the FA core complex, we began with a protein BLAST search (which detects short, nearly exact matches) for gene products that have sequence homology to the highly conserved human FANCD2 monoubiquitination site, LVIRK. Although this search method originally yielded a large number of putative targets, we reduced this list to several dozen human proteins on the basis of protein domain architecture and putative function (**Supplementary Fig. 1** online), placing emphasis on chromatin-associated factors and proteins implicated in ubiquitin-related activities. We used a 'back search' to determine whether any of the proteins have additional homology to FANCD2 extending beyond the LVIRK motif. Notably, only one protein, KIAA1794, has substantial homology to FANCD2. KIAA1794 is a previously uncharacterized protein of 1,328 amino acid residues with a predicted molecular weight of ~140 kDa. Comparison between the two proteins in the region surrounding the FANCD2 monoubiquitination site shows approximately 40% sequence similarity (**Fig. 1a**), but the remainder of the primary amino acid sequences show little similarity (**Supplementary Fig. 2** online). In this study, we identified biallelic mutations in the *KIAA1794* gene in an FA-I reference cell line (see below). Thus, we will refer to *KIAA1794* as *FANCI*.

To test whether FANCI is a DNA damage–responsive protein that functions similarly to FANCD2, we measured FANCI protein levels in cells after treatment with various DNA-damaging agents. A more slowly migrating species of FANCI appeared in a time-dependent manner, mimicking the DNA damage–inducible monoubiquitination of FANCD2 (**Fig. 1b**). To determine whether the more slowly migrating band is a monoubiquitinated form of FANCI, we transfected hemagglutinin (HA)-tagged ubiquitin into HeLa cells. After FANCI immunoprecipitation, we observed that the upper FANCI band reacted with anti-HA, indicating that it is indeed monoubiquitinated (**Fig. 1c**). To determine whether the conserved Lys523 of FANCI (**Fig. 1a**) is crucial for its monoubiquitination, HA-tagged wild-type FANCI or a K523R mutant was transiently expressed in HeLa cells and exposed to DNA damage. HA-FANCI was modified in wild-type but not K523R mutant–expressing cells, suggesting that the ubiquitin modification of FANCI occurs on a specific lysine residue (**Fig. 1d**). Monoubiquitination of FANCD2 is required for its localization to DNA damage and repair nuclear foci. Upon DNA damage, FANCI also formed nuclear foci and resided with FANCD2 and phosphorylated histone H2A.X (**Fig. 1e** and data not shown). The monoubiquitination of FANCI was crucial for its recruitment to nuclear foci (**Fig. 1f**). Thus, our data suggests that, like FANCD2, FANCI is activated by site-specific monoubiquitination upon DNA damage, which then enables it to localize to DNA damage nuclear foci.

To determine whether FANCI monoubiquitination is also regulated by the FA core complex, we examined patient-derived cell lines that were deficient in FA core complex components for FANCI monoubiquitination[14]. We found that in FANCA- and FANCG-deficient cell lines, FANCI monoubiquitination was completely abrogated, whereas FANCI ubiquitination was restored in complemented cells containing an intact FA core complex (**Fig. 2a**). This suggests that the FA core complex is the ubiquitin ligase that activates FANCI upon DNA damage. Notably, the monoubiquitination of FANCI also depends on the presence of the FANCD2 protein and its ability to be monoubiquitinated

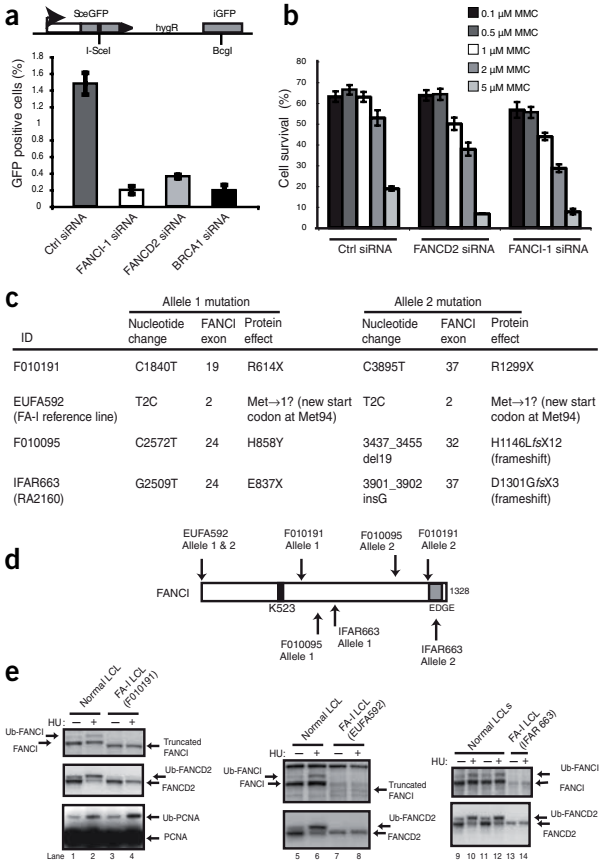


Figure 3 Biallelic mutations in KIAA1794 found in FA-I reference line and other unknown FA cells. (**a**) DR-U2OS cells were treated with the indicated siRNAs for 48 h and then transfected with an I-SceI expression plasmid (pCBASce) to induce double-strand breaks, as described[16]. GFP-expressing cells were analyzed by flow cytometry as described in **Supplementary Methods**. Error bars show s.d. from three independent experiments. (**b**) HeLa cells were exposed to the indicated siRNAs for 48 h and treated with MMC at indicated doses for 96 h. Cytotoxicity levels are plotted as averages from three independent experiments; error bars show s.d. (**c**) *FANCI* mutations in both alleles of individuals with FA. *fs*X# denotes a frameshift mutation, were X# indicates at which codon position the new reading frame ends in a stop (X). Clinical features of FA-I patients are described in **Supplementary Fig. 4**. (**d**) Schematic representation of the FANCI protein, highlighting positions of mutations in different alleles. (**e**) Whole-cell extracts were prepared from exponentially growing lymphoblastoid cell lines from normal and FA-I individuals and analyzed for FANCI (upper gels) and FANCD2 (lower gels) protein levels as in **Figure 1b**.

(**Fig. 2b**). As expected, restoring wild-type FANCD2 to the FANCD2-deficient cells rescued FANCI monoubiquitination. This effect was also reproduced in HeLa cells subjected to small interfering RNA (siRNA)-mediated FANCD2 knockdown (**Fig. 2c**). FANCI protein levels were screened in patient lymphoblastoid cell lines (LCLs) harboring diverse FANCD2 hypomorphic mutations, from the International Fanconi Anemia Registry[15]. In agreement with the cell-line data above, various *FANCD2*-mutant patient cells containing low levels of FANCD2 were also devoid of FANCI monoubiquitination (**Supplementary Fig. 3** online). Therefore, cells expressing FANCD2 at low levels are probably linked to a secondary defect in FANCI activation. We also found that inhibition of USP1 by siRNA increased FANCI monoubiquitination in the absence of the DNA damage treatment (**Fig. 2d**). These results provide evidence that DNA damage–induced activation of FANCI can be regulated, in part, through the actions of the FA core ubiquitin ligase complex and the deubiquitinating enzyme USP1.

The knockdown of FANCI by siRNAs targeting various sequences (siFANCI-1 through siFANCI-4; see **Supplementary Methods** online) inhibited DNA damage–induced monoubiquitination of FANCD2 and also reduced its protein level (**Fig. 2c,d**). The reduction of FANCD2 protein stability via FANCI knockdown could be a consequence of the association of these two factors in a stable protein complex. We found that the majority of monoubiquitinated FANCI is located in the chromatin-enriched nuclear-pellet fraction of asynchronous growing cells (**Fig. 2e**). We partially purified FANCD2 and FANCI from HeLa cell nuclear pellets (**Fig. 2e**). Coeluting fractions containing FANCD2 and FANCI were tested for physical association by coimmunoprecipitation. These experiments identified a weak, but specific, interaction between FANCD2 and FANCI (**Fig. 2e**). A comparable interaction between FANCD2 and FANCI was observed using HeLa nuclear extracts (data not shown). It remains unclear whether the effect of FANCI knockdown on FANCD2 protein stability is due to the loss of FANCI interaction with FANCD2. We are also currently exploring the possibilities of transcriptional or post-transcriptional regulation of FANCD2 stability by FANCI.

Notably, the DNA damage–induced formation of FANCD2 nuclear foci was also greatly reduced upon *FANCI* siRNA treatment, suggesting a direct role of FANCI in promoting efficient monoubiquitination of FANCD2 or stabilization and recruitment of FANCD2 to proper DNA repair sites (**Fig. 2f**). Inhibition of FANCI by siRNA did not have a general effect on nuclear foci formation of other DNA-repair proteins, as BRCA1 localization to dot structures was unaffected (**Fig. 2f**).

It was recently demonstrated that FANCD2 functions in DNA repair by promoting homologous recombination (HR)[16]. To assess the role of FANCI in HR and double-strand break repair, we used an engineered U20S cell line carrying a single, stably integrated copy of a DR–green fluorescent protein (GFP) HR reporter[16,17]. These cells were transfected with siRNAs against *FANCD2*, *KIAA1794* or *BRCA1*, and their ability to perform HR repair (gene conversion) was tested. The percentage of GFP-positive cells was reduced in BRCA1-, FANCD2- and FANCI-knockdown cells compared with the control-siRNA samples, suggesting that FANCI may have a role in DNA repair (**Fig. 3a**). We next tested whether *FANCI* siRNA–treated cells were sensitive to the DNA cross-linking agent mitomycin C (MMC). Like FANCD2-knockdown cells, cells depleted of FANCI were sensitized to MMC at various doses (**Fig. 3b**). Together, these results suggest that FANCI behaves as a functional homolog of FANCD2 in the coordination and repair of DNA cross-links.

We sequenced all of the exons and intron-exon boundaries of *FANCI* and its corresponding complementary DNAs from individuals with FA that were not defective for any of the known FA genes and narrowed the mutation search to patients whose cells have defective FANCD2 monoubiquitination (see **Supplementary Tables 1** and **2** online). From this set, we identified four FA patients with biallelic mutations in the *FANCI* gene (**Fig. 3c,d**). We also sequenced *FANCI* in 100 control alleles (50 normal individuals) and did not find these particular missense or nonsense mutations, providing further evidence that such mutations are probably pathogenic in the individuals with FA. One of the individuals (EUFA592) belonged to the previously uncharacterized FA-I complementation group[13]. This enabled us to directly link the gene product of *KIAA1794* to the elusive FANCI protein. Our data are in agreement with recently published work on FANCI[18].

The FANCI reference line EUFA592 was found to contain a T→C transition in *FANCI* that alters the first methionine codon of the *FANCI* coding sequence. The predicted outcome of this mutation is a new start codon at Met94. Sequencing also confirmed the presence of this mutation in both alleles (**Supplementary Fig. 4** online). Homozygosity at the disease locus probably comes from a consanguineous family. We detected a truncated FANCI protein in LCLs from this individual, which roughly corresponds to a loss of the N-terminal 93 amino acid residues of FANCI owing to the new start site (**Fig. 3e**). We also identified truncating mutations in *FANCI* from a second individual (F010191), who previously had been found not to have mutations in any known FA gene. In this heterozygous patient, we saw an amino acid change of R614X (where X denotes a stop codon) in one allele and R1299X in the other (**Supplementary Fig. 4**). The R1299X mutation predicts a shorter form of the FANCI protein, which was detected in the F010191 LCLs (**Fig. 3e**). However, we were unable to detect any protein product from the other mutant allele. This may be due to nonsense-mediated RNA decay or to the instability of the truncated protein product.

Of note, the last 30 residues of FANCI constitutes a putative EDGE motif[19], which would be deleted in FANCI encoded by the R1299X mutant allele (**Supplementary Fig. 2**). The EDGE motif is defined by the EDGE sequence, originally characterized in a splice variant of FANCD2 containing exon 44 (FANCD2-44)[19]. *FANCD2*-deficient cells expressing FANCD2-44 are capable of restoring FANCD2 monoubiquitination and correcting the MMC sensitivity. Another splice variant containing exon 43 (FANCD2-43) differs only in the C-terminal 24 residues and does not contain the EDGE motif. FANCD2-43 is still capable of rescuing monoubiquitination but cannot correct for the MMC sensitivity[19]. This implies that the EDGE motif may be crucial in DNA damage sensing or DNA repair for both FANCD2 and FANCI. Notably, the F010191 cells were defective for both FANCD2 and FANCI monoubiquitination, suggesting that the extreme C-terminal region of FANCI may be especially important for activation of the FA pathway. However, it is unlikely that the EDGE motif of FANCI is required for FANCD2 protein stability, as the level of FANCD2 in F010191 cells was comparable to that in the normal LCL control (**Fig. 3e**). It is also likely that FANCI has a monoubiquitination-independent role in DNA repair, such as in the regulation of FANCD2 protein stability. In agreement with this, we have yet to observe complete loss-of-function mutations in *FANCI* from any FA-I individual. All of the patients identified as mutated in *FANCI* express some FANCI protein, as detected with our antibodies (**Fig. 3e**). We predict that null mutations of *FANCI* may be lethal in humans. Key questions regarding how DNA cross-links are directly sensed and relayed to the downstream components of the FA pathway will undoubtedly be facilitated by the identification of FANCI and its potential role as a functional counterpart to FANCD2.

Accesssion codes. GenBank: FANCI, EF567077.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

ACKNOWLEDGMENTS

We thank the affected individuals and their families and the many physicians that have referred families to the International Fanconi Anemia Registry for participation in the research. We thank H. Joenje (VU University Medical Center) for providing the FA-I reference cell line EUFA592, K. Nakanishi, M. Jasin (Memorial Sloan-Kettering Cancer Center), T. Taniguchi (Fred Hutchinson Cancer Research Center) and A. D'Andrea (Dana-Farber Cancer Institute) for cell lines and reagents, Deutsche FA-Hilfe and R. Dietrich for research support, members of T.T.H.'s, D.Bar-Sagi and D. Reinberg laboratories (New York University) for technical assistance, equipment and helpful discussions, and H. Wang (SeqWright) for providing technical expertise. This work was supported in part by Deutsche Forschungsgemeinschaft grant Ha2322/2-1 and German Jose Carreras Leukemia Foundation R06/21 (H.H.), US National Institutes of Health grant R37HL32987 (A.D.A.) and New York University School of Medicine start-up funding (T.T.H.).

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

Published online at http://www.nature.com/nsmb/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Kutler, D.I. *et al. Blood* **101**, 1249–1256 (2003).
2. Kennedy, R.D. & D'Andrea, A.D. *Genes Dev.* **19**, 2925–2940 (2005).
3. Taniguchi, T. & D'Andrea, A.D. *Blood* **107**, 4223–4233 (2006).
4. Niedernhofer, L.J. *Mol. Cell* **25**, 487–490 (2007).
5. Levran, O. *et al. Nat. Genet.* **37**, 931–933 (2005).
6. Howlett, N.G. *et al. Science* **297**, 606–609 (2002).
7. Reid, S. *et al. Nat. Genet.* **39**, 162–164 (2007).
8. Xia, B. *et al. Nat. Genet.* **39**, 159–161 (2007).
9. Nijman, S.M. *et al. Mol. Cell* **17**, 331–339 (2005).
10. Huang, T.T. *et al. Nat. Cell Biol.* **8**, 339–347 (2006).
11. Huang, T.T. & D'Andrea, A.D. *Nat. Rev. Mol. Cell Biol.* **7**, 323–334 (2006).
12. Matsushita, N. *et al. Mol. Cell* **19**, 841–847 (2005).
13. Levitus, M. *et al. Blood* **103**, 2498–2503 (2004).
14. Garcia-Higuera, I. *et al. Mol. Cell* **7**, 249–262 (2001).
15. Kalb, R. *et al. Am. J. Hum. Genet.* **80**, 895–910 (2007).
16. Nakanishi, K. *et al. Proc. Natl. Acad. Sci. USA* **102**, 1110–1115 (2005).
17. Xia, B. *et al. Mol. Cell* **22**, 719–729 (2006).
18. Smogorzewska, A. *et al. Cell*, published online 3 April 2007.
19. Montes de Oca, R. *et al. Blood* **105**, 1003–1009 (2005).

A detailed description of the BLAST search used to identify homologous FANCD2 is now provided in the legend of **Supplementary Figure 1**.

Accession	Gene	Motif (Lys position)	Comments
EAX02042	KIAA1794	LVLRK (523)	Homology to FANCD2
EAW77558	RNF111	LVVRK (93)	Ring finger domain
NP_003763	JRKL	LVIRK (508)	Endonuclease and DNA binding domain
EAW63975	BRPF1	ILLRK (639)	Contains bromo- and chromodomains
EAW94337	RNF190	VLLRK (138)	Ring finger domain
Q96EP1	CHFR	VLLRK (35)	E3 ligase with homology to RAD18
BAA75785	DPP3	VLLRK (422)	Peptidase
AAX83871	PA200	IVVRK (1202)	Implicated in DNA repair, spermatogenesis
EAW91845	EDD1	LLGRK (1410)	E3 ubiquitin protein ligase
BAA86497	KIAA1183	VVLRK (112)	Similar to RecN
EAW53522	HORMAD1	LLIRK (156)	Contains a domain implicated in recognizing DNA adducts
AAA52651	Histone H3	LLIRK (65)	Ubiquitination induced upon DNA damage
EAW61271	OTUD7A	LVLRK (86)	Cysteine protease domain
Q9BXT8	RNF17	LVLRK (557)	Ring finger domain
EAW89220	C17orf28	ILIRK (753)	Contains homology to the DNA repair protein Pso2/Snm1
EAW92491	RTF1	LVVRK (407)	Regulates histone ubiquitination and H3K4 and H3K79 methylation
EAW68049	PRDM11	LVIRK (383)	Putative histone methyltransferase

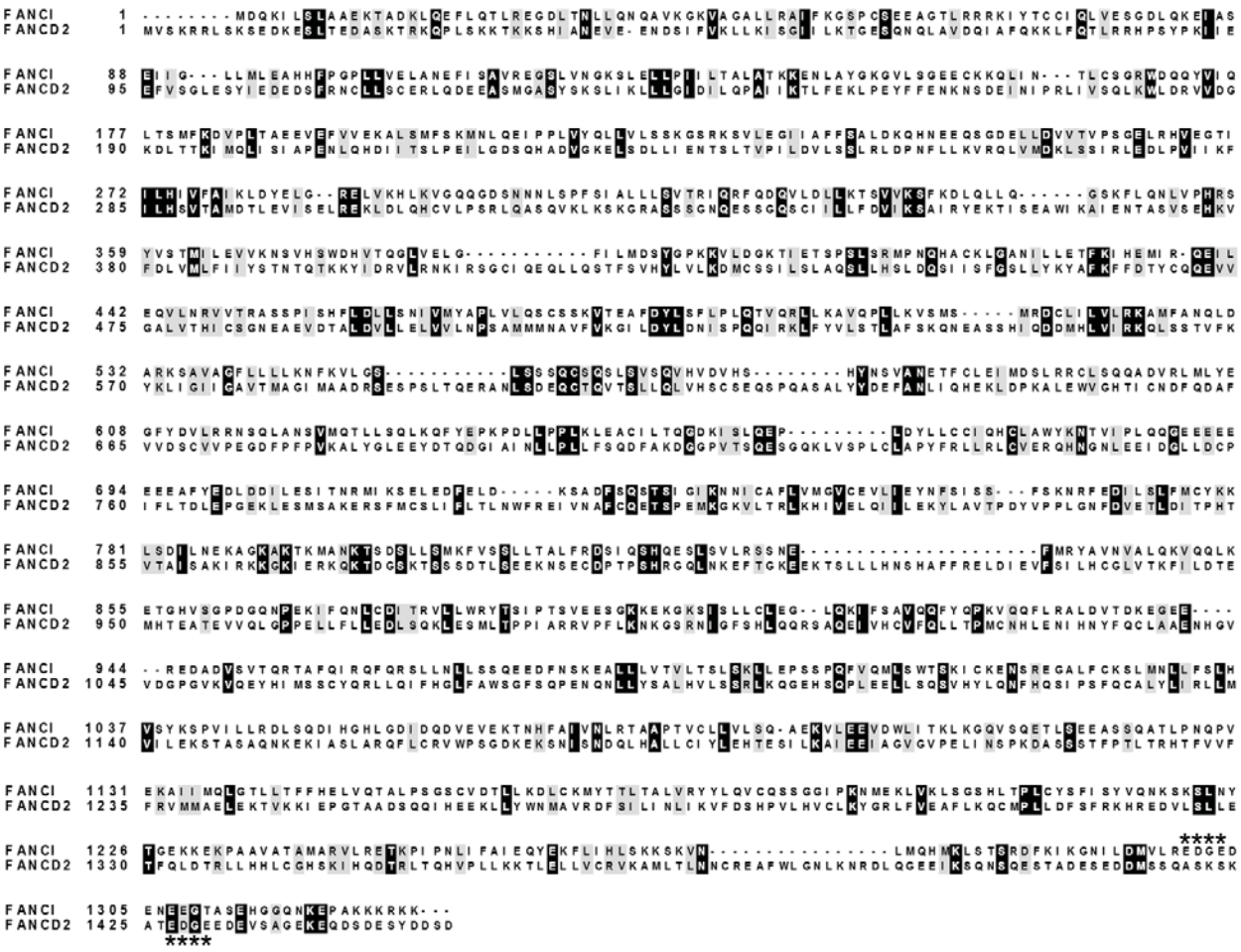
Supplementary Fig. 1
Top candidates from sequence-based homology search using the conserved FANCD2monoubiquitination site LVIRK. From this list, we have tested PA200, histone H3,OTUD7A, and RTF1 for DNA damage-induced monoubiquitination. We were unable to confirm whether these four candidates were indeed monoubiquitinated in an FA-dependent manner.

Identification of candidate substrates of the FA core complex began with a human protein BLAST search (under Search for short, nearly exact matches; http://www.ncbi.nlm.nih.gov/BLAST/) for gene products that have exact sequence homology to the highly conserved human FANCD2 monoubiquitination site, LVIRK. The sequences tested had conservative changes to the hydrophobic residues [L,I,V] with basic amino acids kept constant. We focused on eight variations of this motif that maintained high similarity with the LVIRK sequence in FANCD2; LVIRK, LVLRK, LLLRK, LLIRK, IVLRK, IVIRK, ILLRK, and ILIRK. This search resulted in a large but manageable number of protein hits. We have since expanded and modified our search to include additional LVIRK-like motifs other than those mentioned above and have identified other potential targets for monoubiquitination (data not shown).

The following is an example of our methodology using the LVLRK motif as a search, which yielded KIAA1794 as a putative target for monoubiquitination. The Homo sapiens protein database was chosen to limit the search to human proteins. The PAM30 matrix was used with Gap Costs of Existence: 10; Extension: 1, to minimize the number of hits with gaps. A limit of 500 descriptions and alignments were selected. Upon the search, 106 protein hits were identified. Many of these hits were easily eliminated as putative monoubiquitination targets of the FA pathway based on their protein function (for example, Carnitine O-palmitoyltransferase II, accession BAD9677; seven transmembrane helix receptor, accession BAC45265; kinesin family member 13B, accession EAW63489; vacuolar protein sorting 13B, accession NP_858047; etc.). Proteins that did not fall into the above group were further analyzed by two modes. One method of analyses included examining the protein domain architecture of potential targets to identify putative enzymatic activities or other chromatin-targeting domains, given the nature of FANCD2 targeting to chromatin upon monoubiquitination. Emphasis was also placed on proteins containing domains implicated in ubiquitin-related activities. Hits using this criteria included OTU domain containing 7 (putative cysteine protease, accession EAW61269), and RING finger protein 17 (putative ligase, accession Q9BXT8), etc.

Proteins that lacked protein domains or had few recognizable features were analyzed by BLINK, which "back-searches" (i.e. BLASTs) the entry to a wide range of species. BLINK is accessible from any accession page obtained through ENTREZ Protein (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein). These hits were examined for potential connections to enzymatic activities, chromatin-targeting, or ubiquitin-related activities as described above. This is how KIAA1794 was identified as containing extended homology to FANCD2 (Strongylocentrotus purpuratus). A list of putative targets was then assembled and the sequences were compared to FANCD2 to identify additional homologies. From this list, KIAA1794 was the only protein that contained additional homology to FANCD2. Similar analyses were performed for the additional targets mentioned above. The number of hits for each sequence were as follows: LVIRK: 100; LVLRK: 106; LLLRK: 115; LLIRK: 109; IVLRK: 114; IVIRK: 107; ILLRK: 136; and ILIRK: 102.

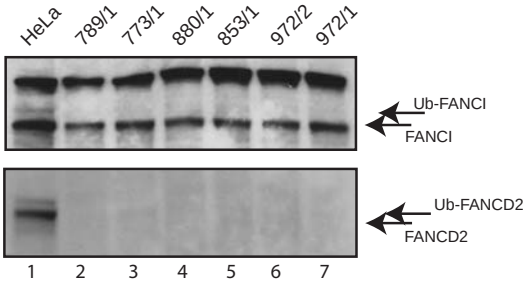
Sims et al.



Supplementary Fig. 2
Protein sequence alignment of human FANCI and FANCD2. Exact and similar sequences are highlighted with dark or light colors, respectively. Asterisks denote semiconserved EDGE motif in both FANCI and FANCD2.

Sims et al.

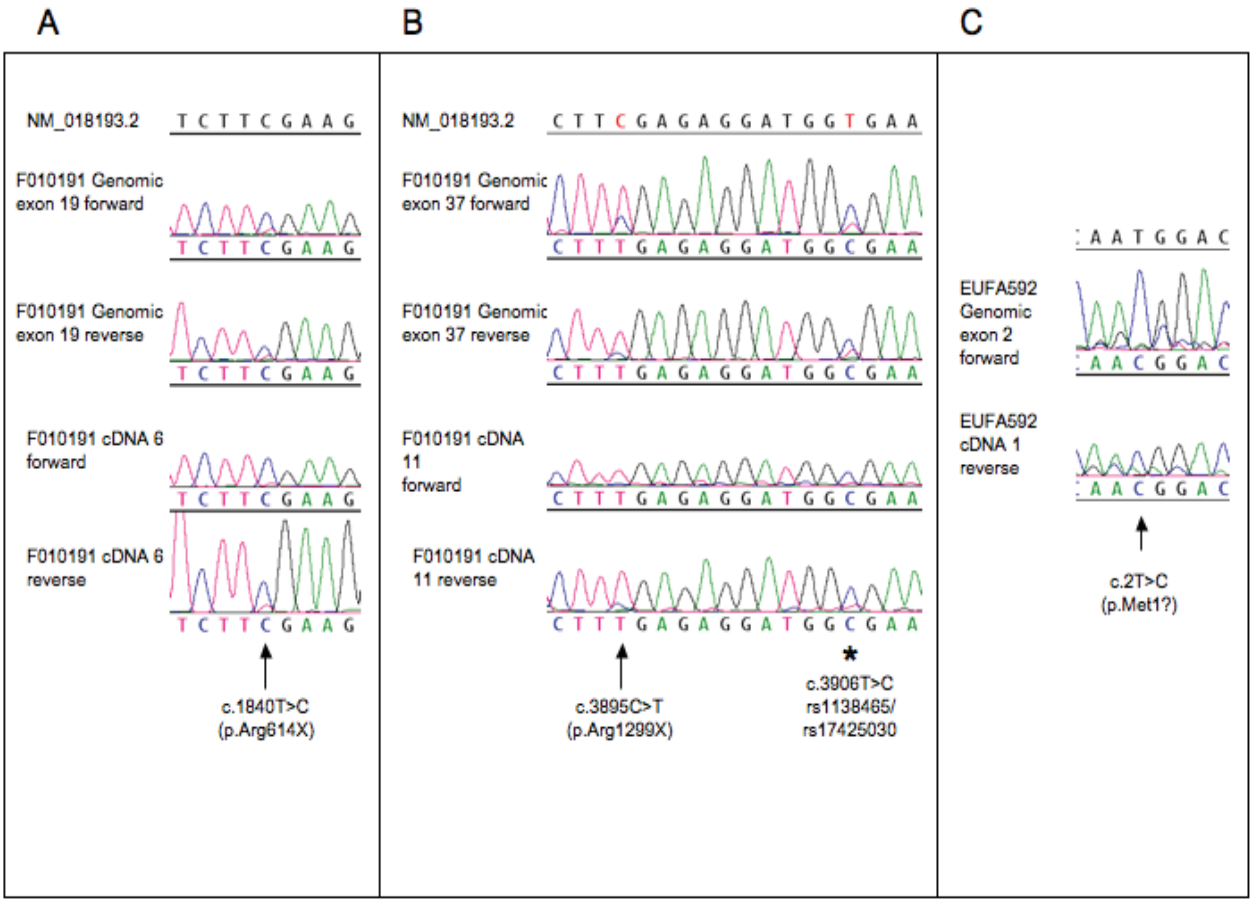
Individual IFAR#	Paternal FANCD2 mutation	Maternal FANCD2 mutation
789/1	c.2660delA	c.1948-6C>A
773/1	c.2T>C	c.3706>A
880/1	c.2444G>A	c.2715+1G>A
853/1	c.2444G>A	c.2404C>T
972/2	c.1367T>G	c.757C>T
972/1	c.1367T>G	c.757C>T



Supplementary Fig. 3
Determining the monoubiquitination status of FANCI in FA patients with low FANCD2 protein levels. Individuals from IFAR harboring known FANCD2 mutations were analyzed for FANCD2 protein expression (lower panel) and FANCI monoubiquitination (upper panel). Whole-cell extracts from exponentially growing lymphoblastoid cell lines (LCLs) from patients were used in this study.

Sims et al.

Mutation detection in genomic and cDNA sequences and Clinical features of FA-I patient-derived cells



Supplementary Fig. 4

Sequence traces for patients F010191 and EUFA592. Genomic DNA and transcripts represented in cDNA isolated from F010191 (A and B) and EUFA592 (C) were amplified by PCR and sequenced. The heterozygous mutations c.1840C>T and c.3895C>T identified in F010191 and the homozygous mutation c.2T>C identified in EUFA592 are indicated by arrows and found in both genomic and expressed transcripts. The location of an SNP in F010191 exon 37 is indicated by and asterisk. Clinical features of FA-I patients are : (F01019)Growth retardation and microophthalmia, thumb and radial aplasia, hearing loss, anemia at 1 year of age, transfusion dependent, alive at 31 years of age . (F010095) Growth retardation and microophthalmia, multiple congenital malformations including thumb, kidney, hearing loss, BM failure at age 6 years, died at age 21 during HCT. (IFAR663) Growth retardation and microophthalmia, multiple congenital malformations including thumb, kidney, heart, brain, hearing loss, HCT at age 6 years, currently alive at age 10 years.

FANCI Genomic primers 5'> 3'	
FANCI ex1-F	CTCCCTCAACAGGACAGGAG
FANCI ex1-R	TCCACGCCAGAGAAAATACC
FANCI ex2-F	TTCCAGGGAATTTTCAGGATT
FANCI ex2-R	GGTCACAAATGCCCTCAAGT
FANCI ex3-F	GGTGTTTGTAATACTTGGCGTCT
FANCI ex3-R	CAGCAACGCAATCTCAGGTA
FANCI ex4-F	TCAAAGCCCTTAACCATTGC
FANCI ex4-R	ATGTTGCAGAGGGTGGTCTC
FANCIex5-F	CATCAGGGAACCGATACAGG
FANCI ex5-R	TTTTCCGCCACTTGTATTCC
FANCI ex6-F	CACCATTTTCCAGGACCATT
FANCI ex6-R	TTTTCCGCCACTTGTATTCC
FANCI ex7-F	TTTGCGAGTCGGGTAGGTTA
FANCI ex7-R	AAGGAGGCAGGAGTGAGGA
FANCI ex8-F	TTCTCTGCTCCCAAGTTTCA
FANCI ex8-R	TGCATGTTTTCCTTGGATGA
FANCI ex9-F	TGCGGAGGTAAAAGGGATAA
FANCI ex9-R	TTCCTGGAAAGGTGAAAATG
FANCI ex10-F	ACCACATCGGGCTGATTTT
FANCI ex10-R	GGCTTGCTTAAGGATGTGGT
FANCI ex11-F	GTTGAGCCTGGGAAATCAAG
FANCI ex11-R	TCCATCAATCCCCAGAGAAC
FANCI ex12-F	TGTGAATGGCAGCTCATAGG
FANCI ex12-R	AGGGCCTGAGATTCAAGGAT
FANCI ex13-F	GAGACCACTTGCCTGCTGTT
FANCI ex13-R	CGTAAGTAAGTATTCCATCAAACTGG
FANCI ex14-F	AAGCACAGAACAAGGGGAAA
FANCI ex14-R	TTGACAACTGACAATCCCAGA
FANCI ex15-F	TCGGTTTCTTCCTTTCTTTTAGG
FANCI ex15-R	TGCTCTTGCCCTTTTAATGC
FANCI ex16-F	GAGGCATTTGCAACCAGTTT
FANCI ex16-R	CTCTGCGGGGCTAGGAAT
FANCI ex17-F	TTTGTTTTGCTCTACGCTTCA
FANCI ex17-R	CACTGAGGACAGTGGCTACC
FANCI ex18-F	TGTCAAGGAAACCTTAAGGAATATG
FANCI ex18-R	TGCCCTGCCTTAAACAACTT
FANCI ex19-F	GACATTTTGGGTGGGAAAAA
FANCI ex19-R	ACCCACAGCATGTTCACAAA

FANCI ex20-F	TTGGCTGCATTGTTCTTTGA
FANCI ex20-R	GGAAGTGATGGAAGCCTTGA
FANCI ex21-F	GGGGGAAGCATTAGAAAAGG
FANCI ex21-R	CCCATCACAAGAAAAGCACA
FANCI ex22-F	TACAGCAGGGAGAGGAGGAA
FANCI ex22-R	TCAGGGTCTGGGAAATTCTG
FANCI ex23-F	GATTGCTGTGACCTGGGAGT
FANCI ex23-R	CCTGCTGGAATTCATCCCTA
FANCI ex24-F	GAGAGTCTGCCAGTCGGAAC
FANCI ex24-R	CTGCCACCTGGCTAATGTTT
FANCI ex25-F	CTTGGAAATGGGAGGAGAGA
FANCI ex25-R	CAGCCACTCTTTGTGGTTGA
FANCI ex26-F	AGAGTGGCTGGAAAATGGAA
FANCI ex26-R	TGTTGCCTGTATGACCCAGA
FANCI ex27-F	GGTTGGAACGGAAAGAGTCA
FANCI ex27-R	CCCTGTATGGCTGCATGTAA
FANCI ex28-F	CCCTAAAACTTTTGGACCTCA
FANCI ex28-R	ACCCCCAGTGAATGAAAGGT
FANCI ex29-F	TGGTGAGGCTGGTCTTGAAT
FANCI ex29-R	TATTCCCCATGGCTCAGGTA
FANCI ex30-F	CATTCTGCTGCGTGACTTGT
FANCI ex30-R	AGAACCTTCTCGGCCTGACT
FANCI ex31-F	TCCTATAGCGGCTTGTGTCC
FANCI ex31-R	TCAGGGGCTCCATTTTTATG
FANCI ex32-F	CATAAAAATGGAGCCCCTGA
FANCI ex32-R	AAACCATGACCTGCCAAGAT
FANCI ex33+34-F	GGGAAGCTTAGGAGCTTTGG
FANCI ex33+34-R	CTTGGCTTGGTCTCTGATGG
FANCI ex35-F	GGTTACAGTGGGTCGACATC
FANCI ex35-R	CAACCCACCTAGCTCTACGC
FANCI ex36-F	TACTGGCATTTGGGTCCTTC
FANCI ex36-R	AGGCCAGAGGATCACTTGAA
FANCI ex37-F	CACACTCAGCCTCTTTGCTG
FANCI ex37-R	TGCCTGATCACCTTTTAGCC
FANCI ex38 a-F	GAAAGTGGGGAAAGCATGAC
FANCI ex38a-R	CAGGTGAAGCGCTGGATATT
FANCI ex38b-F	AAGGAATCTTCTTGGCAGGTC
FANCI ex38b-F	GCCCCAGAGAATCCACTCTT

KIAA1794 cDNA primers 5'> 3'	
cDNA -1F	GGCTTTTTGGAAGTTTGTGG
cDNA-1R	ATGGTCCTGGAAAATGGTGA
cDNA-2F	TCATAGGATTACTGATGCTGGAG
cDNA-2R	GAGGAGAGAACCAGAAGCTGA
cDNA-3F	TCTTCAAGAAATACCACCTTTGG
cDNA-3R	AAAGCTCTTTACAACCGAAGTCT
cDNA-4F	CAGGACCAGGTGCTTGATCT
cDNA-4R	TGGGAGAAGATGCTCTGGTAA
cDNA-5F	TGGAGCAGGTCCTCAACAG
cDNA-5R	GGCTGTGAACATCCACATGA
cDNA-6F	GGTTTTTGCTGCTCCTGAAG
cDNA-6R	TTCGTAGAATGCCTCTTCCTC
cDNA-7F	AACCACTGGATTATCTGCTGTG
cDNA-7R	GAAAAGAGCAGTGAGAAGACTGG
cDNA-8F	GATAGTCTTTTGTCCATGAAATTTGT
cDNA-8R	CTTGGACAAACTGGTAAGAACC
cDNA-9F	GGCAATTTCAGAGGTCCTTGT
cDNA-9R	TGGTAGGGTTGCCTGAGAAG
cDNA-10F	AGGGACAAGTGAGCCAAGAA
cDNA-10R	TGGCAAAGATGAGGTTAGGG
cDNA-11F	GCCAGAGTTCTTCGGGAAAC
cDNA-11R	GCCAGCCATTTTTCAGTAGC
cDNA-12F	TACCCAACAAGCAACAATGC
cDNA-12R	TTGCACTCTATAGGTGACAGAAAA

Sims et al.

Supplementary Methods

Cell culture and transfection. HeLa, U20S (ATCC), and Fanconi anemia patient-derived lines PD20, PD316, and GM6914 cells (Coriell Cell Repository) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS), glutamine, and antibiotics. All lymphoblastoid cell lines (LCLs) derived from the affected patients were established according to standard procedures (*1*). LCLs were maintained in RPMI 1640 medium supplemented with 20% FBS, glutamine, and antibiotics. All cells were grown at 37°C in a humidified incubator containing 5% CO2. SiRNA transfections were done using HiPerfect (Qiagen) and plasmid transfections were done using Fugene6 (Roche) according to the manufacturer's protocol.

Antibodies, Western Blotting and Immunofluorescence Staining. Anti-KIAA1794 antibodies were purchased from Abcam (ab15344 and ab15346). Anti-FANCD2, -BRCA1 and PCNA antibodies were purchased from Santa Cruz Biotech (sc-56, sc-6954 and sc-20022, respectively). Monoclonal anti-HA (HA.11) was purchased from Covance. Anti-tubulin was purchased from Sigma. Anti-GFP antibody (JL-8) was obtained from BD Biosciences.. Immunostaining was performed as described (*2*), using paraformaldehyde fixation. When necessary, cells were pre-extracted with PBS containing 0.5% Triton X-100 for 5 min at room temperature. Briefly, untransfected or transfected cells were split into 4-well chamber slides for foci analysis. Immunoprecipitations were performed in RIPA buffer with specific antibodies and protein G beads and incubated at 4°C overnight with gentle rotation. Western blots were performed with whole-cell extracts and separated on Nupage 3-12% Tris-Acetate or 4-12% Bis-Tris gradient gels (Invitrogen). Solubilization and partial fractionation of HeLa nuclear pellets were performed as described in (*3*).

Plasmids and siRNAs. Full-length human KIAA1794 (FANCI) cDNA was purchased from Origene and verified by sequencing (SeqWright). GenBank accession number for FANCI is EF567077. KIAA1794 cDNA was subcloned into the pIRESpuro3 vector (Clontech) that was modified to contain a 5' sequence coding for 2X HA-epitope tag. The point mutation K523R was generated by two-step PCR mutagenesis from the original KIAA1794 template and verified by DNA sequencing (SeqWright). The siRNAs were synthesized by Qiagen: KIAA1794 (FANCI)-1 (CTGGCTAATCACCAAGCTTAA), KIAA1794-2 (TTGAATTTACTTAGCAGTCAA), KIAA1794-3 (CACGGGCATCTGGGAGATATA), KIAA1794-4 (TACGAAGACCTAGATGATATA), AllStars Neg. Control siRNA, FANCA (AAGGGTCAAGAGGGAAAAATA), FANCD2 (AACAGCCATGGATACACTTGA), and USP1 (TCGGCAATACTTGCTATCTTA)

Homologous Recombination Assay. To test the effect of siRNA knockdown in gene conversion-type DNA repair, DR-U2OS reporter cells (*4*) were first transfected with indicated siRNAs in 6-well plates at low density. 48 hours later, cells were again transfected with I-SceI expression plasmid (pCBASce, 1 microgram per well). 48 hours

after the second transfection, cells were trypsinized and single cell suspensions were analyzed by flow cytometry in a Becton Dickinson FACScan.

Mitomycin C (MMC) sensitivity assay. HeLa cells were seeded at 1000 cells per well in 96-well plates. Eight hours later, cells were treated with MMC (Sigma) at the desired final concentrations. Cell were incubated with the drug for 96 hours and their survival rates were measured using a CellTiter Glo kit (Promega) following the manufacturer's instructions.

Subjects and cell lines. Cell lines and genomic DNA samples were derived by standard methods from FA patients registered in the IFAR after obtaining informed written consent. The studies were approved by the Institutional Review Board of The Rockefeller University. MMC sensitivity was performed by cell cycle analysis of phytohemagglutinin (PHA)-stimulated peripheral blood T cells or of skin derived fibroblast cells according to (*5*).The diagnosis of FA was made by study of DEB-induced chromosomal breakage in PHA-stimulated cultured peripheral blood lymphocytes (PBLs) as described (*6*). Complementation studies with retroviral vectors containing normal FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG and FANCL cDNAs were as described (*7*). Immunoblotting for detection of monoubiquitinated FANCD2 was also performed as described (*8*). FA-M (*9*) and FAAP100 (*10*) were ruled out by immunoblotting for those proteins. Laboratory diagnosis of F010095 and F010191 were also done by spontaneous and MMC-induced G2-phase cell cycle arrest in PBLs.

Isolation of cDNA. RNA was extracted from cells using the RNAqueous Midi kit (Ambion, Austin Texas) following manufacturers directions. RNA was transcribed to cDNA using the Cells-to-cDNA II kit (Ambion) following manufacturers directions.

PCR primers and conditions. KIAA1794 exons were amplified from genomic DNA with the conditions: 94 degrees for 5 minutes, 34 cycles of 94 degrees for 30 seconds, 58 degrees for 30 seconds, and 72 degrees for 30 seconds, followed by one cycle of 72 degrees for five minutes. cDNA was amplified with the same conditions for 40 cycles. Primer sequences are listed in **Supplementary table 1** (genomic) **and 2** (cDNA).

Sequencing. Direct genomic and cDNA sequencing was performed for FANCA/B/C/D2/E/F/G/J according to standard protocols. PCR products were cleaned using ExoSAP-IT (USB, Cleveland, OH) according to manufacturer's protocol. Most of the 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 was generated by means of Sequencing Analysis v.5.1 (Applied Biosystems, Forest City, CA) and analyzed with Lasergene (DNASTAR inc., Madison, Wisconsin).

1. H. Neitzel, *Hum Genet* **73**, 320 (Aug, 1986).
2. T. Taniguchi *et al.*, *Blood* **100**, 2414 (Oct 1, 2002).
3. D. Reinberg, R. G. Roeder, *J Biol Chem* **262**, 3310 (Mar 5, 1987).

4. B. Xia *et al.*, *Mol Cell* **22**, 719 (Jun 23, 2006).
5. H. Seyschab *et al.*, *Blood* **85**, 2233 (1995).
6. A. D. Auerbach, A. Rogatko, T. M. Schroeder-Kurth, *Blood* **73**, 391 (1989).
7. H. Hanenberg *et al.*, *Exp Hematol* **30**, 410 (2002).
8. I. Garcia-Higuera *et al.*, *Mol Cell* **7**, 249 (Feb, 2001).
9. A. R. Meetei *et al.*, *Nature Genetics* **37**, 958 (Sept, 2005).
10. C. Ling *et al.*, *Embo J* (Mar 29, 2007).

3

General Discussion

Fanconi anemia is a disease which is very heterogeneous regarding the clinical and cellular phenotypes of the patients but also with respect to the different genes and proteins of the FA/BRCA pathway. The only common ground is that all these FA/BRCA genes belong to a group of so called “caretaker” genes. Unlike gatekeeper genes, caretakers do not regulate directly cell proliferation but have the task to defend the integrity of the genome (Levitt and Hickson, 2002). The integrity of the genome is threatened by various kinds of DNA damage from endogenous and exogenous sources. DNA-damaging agents include chemicals, ionizing radiation, collapsed replication forks or reactive oxygen species. The resulting DNA-lesions involve loss of purines and pyrimidines, single strand breaks, double strand breaks, or crosslinks. Under the conditions of body temperature of 37 degrees and unavoidable ROS generation due to oxidative phosphorylation, these different types of DNA alterations can amount to several thousand lesions per cell per day. Therefore, an intact DNA repair machinery which includes a variety of caretaker genes, is one of the most important defense- and longevity assurance systems. Mutations in caretaker genes can have devastating effects. In the case of DNA damage, a decision must be taken whether to repair the damage or to channel the damaged cell into apoptosis. Mutational inactivation of DNA caretaker genes automatically leads to severe impairment of this complicated defense system. Loss of the ability to repair DNA damage can result in genomic instability and in the accumulation of mutations, which in turn often results in cancer development. This chain of events implies a close connection between DNA damage, DNA repair, genetic instability and tumorigenesis.

3.1 The FA/BRCA pathway and DNA repair

One recently described group of important caretaker genes consists of genes belonging to the FA/BRCA pathway. This pathway is activated in response to DNA damage and regulates in particular the repair of DNA interstrand crosslinks (ICLs). In bacteria, ICL repair is mediated by a combination of nucleotide excision repair (NER) and homologous recombination (HR) (Dronkert and Kanaar, 2001). Eukaryotes use at least the three different repair mechanisms: NER, translesion synthesis (TLS) and homologous recombination (HR). The current notion is that an ICL which is produced for example by one of the crosslinking agents MMC, DEB, or cisplatin, might be recognized by the inability of the replication fork to progress beyond the DNA lesion. Stalled replication forks are thought to activate the ataxia-telangiectasia and Rad3-related protein (ATR) and its downstream target CHK1. In turn, these “sensor” proteins phosphorylate, and thereby activate the FA core complex and the ID complex. The FA core complex consists of at least four subcomplexes. The first subcomplex, established in the cytoplasm, comprises FANCB, FAAP100 and the catalytic E3 ligase subunit FANCL. The second subcomplex is also located in the cytoplasm and consists of heterodimeric FANCA and FANCG proteins. Nuclear transport of these two subcomplexes probably is independent, but nuclear accumulation appears to be mediated by FANCA. In the nucleus, both subcomplexes form a complex around FANCM and are joined by another subcomplex consisting of FANCC and FANCE. FANCF serves as an adaptor protein, presumably linking and stabilizing the respective subcomplexes. After complete assembly, the FA core complex is recruited to the site of DNA damage by FANCM and its interacting partner FAAP24, where it subsequently monoubiquitinates the phosphorylated proteins FANCI and FANCD2 which form the ID complex (Medhurst et al., 2006; Wang, 2007). E1 and E2 ligase function of the ubiquitin ligase are provided by an ubiquitin-activating enzyme (E1) and a conjugating enzyme (E2). The latter was recently identified as UBE2T (Machida et al., 2006). Other substrates phosphorylated by ATR are H2AX, BRCA1 and BRCA2, which are also recruited to the ICL. At the site of DNA damage, one of the first steps of DNA repair is the incision of the DNA on both sides of the crosslink. This first step is regulated by proteins belonging to the NER machinery. In the case of a TMP crosslink, for example, incisions are made at the fourth phosphodiester bond 3’ to the adducted thymine and at the sixth phosphodiester bond 5’ to the modified nucleotide. Both incisions are made by the Xeroderma pigmentosum endonuclease XPF whose activity might be modulated by its heterodimer-partner ERCC1 (Kumaresan et al.,

2007). The enzymatic cleavage of DNA at both sides of the ICL is followed by “unhooking” of the crosslink and translesion synthesis (TLS). TLS is an error-prone DNA repair mechanism that permits to bypass the gap in damaged DNA. In analogy to monoubiquitinated PCNA, monoubiquitinated ID complex might recruit translesion polymerases to the site of DNA damage. Thereby the monoubiquitinated ID complex would release the replicative polymerase POLδ and load the translesion polymerases REV1 and REV3 (Wang, 2007). Both, monoubiquitinated PCNA and the monoubiquitinated ID complex are de-ubiquitinated by the ubiquitin specific protease USP1 (Nijman et al., 2005). A second set of incisions is thought to be necessary for removing the unhooked ICL, and this step might also include XPF. As a consequence of DNA incisions and unhooking, a DNA double strand break (DSB) is produced. Repair of this DSB is mediated by homologous recombination (HR). Phosphorylation of H2AX, BRCA1 and BRCA2 by ATR might trigger the recruitment of these and other DNA repair proteins like RAD51 to the site of the DSB. BRCA2 and its partner PALB2 can promote homologous recombination and the 5’-3’ helicase BRIP1/FANCJ might promote translesion bypass (Niedernhofer et al., 2005; Wang, 2007) to enable restart of the blocked replication fork. The exact role of the individual FA proteins in this complex series of events leading to DNA repair has long been elusive, but recent data suggest that FA deficient cells have a defect in unhooking the ICL via XPF (Kumaresan et al., 2007). It is conceivable that the FA core complex is involved in this early step of ICL repair, and that the other FA proteins act in the recruitment of translesion polymerases and other DNA repair proteins (ID complex). They may also be directly (BRCA2, PALB2) or indirectly (FANCJ) involved in the execution of DNA repair. This notion would fit with the observation of differences between the upstream and the downstream FA proteins, and also explain in part phenotypes of FA patients belonging to different FA subgroups.

The FA/BRCA network of proteins is not only involved in the resolution of ICLs via NER, TLS and HR repair, but is also linked closely to proteins responsible for other diseases that are similar to FA, sharing with FA the features of genomic instability and cancer. For example, the FA core complex was initially identified as part of a larger subcomplex called BRAFT complex (Meetei et al., 2003b). The BRAFT complex is composed from at least 17 different proteins, including all FA core complex proteins, FAAP100, FAAP24, BLM, Topoisomerase IIIα, BLAP75, RPA70, RPA34, RPA14 and BLAP250 (Wang, 2007). The presence of the BLM helicase and BLM associated proteins (BLAPs) suggests a close connection between FA and Bloom syndrome. Indeed, these syndromes show some clinical overlap, although they have strikingly different cellular phenotypes. For example, SCE (sister chromatid exchange) levels are high in Bloom Syndrome but low in FA. Conversely, MMC sensitivity is high in FA but low in Bloom Syndrome. There is, however, a closely related function in DNA repair, since the BLM complex stabilizes stalled replication fork and facilitates a HR dependent processs to restart stalled replication forks. Other proteins closely related to the FA/BRCA pathway are proteins like ATR, CHK1, RPA, ATRIP, NBS1, MRE11, RAD50, or HCLK2. These proteins are involved in the activation of the FA core complex or the ID complex via phosphorylation events. The downstream FA proteins such as FANCD1, FANCN and FANCJ work also in close contact to other DNA repair proteins. This is very obvious by the direct interaction of BRCA2 and the recombinase RAD51, one of the key players in homologous recombination. Most recently, an interaction between FANCJ and proteins of the mismatch repair pathways was described (Peng et al., 2007). FANCJ appears to interact directly with MLH1 through its helicase domain. This interaction is BRCA1 independent, but appears to be necessary for ICL induced cellular response to DNA repair. These recent observations link the FA/BRCA network of proteins to mismatch repair (MMR). This is a very important observation, since it shows that DNA repair is not an isolated process, but that different kinds of DNA repair mechanisms must interact and work together to preserve genomic integrity. DNA repair thus is not a fixed process depending on the respective type of DNA damage, but is a flexible process that involves multiple strategies that are buffered against failure by a certain degree of redundancy.

3.2 Categories of FA Genes

Although it seems clear that all FA genes belong to the group of caretaker genes, there is a conspicuous difference between FA genes belonging to the FA core complex and the genes whose products are located downstream of FANCD2. So far, there are eight upstream proteins known and necessary for FANCD2 and FANCI monoubiquitination. Only *FANCM* and *FANCL* are highly conserved in evolution and can be found in vertebrates, insects and slime mould (*FANCL*) or vertebrates, invertebrates, yeast and archeae (*FANCM*). All other core complex genes have only vertebrate orthologs (Titus et al., 2006; Wang, 2007). FA genes that are more highly conserved in evolution code for proteins having enzymatic domains and functions. One of the conserved genes is *FANCL*, the gene coding for the catalytic subunit of the FA core complex. Mutations in *FANCL* were thought to be mostly lethal, since for a long time only a single FA-L patient was known. This patient was severely affected and died at young age (Meetei et al., 2003a). Within the framework of this thesis I was able to analyze three further FA-L patients. The clinical phenotype of these three patients turned out to be highly variable, probably as a consequence of the underlying different types of mutations. This leads to the conclusion that the clinical phenotype of FA-L patients is not likely to be fundamentally different from the phenotypes from other FA patients with mutations in the core complex genes. The rareness of FA-L patients must thus be due to other factors. Indeed, the clinical phenotypes were comparable to patients belonging to subgroup FA-E. Patients belonging to this likewise rare complementation group exhibit a variable spectrum of clinical phenotypes that are typical for most of the FA complementation groups. Relative to most other FA genes, *FANCE* stands out because all patients have either truncating mutations or a mutation leading to the disruption of the protein structure. FANCE therefore seems to function as an adaptor protein, connection the FA core complex via FANCC with its substrate FANCD2. This is similar for FANCF, the protein that connects the subcomplexes FANCA/FANCG and FANCC/FANCE. These observations would suggest that FANCL and FANCE share similar consequences of disruption. The two proteins of the ID complex, FANCD2 and FANCI, are also conserved in evolution. They have been referred to as “fraternal twins” and can be found in vertebrates, worms, insects and slime moulds (Grompe and van de Vrugt, 2007; reviewed in Wang, 2007). For *FANCD2* we could show that all patients exhibit hypomorphic mutations leading to detectable residual protein, while biallelic null mutations were not found in any of our patients. Together with the relative severity of the FA-D2 clinical phenotype this observation suggests that patients without traces of functional FANCD2 protein are not viable. Although data concerning the recently identified *FANCI* are not that clear and patients are too rare to make a definitive statement, the situation appears to be very similar in that all FA-I patients so far examined show residual protein resulting from hypomorphic mutations. The situation in the third group of FA proteins, the proteins downstream of FANCD2 and FANCI, is again different. This downstream group must be divided into two subgroups. One is represented by FANCJ, a helicase found in vertebrates, invertebrates and yeast. Although not necessary for FANCD2 and FANCI monoubiquitination, FANCJ resembles the core complex members rather than the other two downstream FA proteins FANCD1/BRCA2 and FANCN/PALB2. Accordingly, the clinical phenotype of FA-J patients corresponds to the “average” FA patient, while FA-D1 and FA-N patients are much more very severely affected. The discovery of additional “missing link” FA-genes might explain the phenotypic discrepancy between these two subgroups of genes that appear to operate downstream of the ID complex.

3.3 FA and Cancer

A high susceptibility for malignant cell growth is one of the main threads faced by FA patients. Most FA patients are at high risk for MDS, AML and squamous cell carcinomas. Regarding tumor types, there is a surprising difference between patients belonging to the upstream and the downstream FA subgroups. All three known genes whose proteins act downstream of FANCD2 and FANCI were shown to be are breast cancer susceptibility genes. The most important of these is the breast cancer susceptibility protein BRCA2. Patients with heterozygous mutations in *BRCA2* have

a tenfold higher risk to develop breast or ovarian cancer than the average population (Rahman et al., 2007). Patients with biallelic mutations in *BRCA2* develop a very severe form of FA, with early childhood cancers like Wilms tumor and medulloblastoma. These tumors frequently lead to the patients‘ death prior to the occurrence of bone marrow failure (Offit et al., 2003; Wagner et al., 2004; Reid et al., 2005; Alter et al., 2007). In contrast to *BRCA2*, *FANCJ* and *FANCN* are low penetrance breast cancer susceptibility genes (Seal et al., 2006; Rahman et al., 2007). FA patients with biallelic mutations in *FANCN* are as severely affected as *BRCA2* patients, but individuals with heterozygous mutations have only a 2.3 fold higher risk for getting breast and ovarian cancer (Rahman et al., 2007). FA patients with biallelic mutations in *FANCJ* are not that severely affected, but rather resemble patients with mutations in the FA core complex (Levitus et al., 2005; Levran et al., 2005). Individuals with monoallelic mutations in *FANCJ* have a 2 fold elevated risk to develop breast and ovarian cancer (Seal et al., 2006). Differences between clinical phenotypes as a function of the particular affected gene illustrate major functional differences between upstream and downstream FA genes. These differences might be explained in part by the fact that BRCA2 and PALB2 are proteins that are directly involved in homologous recombination repair (reviewed in Wang, 2007) while FANCJ, like the upstream members, seems to be only indirectly involved.

An important question is whether carriers of heterozygous FA core complex gene mutations have a higher risk for the development of cancers than non-carriers. However, there seem to be no obvious increased cancer risk for heterozygous carriers, although there are some data suggesting that *FANCC* mutations might be associated with an increased risk for breast cancer (Berwick et al., 2007). If FA genes were to function as classical tumor suppressor genes, one would expect frequent mutations in FA genes in tumors of non-FA patients. One of the studies investigating this point is part of this thesis. The aim of said study was to investigate whether there are mutations in *FANCC* or *FANCG* in bladder tumors of non-FA patients. The results showed that the only example of disruption of the FA/BRCA pathway involved silencing of the *FANCF* gene via hypermethylation, a mechanism that has been observed in several kinds of non-FA tumors (reviewed in Neveling K, 2007c). In such non-FA tumors, *FANCF* promoter methylation never affects all tumor specimens. Most notably, localization of *FANCF* near a hot spot region of chromosome 11p15 suggests that inactivation of *FANCF* via hypermethylation may not be a causal event leading to genetic instability and tumor development, but rather a consequence of tumor progression. Another important conclusion of this study was that the FA/BRCA pathway seems to be rather intact in most tumors investigated. The integrity of the FA/BRCA pathway is therefore maintained in most tumor types as if even such genetically compromised cells need to maintain a certain degree of DNA-damage recognition and repair.

3.4 Identification of New FA Genes

As of today, there still are patients with FA that are not assigned to any of the known complementation groups. To the best of our knowledge, all remaining patients are typical FA patients, meaning that they are not as severely affected like FA-D1 or FA-N patients. The remaining patients can be separated at least in two different groups, according to whether monoubiquitination of FANCD2 is intact or defective. One of the obvious candidates for a missing FA protein was the tumor suppressor protein BRCA1. BRCA1 is connected to the FA/BRCA pathway by the interaction with the 5’-3’ helicase FANCJ, which is also referred to as BRIP1 (BRCA1 interacting protein). BRCA1 also interacts with BRCA2. Although intensely investigated and discussed, BRCA1 itself is probably not a bona fide FA protein, since there are no FA patients with mutations in *BRCA1* and *BRCA1*-/- mice are not viable. Another point is that monoallelic *BRCA1* mutations are relatively frequent in familial breast cancer patients, but none of these patients is known have children with FA and biallelic mutations in *BRCA1*. This suggests that biallelic mutations in *BRCA1* are not viable. Another recent candidate protein was the histone H2 variant protein H2AX. Like the members of the FA core complex, H2AX is a subtrate the kinase ATR, resulting in the phosphorylated form

γH2AX. It was shown that γH2AX is functionally connected to the FA/BRCA pathway by resolving stalled replication forks and preventing chromosomal instability (Bogliolo et al., 2007). It has been demonstrated that phosphorylated H2AX is required for recruiting FANCD2 to chromatin at stalled replication forks. This indicates that γH2AX and FANCD2 function in the same pathway in response to DNA damage. γH2AX is required for FANCD2 relocation to damaged sites but not for FANCD2 activation. Binding of FANCD2 to γH2AX is BRCA1 dependent. Furthermore, cells deficient in γH2AX show an FA like phenotype with chromosomal aberrations and MMC hypersensitivity. Despite this highly suggestive evidence, FA patients with mutations in *H2AX* have not been identified to date. We conclude that H2AX interacts with the FA pathway to prevent MMC induced damage, but H2AX itself is unlikely to be a genuine FA gene (Bogliolo et al., 2007). So far, FAAP100 is one of the best candidate proteins identified by co-immunoprecipitation with an antibody against FANCA protein. It is identical to LOC80233, a predicted hypothetical protein with unknown function (Ling et al., 2007). FAAP100 was shown to be an integral component of the FA core complex and is essential for the stability of this complex. Together with FANCB and FANCL, FAAP100 forms a stable subcomplex which is coregulated by FANCA and FANCM during nuclear localization. FAAP100 is conserved in vertebrates and contains a putative coilded coil domain required for the interaction with FANCB and FANCL. *FAAP100* deficient cells show hallmark features of FA with defective FANCD2 monoubiquitination, hypersensitivity to DNA crosslinking agents, and genomic instability (Ling et al., 2007). Depite intensive efforts, we have so far failed to identify individuals with *FAAP100* mutations, but *FAAP100* is still one of the most convincing candidates for one of the missing FA genes. Several other candidate genes have been tested during the time of this PhD thesis, but most of them turned out to be negative. Candidate gene approaches are still pursued and unassigned FA patients are permanently tested for any of the plausible candidates, including for example the group of *FAAP10*, *FAAP16* and *FAAP24*. Novel candidate genes include *RAP80*, *Abraxas* and *hCLK2*. The human homologue of the C.elegans biological clock protein hCLK2 is a protein that interacts with the S-phase checkpoint components ATR, ATRIP, Claspin and CHK1 (Collis et al., 2007). hCLK2 prevents spontaneous DNA damage and is required for intra S phase arrest after DNA damage. hCLK2 promotes activation of the S phase checkpoint and downstream repair processes by preventing unscheduled Chk1 degradation by the proteasome. The connection to the FA/BRCA pathway is suggested by the observation that *hCLK2* deficient cells are defective in damage-induced monoubiquitination of FANCD2, and fail to recruit RAD51 and FANCD2 to sites of DNA damage. *hCLK2* depleted cells share many phenotypes abnormalities with cells derived from FA and Seckel syndrome patients. This raises the possibility that *hCLK2* may be inactivated in human disease (Collis et al., 2007). However, so far we have failed to detect any mutations in the *hCLK2* gene in our unassigned FA patients. Additional candidate genes that have recently emerged are *Abraxas* and *RAP80*. *Abraxas* codes for a protein that was described as interaction partner of BRCA1 (Wang et al., 2007). It binds BRCA1 to the exclusion of the FA protein BRIP1/FANCJ and another protein called CtIP. Abraxas also recruites the ubiquitin interacting motif (UIM)-containing protein RAP80 (receptor associated protein 80) to BRCA1. Both Abraxas and RAP80 are phosphorylation substrates of ATR or ATM. They are both required for DNA damage resistance, G2-M checkpoint control and DNA repair (Kim et al., 2007; Sobhian et al., 2007). These facts in combination with the ubiquitin interacting motif of RAP80 might imply that defects in one of these genes might result in FA. Therefore both RAP80 and Abraxas are currently tested for defects in unassigned FA patients, with special emphasis on RAP80 because this might be the long searched protein which interacts with the monoubiquitinated ID complex. In summary, it is quite clear that there must be at least two further FA genes, and *FAAP100* and *RAP80* seem to be the most promising candidates. Other candidates that one must keep in mind are *hCLK2*, *Abraxas* and the group of FAAPs, including *FAAP10*, *FAAP16* and *FAAP24*. In contrast, the genes *BRCA1* and *H2AX* are less likely to function as genuine FA genes.

The only curative therapy regarding bone marrow failure is hematopoetic stem cell transplantation (HSCT). Despite considerable progress in recent years, HSCT still is a high risk procedure if no matching sibling donors are available. Although gene therapy has not been successful in FA, knowledge about the affected genes and mutations will be required for further effort in this direction. As an alternative strategy, there are exciting experiments using an agent called PTC124, a chemical that induces ribosomal readthrough of premature but not of terminal stop codons (Welch et al., 2007). Stop codon read-through has been applied in mammalian cells and mouse models for Duchenne muscular dystrophy, and cystic fibrosis. Unlike aminoglycosides such as gentamicin, treatment with PTC124 has no severe side effects such as renal and otic toxicities and need for intravenous or intramuscular administration. The drug is orally bioavailable when prepared in aqueous suspension. However, PTC124 can only be used for patients with mutations resulting in premature truncating codons. This requires a priori knowledge about the underlying mutation. Other strategies for gene therapy involve the rescue of a dystrophic muscle by U7 snRNA-mediated exon skipping, the usage of single-stranded short fragment homologous replacement (ssSFHR) and oligonucleotid-directed mismatch repair, and adeno-associated virus-mediated gene transfer (Goyenvalle et al., 2004; Wells, 2006; Kügler et al., 2007).

Improved understanding of the FA/BRCA pathway will not only be helpful for patients and their relatives. The special function of the FA/BRCA pathway in the repair of ICLs by combining different kinds of DNA repair mechanisms is a model for basic research on DNA repair in mammalian cells. Understanding the FA/BRCA pathway and more detailed examination of the FA-cancer connection will improve our understanding of DNA repair and is likely to reveal basic principles of tumorigenesis.

4

Summary/Zusammenfassung

Summary

In the context of this thesis, I investigated the molecular causes and functional consequences of genetic instability using a human inherited disease, Fanconi anemia. This rare disorder uniquely exemplifies the close connection between genetic instability, organ malformation or malfunction, and neoplastic cell growth. FA patients display a highly variable clinical phenotype, including congenital abnormalities, progressive bone marrow failure and a high cancer risk. The FA cellular phenotype is characterized by spontaneous and inducible chromosomal instability, and a typical S/G2 phase arrest after exposure to DNA-damaging agents. FA is a heterogeneous disease. So far, 13 genes have been identified, whose biallelic (or, in the case of X-linked *FANCB*, hemizygous) mutations cause this multisystem disorder. The FA proteins interact in a multiprotein network, instrumental and essential in the cellular response to DNA damage. With each newly discovered gene, there is improved understanding of the many and intertwined functions of the FA/BRCA caretaker gene network. Since there are FA patients without any defects in any of the known FA genes, it is likely that not all FA genes have been identified to date. A more comprehensive summary of Fanconi anemia and its myriad clinical, cellular and molecular manifestations is provided in the **introduction** section of this thesis.

The results of my experimental work are presented as published papers and manuscripts ready to be submitted. In the **first publication**, I investigated the connection between FA genes and bladder tumors. Bladder cancers frequently show deletions of regions on chromosomes 9p and/or 9q, potentially including the FA genes *FANCC* and *FANCG*. Chemotherapy for bladder cancers includes the DNA-crosslinker cisplatinum, to which FA cells are highly sensitive. The question I tried to answer was whether a disruption of the FA/BRCA pathway may be a frequent and possibly causal event in bladder cancer, explaining the hypersensitivity of these cells to DNA-crosslinking agents. There were no defects in *FANCC* or *FANCG* in 23 bladder cancer cell lines and ten primary bladder cancers. However, a single bladder cancer cell line, BFTC909, displayed a disruption of the FA/BRCA pathway which was shown to be due to hypermethylation of the *FANCF* promotor region. The *FANCF* methylation status was investigated in additional 41 primary bladder cancers, but in these native materials there was no evidence for promoter hypermethylation as a frequent cause of *FANCF* silencing. Inactivation of *FANCF* via hypermethylation has been reported in a number of different tumors. Some authors postulate that disruption of the FA/BRCA pathway by *FANCF* hypermethylation might result in genomic instability and thereby promote tumor initiation and progression. However, the results of my own studies of bladder tumors suggest that silencing of *FANCF* via hypermethylation is a rather rare event in bladder cancers. If it occurs at all, it emerges rather late during tumor progression and might occur only by chance due to the location of *FANCF* near a known hot spot region for hypermethylation on human chromosome 11p15. On the basis of my experimental data and by reviewing the literature I arrived at the conclusion that disruption of the FA/BRCA pathway might be detrimental rather than advantageous for the majority tumor types by rendering them vulnerable towards DNA damaging agents and oxidative stress. In order to survive under unfavorable conditions, tumor cells may require functional FA genes just like normal cells.

The **second publication** deals with the gene coding for the core complex protein FANCE and tries to answer the question why *FANCE* is so rarely affected among FA-patients. Within the FA/BRCA network of proteins, FANCE has the important task to mediate the necessary physical proximity between the catalytic subunit FANCL of the E3 ubiquitin ligase and its substrate FANCD2. The paper represented in this thesis is the first one that investigates a relatively large cohort of these individually rare *FANCE* patients. In contrast to most other FA-genes, genetic alterations in *FANCE* result in most cases in premature protein truncation. A remarkable exception was a single missense mutation (c.1111 C>T) which was detected in more than 50% of the patients. Using complementation studies with retroviral vectors, I was able to confirm that this particular mutation severely affects FANCE function. A number of other types of missense mutations were tested in

a similar way. All were shown to restore MMC sensitivity in *FANCE*-defective cells, thus proving their innocuous nature. The conclusion from these studies is that like FANCF, FANCE functions as a probable adaptor protein with a high tolerance towards amino acid substitutions which would explain the relative rareness of FA-E patients.

In addition to the FA core complex members FANCF and FANCE, I have also investigated the *FANCL* gene whose product functions as the catalytic subunit of the E3 ligase. Since the only FA-L patient reported so far in the literature is deceased, there is virtually no information concerning the cellular and phenotypic consequences of biallelic loss of FANCL function. The **third publication** addresses this issue by providing the first comprehensive description of genetic alterations and phenotypic manifestations in a series of three FA-L patients. There were variable mutations, including a large deletion of more than 37.000 nucleotides at the 5' portion of the gene, one short in-frame deletion of three nucleotides, one small but out-of-frame deletion (five nucleotides), one splice mutation leading to exonisation of an intronic sequence, and one base substitution affecting splicing and resulting in skipping of two exons. Additionally, one of the patients developed a somatic reversion, adding *FANCL* to the list of genes in which natural gene therapy occurs. The clinical phenotypes of our FA-L patients were as variable as their pattern of genetic alterations, ranging from mildly to severely affected. Given the key role of FANCL in the activation of the ID protein complex, one might have expected lethality of biallelic *FANCL* null mutations. However, the results of my study show that (a) genetic alterations of *FANCL* are compatible with survival, (b) these alterations may include large deletions such as so far common only in the *FANCA* gene, (c) FA-L phenotypes can be mild to severe, and (d) *FANCL* belongs to the group of FA genes that may undergo somatic reversion.

The central protein of the FA/BRCA network, FANCD2, is the subject of the **fourth publication** presented in this thesis. Together with my colleague Reinhard Kalb, I assigned a cohort of 33 FA patients to complementation group FA-D2 by complementation assays and immunoblotting. We have shown that the majority of mutations detected in FA-D2 patients result in aberrant splicing. We uncovered a number of ethnic associations by investigating haplotype structures. Most importantly, we were able to show that there are no biallelic null mutations in *FANCD2*. Correspondingly, residual protein of both FANCD2-isotypes (FANCD2-S and FANCD2-L) was present in all available patient cell lines. This suggests that complete abrogation of the FANCD2 protein cannot be tolerated and causes early embryonic lethality. Compared to other FA subgroups (with the exception of FA-D1 and FA-N, see below), *FANCD2* patients are more severely affected. Malformations are frequent and hematological manifestations appear earlier and progress more rapidly when compared to other FA subgroups. We have further shown that *FANCD2* is flanked by two pseudogenes. *FANCD2*-P1 is spanning 16 kb and is located ~24 kb upstream of *FANCD2*, and *FANCD2*-P2 is spanning 31.9 kb and is located 1.76 Mb downstream of *FANCD2*. Both pseudogenes are in the same orientation as the functional gene. They display a high degree of sequence homology, and they reveal recognizable patterns of the conserved gene structure.

Defects in any of the FA core complex genes, including the above described genes *FANCF*, *FANCE* and *FANCL,* lead to defective FANCD2 monoubiquitination. However, there are at least three FA proteins that are not required for the posttranslational modification of FANCD2. One of these proteins is the 5'-3' helicase BRIP1 (BRCA1-interacting protein 1), a protein that interacts directly with the breast cancer susceptibility protein BRCA1. I participated in the identification of BRIP1 as the FA protein FANCJ. This discovery is described in the **fifth publication** of this thesis. *BRIP1* was identified as a FA-causing gene by homozygosity mapping of five individuals from four consanguineous families. The pathogenetic nature of genetic alterations of *FANCJ* was confirmed by mutation analysis and western blot data in these and 7 additional patients. Since all these patients had biallelic truncation mutations in the *BRIP1* gene, and one of them had been assigned previously to complementation group FA-J by somatic cell fusion, *BRIP1* was designated *FANCJ*. Monoubiquitinated FANCD2 and BRCA1 interact at presumptive sites of DNA

damage, and the newly discovered protein BRIP1/FANCJ seems to act as one of the mediators of genomic maintenance downstream of FANCD2.

Another protein identified downstream of FANCD2 is PALB2. PALB2 was originally discovered as “partner and localizer of BRCA2”. FA patients with biallelic mutations in the breast cancer susceptibility gene *BRCA2* (also known as *FANCD1*) represent the rare FA subgroup FA-D1. FA-D1 patients are much more severely affected than patients from other FA subgroups. These very young patients suffer from early childhood cancers like Wilms tumor and medulloblastoma. PALB2 was described as a nuclear binding partner of BRCA2, to promote its stabilization, its localisation and its function in DNA repair via homologous recombination. Furthermore, knockdown of PALB2 by siRNA resulted in MMC sensitivity, leading to DNA-interstrand crosslinks and DNA double strand breaks. In a candidate gene approach we tested patients with early childhood cancers but without mutations in *BRCA2* for mutations in *PALB2* (**publication 6**). We discovered biallelic mutations in seven patients, all resulting in premature protein truncation. The *PALB2*-deficient cells had no detectable PALB2 protein, very high chromosomal breakage rates, and lack of MMC-inducible RAD51 foci formation. Using retroviral transduction of *PALB2*-deficient cells with wildtype *PALB2* cDNA we were able to complement the defective cellular phenotype. Therefore, *PALB2* was identified as a novel FA gene and designated *FANCN*. Like FA-D1 patients, FA-N patients are very severely affected: in the seven patients, we observed five medulloblastomas, three Wilms tumors, one neuroblastoma, and two cases of AML. Only one of the patients was still alive at the time of this gene discovery. All others had succumbed to their disease prior to four years of age.

The last publication included in my thesis describes the identification of the FA gene *FANCI* as the second monoubiquitinated member of the FA/BRCA pathway (**publication 7**). In order to test whether proteins other than FANCD2 might also be substrates of the FA core complex, a BLAST search was performed to find gene products with sequence homology to the highly conserved human FANCD2 monoubiquitination site, LVIRK. The large number of putative targets was reduced on the basis of protein domain architecture and putative function. A back search for homology to FANCD2 extending beyond the LVIRK motif finally resulted in the identification of only a single protein, KIAA1794. KIAA1794 was a previously uncharacterized protein with 1328 amino acids and a molecular weight of 140 kDa. Monoubiquitination was shown to occur at Lys523, and required function of the FA core complex and simultaneous modification of FANCD2. KIAA1794 also forms nuclear foci in response to DNA damage, together with FANCD2 and phosphorylated H2AX. Like FANCD2, deubiquitination of KIAA1794 is regulated by USP1. KIAA1794 and FANCD2 show a weak but specific interaction. It thus became clear that KIAA1794 behaves as a functional homolog of FANCD2 and is likely to function in the coordination and repair of DNA-interstrand crosslinks. We identified biallelic mutations in *KIAA1794* in four FA patients, thus proving the genuine FA-nature of this candidate sequence. One of the tested patients belonged to the previously uncharacterized FA-I complementation group, the only FA subgroup that had not been assigned to a specific gene. In analogy to what we observed with genetic alterations in the *FANCD2* gene, all FA-I patients could be shown to express residual FANCI protein. This suggests that null mutations in *FANCI* might be likewise lethal in our species.

The **general discussion** provides a synopsis of the results and conclusions of my work with the state of art of FA research. Point by point, important issues in the area of FA (the FA/BRCA pathway and DNA repair, categories of FA genes, FA and cancer, identification of new FA genes) are discussed.

Zusammenfassung

Im Rahmen der vorliegenden Dissertation wurden molekulare Ursachen und funktionale Konsequenzen genetischer Instabilität am Beispiel der menschlichen Erbkrankheit Fanconi Anämie (FA) untersucht. Diese seltene Krankheit zeigt auf eine einzigartige Weise den engen Zusammenhang zwischen genetischer Instabilität, organischen Fehlbildungen oder Fehlfunktionen und entartetem Zellwachstum. FA Patienten zeigen einen sehr variablen klinischen Phänotyp, der in der Regel angeborene Fehlbildungen, progressives Knochenmarkversagen und ein hohes Risiko für Tumorerkrankungen beinhaltet. Der zelluläre Phänotyp der FA ist durch eine spontane und induzierbare chromosomale Instabilität und einen typischen S/G2-Phasen-Arrest nach Exposition mit DNA-schädigenden Agentien charakterisiert. FA ist eine heterogene Erkrankung. Biallelische oder -im Fall des X-chromosomalen *FANCB*- hemizygote Mutationen, die zu dieser Erkrankung führen, wurden in bislang 13 Genen identifiziert. Die FA Proteine arbeiten in einem gemeinsamen Netzwerk und sind essentiell beteiligt an der zellulären Antwort auf DNA Schädigung. Mit jedem neu entdeckten Gen verbessert sich das Verständnis für die vielen und vernetzten Funktionen des FA/BRCA „caretaker“ Netzwerks. Da es immer noch Patienten ohne nachgewiesene Defekte in einem der bekannten FA Gene gibt, ist es wahrscheinlich, dass noch nicht alle FA Gene identifiziert wurden. Eine umfassendere Übersicht über Fanconi Anämie und ihre vielfältigen klinischen, zellulären und molekularen Erscheinungsformen ist in der **Einleitung** dieser Dissertation gegeben.

Die Ergebnisse meiner experimentellen Arbeiten sind in Form von publizierten Fachartikeln und fertigen Manuskripten dargestellt. In der **ersten Publikation** habe ich den Zusammenhang von FA Genen und Harnblasentumoren untersucht. Harnblasentumore zeigen oft Deletionen in Regionen des Chromosoms 9p und/oder 9q, in denen sich die FA Gene *FANCC* und *FANCG* befinden. Die Chemotherapie für die Behandlung von Harnblasentumoren beinhaltet meist das DNA-quervernetzende Agens Cisplatin, auf das FA Zellen hoch-sensitive reagieren. Die Frage, die ich zu beantworten versucht habe, war, ob ein Defekt im FA/BRCA Weg eine mögliche Ursache für die Entstehung von Blasentumoren sein könnte, was die Hypersensitivität dieser Zellen gegenüber Cisplatin erklären würde. Ich habe in Zelllinien von 23 Harnblasentumoren und in DNA aus 10 primären Tumoren keine Defekte in *FANCC* oder *FANCG* finden können. Allerdings zeigte eine Zelllinie, BFTC909, einen Defekt im FA/BRCA Weg, der auf einer Hypermethylierung der Promotor-Region von *FANCF* beruhte. Eine mögliche *FANCF*-Methylierung wurde daraufhin noch in Material aus 41 weiteren primären Harnblasentumoren untersucht, aber auch in diesem Material gab es keinen Hinweis darauf, dass eine Promotor-Methylierung eine häufige Ursache für die Inaktivierung von *FANCF* könnte. Die Inaktivierung von *FANCF* durch Methylierung des Promotors wurde schon für eine Reihe von verschiedenen Tumoren gezeigt. Einige Autoren haben postuliert, dass eine *FANCF* Hypermethylierung in genomischer Instabilität resultieren und somit Tumorentstehung und –progression begünstigen könnte. Allerdings haben die Ergebnisse meiner Arbeit gezeigt, dass die Hypermethylierung von *FANCF* in Harnblasentumoren ein eher seltenes Ereignis ist, das –wenn es überhaupt vorkommt- eher spät in der Tumorprogression auftritt und vermutlich durch die Nähe zu einer Region auf Chromosom 11p15 begünstigt wird, die als „Hot-Spot“ für Hypermethylierung bekannt ist. Aufgrund meiner experimentellen Daten und ausgiebigen Literaturrecherchen bin ich zu dem Schluss gekommen, dass ein Defekt im FA/BRCA Weg für einen Tumor vermutlich eher schädlich als vorteilhaft ist, da so ein Defekt den Tumor gegenüber DNA-schädigenden Agentien und oxidativem Stress anfällig machen würde. Um unter ungünstigen Bedingungen überleben zu können, scheinen Tumorzellen den FA/BRCA Weg genauso wie andere Zellen zu benötigen.

Meine **zweite Publikation** befasst sich mit dem Kern-Komplex Protein FANCE und versucht die Frage zu beantworten, warum das *FANCE* Gen in so wenigen FA Patienten betroffen ist. Innerhalb des FA/BRCA Netzwerks hat FANCE die wichtige Funktion, die notwendige räumliche Nähe zwischen der katalytischen Untereinheit der E3-Ligase, FANCL, und dessen Substrat, FANCD2,

herzustellen. Mein Artikel über *FANCE* beschreibt die Untersuchung einer relativ großen Gruppe dieser vergleichsweise seltenen FA-E Patienten und zeigt, dass -im Gegensatz zu anderen FA Genen- Mutationen in *FANCE* fast immer zu vorzeitigen Stop-Codons führen. Eine Ausnahme bildet die Missense-Mutation c.1111C>T, die in mehr als 50% der Patienten detektiert wurde. Mit Hilfe von retroviralen Komplementationsstudien war ich in der Lage zu zeigen, dass diese Mutation die Funktion von FANCE erheblich beeinflusst. Eine Vielzahl von *FANCE*-Vektoren mit anderen putativen Missense-Mutationen wurde auf die gleiche Weise untersucht und alle waren in der Lage, die MMC-Sensitivität einer *FANCE*-defizienten Zelllinie zu korrigieren, was auf einen harmlosen Charakter dieser Basensubstitutionen schließen ließ. Die Schlussfolgerung dieser Arbeit war, dass FANCE vermutlich genauso wie FANCF im Kern-Komplex die Rolle eines Adaptor-Proteins mit einer hohen Toleranz gegenüber Aminosäure-Austauschen innehat, was die relative Seltenheit von Patienten dieser Untergruppe erklären könnte.

Zusätzlich zu den FA Kern-Komplex Mitgliedern FANCF und FANCE habe ich auch das *FANCL* Gen untersucht, dessen Produkt als katalytische Untereinheit der E3-Ligase fungiert. Da der bisher einzige publizierte FA-L Patient früh verstorben ist, gab es nahezu keine Informationen bezüglich zellulären und klinischen Konsequenzen von biallelischen *FANCL* Mutationen. Die **dritte Publikation** in dieser Dissertation befasst sich mit diesem Thema und enthält eine umfassende Beschreibung von genetischen Veränderungen und phänotypischen Auswirkungen in einer Gruppe von 3 FA-L Patienten. Die detektierten Mutationen waren sehr variabel und beinhalteten eine große Deletion von mehr als 37.000 Nukleotiden am 5' Ende des Gens, eine kurze Leseraster-haltende Deletion von drei Nukleotiden, eine kleine Leseraster-verschiebende Deletion (5 Nukleotide), eine Spleiß-Mutation, die zur Exonisierung einer intronischen Sequenz führt und einer Basen-Substitution, die im Skipping von 2 Exons resultiert. Zusätzlich hat einer der Patienten eine somatische Reversion entwickelt, wodurch *FANCL* nun zu den Genen gehört, in denen eine „natürliche Gentherapie" detektiert wurde. Die klinischen Phänotypen der FA-L Patienten waren genauso variable wie ihre Mutationen und reichte von mild betroffen bis schwer betroffen. Wenn man die Schlüsselrolle von FANCL in der Aktivierung des ID (FANCD2/FANCI) Komplexes bedenkt, könnte man glauben, dass biallelische Nullmutationen in *FANCL* lethal sein müssten. Die Ergebnisse meiner Arbeit haben allerdings gezeigt, dass (a) genetische Veränderungen in *FANCL* mit dem Leben vereinbar sind, (b) dass diese Veränderungen sehr große Deletionen beinhalten können, was bisher nur für *FANCA* gezeigt werden konnte, (c) dass FA-L Phänotypen von mild bis schwer betroffen reichen können und d) dass *FANCL* zu den Genen gehört, in denen somatische Reversionen stattfinden.

Das Schlüsselprotein des FA/BRCA Netzwerks, FANCD2, ist das Thema der **vierten Publikation** in dieser Dissertation. Gemeinsam mit meinem Kollegen Reinhard Kalb habe ich 33 FA Patienten mittels Komplementationsstudien und Immunoblotting zur Untergruppe FA-D2 zugeordnet. Wir haben gezeigt, dass eine Vielzahl der detektierten Mutationen in aberrantem Spleißen resultiert und haben durch Haplotyp-Untersuchungen ethnische Assoziationen erkannt. Insbesondere konnten wir zeigen, dass es keine biallelischen Nullmutationen in *FANCD2* zu geben scheint. Dementsprechend war Restprotein von beiden FANCD2-Isoformen, FANCD2-L und FANCD2-S, in allen verfügbaren Patienten-Zelllinien nachweisbar. Dies ließ vermuten, dass ein komplettes Fehlen des FANCD2 Proteins nicht tolerierbar ist und frühe embryonale Letalität verursacht. Verglichen mit anderen FA Untergruppen (mit der Ausnahme von FA-D1 und FA-N, siehe unten) scheinen FA-D2 Patienten schwerer betroffen zu sein. Fehlbildungen kommen häufig vor und hämatologische Probleme treten früh auf und verlaufen schwerer als in anderen FA Untergruppen. Wir haben außerdem gezeigt, dass *FANCD2* von zwei Pseudogenen flankiert wird. *FANCD2*-P1 umfasst 16 kb und ist ~24 kb 5' von *FANCD2* lokalisiert, während *FANCD2*-P2 31.9 kb groß ist und sich 1.76 Mb 3' von *FANCD2* befindet. Beide Pseudogene haben die gleiche Orientierung wie *FANCD2* und zeigen ein hohes Maß an Sequenzhomologie und erkennbare Muster der konservierten Genstruktur.

Defekte in jedem der FA Kern-Komplex Gene, einschließlich der oben beschriebenen Gene *FANCF, FANCE* und *FANCL*, führen zu einem Defekt in der FANCD2-Monoubiquitinierung. Allerdings gibt es mindestens drei Proteine, die nicht für diese posttranslationale Modifikation benötigt werden. Eines dieser Proteine ist die 5'-3' Helikase BRIP1 (BRCA1-interagierendes Protein 1), ein Protein, das direkt mit dem Brustkrebs-assoziierten Protein BRCA1 interagiert. Ich war an der Identifizierung von BRIP1 als FA Protein (FANCJ) beteiligt. Diese Entdeckung ist in der **fünften Publikation** meiner Dissertation beschrieben. BRIP1 wurde durch Homozygotie-Studien an fünf Personen aus blutsverwandten Familien identifiziert. Der pathogene Charakter genetischer Veränderungen in *FANCJ* wurde durch Mutationsanalysen und Immunoblot-Daten in diesen und sieben weiteren Patienten bestätigt. Da in allen Patienten biallelische Mutationen in *FANCJ* gefunden wurden und einer dieser Patienten zuvor mittels somatischer Zellfusion der Komplementationsgruppe FA-J zugeordnet worden war, wurde *BRIP1 „FANCJ*" genannt. Monoubiquitiniertes FANCD2 und BRCA1 interagieren an mutmaßlichen Orten von DNA Schäden, und das neu entdeckte Protein BRIP1/FANCJ, das direkt mit BRCA1 interagiert, scheint als einer der Mediatoren zur Aufrechterhaltung genomischer Stabilität downstream von FANCD2 zu wirken.

Ein weiteres Protein downstream von FANCD2 ist PALB2. PALB2 wurde ursprünglich als „Partner und Lokalisierer von BRCA2" entdeckt. FA Patienten mit biallelischen Mutationen im Brustkrebs-Gen *BRCA2* (auch als *FANCD1* bekannt) bilden die Untergruppe FA-D1. FA-D1 Patienten sind viel schwerer betroffen als Patienten aus anderen FA Untergruppen. Diese in der Regel sehr jungen Patienten leiden an frühkindlichen Tumoren wie Wilms Tumor und Medulloblastom. PALB2 wurde beschrieben als ein nukleärer Bindungspartner von BRCA2, der die Stabilisation, die Lokalisation und die Funktion von BRCA2 in der Homologen Rekombination fördert. Außerdem wurde gezeigt, dass eine Inaktivierung von PALB2 durch siRNA in MMC Sensitivität resultiert, was sich in DNA-Quervernetzungen und DNA Doppelstrangbrüchen äußerte. In einer Kandidatengen-Studie haben wir Patienten mit frühkindlichen Tumoren, aber ohne Mutationen in *BRCA2*, auf Mutationen in *PALB2* untersucht (**Publikation 6**). Wir haben in sieben Patienten biallelische Mutationen gefunden, die alle in einem vorzeitigem Abbruch des Transkriptes resultieren. *PALB2*-defiziente Zellen besaßen kein nachweisbares PALB2 Protein, wiesen sehr hohe chromosomale Bruchraten auf und zeigten keine MMC-induzierbaren RAD51 Foci. Mittels retroviraler Komplementation *PALB2*-defizienter Zellen mit wildtypischer *PALB2* cDNA konnte der zelluläre Phänotyp komplementiert werden. Aufgrund dieser Ergebnisse haben wir *PALB2* als ein neues FA Gen identifiziert und haben es *FANCN* genannt. Genauso wie FA-D1 Patienten sind FA-N Patienten sehr schwer betroffen: In den sieben Patienten gab es fünf Medulloblastome, drei Wilms Tumore, ein Neuroblastom und zwei Fälle von AML. Zur Zeit der Gen-Entdeckung war nur noch einer der sieben Patienten am Leben. Alle anderen waren vor dem vierten Lebensjahr an ihrer Erkrankung verstorben.

Die letzte Publikation meiner Dissertation beschreibt die Identifikation des FA Genes *FANCI,* dessen Produkt das zweite monoubiquitinierte Mitglied des FA/BRCA Weges darstellt (**Publikation 7**). Um zu testen, ob es zusätzlich zu FANCD2 noch andere Substrate des FA Kern-Komplexes gibt, wurde eine BLAST Suche durchgeführt, die Proteine mit Sequenz-Homologie zu der hoch konservierten FANCD2-Monoubiquitinierungsstelle, LVIRK, identifizieren sollte. Die große Anzahl möglicher Produkte wurde aufgrund von Protein-Domänen-Architektur und möglicher Funktion eingeschränkt. Eine Rückwärtssuche nach Homologie zu FANCD2, die über LVIRK hinausgeht, resultierte schließlich in der Identifikation eines einzelnen Proteins, KIAA1794. KIAA1794 war ein zuvor nicht charakterisiertes Protein mit 1328 Aminosäuren und einem Molekulargewicht von 140 kDa. Es konnte gezeigt werden, dass die Monoubiquitinierung am Lys523 stattfindet und vom FA Kern-Komplex und einer gleichzeitigen Ubiquitinierung von FANCD2 abhängt. KIAA1794 bildet, gemeinsam mit FANCD2 und phosphoryliertem H2AX, nukleäre Foci in Abhängigkeit von DNA Schäden. Genauso wie bei FANCD2 wird die Deubiquitinierung durch USP1 reguliert. Außerdem zeigen KIAA1794 und FANCD2 eine schwache, aber spezifische Interaktion. Daraus resultierte die Schlussfolgerung, dass KIAA1794 ein funktionales Homolog zu FANCD2 darstellt und

vermutlich genauso in der Koordination und Reparatur von DNA Doppelstrangbrüchen beteiligt ist. Wir haben in vier Patienten biallelische Mutationen in *KIAA1794* gefunden, und so zeigen können, dass *KIAA1794* wirklich ein FA Gen ist. Einer der untersuchten Patienten war zuvor zu der uncharakterisierten FA Untergruppe FA-I zugeordnet worden, der einzigen Untergruppe, in der das zugrunde liegende Gen nicht bekannt war. Daher ist *KIAA1794* gleich „*FANCI*“. In Analogie zu Beobachtungen bei *FANCD2* zeigen alle FA-I Patienten Restprotein, was vermuten lässt, dass Nullmutationen in *FANCI* ebenfalls letal sind.

Die **generelle Diskussion** birgt eine Synopsis der Ergebnisse und Schlussfolgerungen meiner Forschung mit dem aktuellen Wissensstand über FA. Punktweise werden wichtige Themen aus dem Gebiet der FA (der FA/BRCA Weg und DNA Reparatur, Kategorien von FA Genen, FA und Tumore, Identifikation neuer FA Gene) diskuttiert.

5

References

Akkari, Y.M., Bateman, R.L., Reifsteck, C.A., D'Andrea, A.D., Olson, S.B. and Grompe, M. (2001) The 4N cell cycle delay in Fanconi anemia reflects growth arrest in late S phase. Mol Genet Metab, 74, 403-412.

Alter, B.P. (2006) The association between FANCD1/BRCA2 mutations and leukaemia. Br J Haematol, 133, 446-448; author reply 448.

Alter, B.P., Rosenberg, P.S. and Brody, L.C. (2007) Clinical and molecular features associated with biallelic mutations in FANCD1/BRCA2. J Med Genet, 44, 1-9.

Ameziane, N., Errami, A., Leveille, F., Fontaine, C., de Vries, Y., van Spaendonk, R.M., de Winter, J.P., Pals, G. and Joenje, H. (2007) Genetic subtyping of Fanconi anemia by comprehensive mutation screening. Hum Mutat.

Auerbach, A.D. and Wolman, S.R. (1978) Carcinogen-induced chromosome breakage in Fanconi's anaemia heterozygous cells. Nature, 271, 69-71.

Berwick, M., Satagopan, J.M., Ben-Porat, L., Carlson, A., Mah, K., Henry, R., Diotti, R., Milton, K., Pujara, K., Landers, T., Dev Batish, S., Morales, J., Schindler, D., Hanenberg, H., Hromas, R., Levran, O. and Auerbach, A.D. (2007) Genetic heterogeneity among Fanconi anemia heterozygotes and risk of cancer. Cancer Res, 67, 9591-9596.

Blom, E., van de Vrugt, H.J., de Vries, Y., de Winter, J.P., Arwert, F. and Joenje, H. (2004) Multiple TPR motifs characterize the Fanconi anemia FANCG protein. DNA Repair (Amst), 3, 77-84.

Bogliolo, M., Lyakhovich, A., Callen, E., Castella, M., Cappelli, E., Ramirez, M.J., Creus, A., Marcos, R., Kalb, R., Neveling, K., Schindler, D. and Surralles, J. (2007) Histone H2AX and Fanconi anemia FANCD2 function in the same pathway to maintain chromosome stability. Embo J, 26, 1340-1351.

Centra, M., Memeo, E., d'Apolito, M., Savino, M., Ianzano, L., Notarangelo, A., Liu, J., Doggett, N.A., Zelante, L. and Savoia, A. (1998) Fine exon-intron structure of the Fanconi anemia group A (FAA) gene and characterization of two genomic deletions. Genomics, 51, 463-467.

Ciccia, A., Ling, C., Coulthard, R., Yan, Z., Xue, Y., Meetei, A.R., Laghmani el, H., Joenje, H., McDonald, N., de Winter, J.P., Wang, W. and West, S.C. (2007) Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. Mol Cell, 25, 331-343.

Collis, S.J., Barber, L.J., Clark, A.J., Martin, J.S., Ward, J.D. and Boulton, S.J. (2007) HCLK2 is essential for the mammalian S-phase checkpoint and impacts on Chk1 stability. Nat Cell Biol, 9, 391-401.

Daniels, M.J., Wang, Y., Lee, M. and Venkitaraman, A.R. (2004) Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2. Science, 306, 876-879.

de Winter, J.P., Leveille, F., van Berkel, C.G., Rooimans, M.A., van Der Weel, L., Steltenpool, J., Demuth, I., Morgan, N.V., Alon, N., Bosnoyan-Collins, L., Lightfoot, J., Leegwater, P.A., Waisfisz, Q., Komatsu, K., Arwert, F., Pronk, J.C., Mathew, C.G., Digweed, M., Buchwald, M. and Joenje, H. (2000a) Isolation of a cDNA representing the Fanconi anemia complementation group E gene. Am J Hum Genet, 67, 1306-1308.

de Winter, J.P., Rooimans, M.A., van Der Weel, L., van Berkel, C.G., Alon, N., Bosnoyan-Collins, L., de Groot, J., Zhi, Y., Waisfisz, Q., Pronk, J.C., Arwert, F., Mathew, C.G., Scheper, R.J., Hoatlin, M.E., Buchwald, M. and Joenje, H. (2000b) The Fanconi anaemia gene FANCF encodes a novel protein with homology to ROM. Nat Genet, 24, 15-16.

de Winter, J.P., Waisfisz, Q., Rooimans, M.A., van Berkel, C.G., Bosnoyan-Collins, L., Alon, N., Carreau, M., Bender, O., Demuth, I., Schindler, D., Pronk, J.C., Arwert, F., Hoehn, H., Digweed, M., Buchwald, M. and Joenje, H. (1998) The Fanconi anaemia group G gene FANCG is identical with XRCC9. Nat Genet, 20, 281-283.

Dronkert, M.L. and Kanaar, R. (2001) Repair of DNA interstrand cross-links. Mutat Res, 486, 217-247.

Dutrillaux, B., Aurias, A., Dutrillaux, A.M., Buriot, D. and Prieur, M. (1982) The cell cycle of lymphocytes in Fanconi anemia. Hum Genet, 62, 327-332.

Ferrer, M., Rodriguez, J.A., Spierings, E.A., de Winter, J.P., Giaccone, G. and Kruyt, F.A. (2005)

Identification of multiple nuclear export sequences in Fanconi anemia group A protein that contribute to CRM1-dependent nuclear export. Hum Mol Genet, 14, 1271-1281.

Freie, B.W., Ciccone, S.L., Li, X., Plett, P.A., Orschell, C.M., Srour, E.F., Hanenberg, H., Schindler, D., Lee, S.H. and Clapp, D.W. (2004) A role for the Fanconi anemia C protein in maintaining the DNA damage-induced G2 checkpoint. J Biol Chem, 279, 50986-50993.

Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M. and D'Andrea, A.D. (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell, 7, 249-262.

Gordon, S.M., Alon, N. and Buchwald, M. (2005) FANCC, FANCE, and FANCD2 form a ternary complex essential to the integrity of the Fanconi anemia DNA damage response pathway. J Biol Chem, 280, 36118-36125.

Goyenvalle, A., Vulin, A., Fougerousse, F., Leturcq, F., Kaplan, J.C., Garcia, L. and Danos, O. (2004) Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. Science, 306, 1796-1799.

Gregory, J.J., Jr., Wagner, J.E., Verlander, P.C., Levran, O., Batish, S.D., Eide, C.R., Steffenhagen, A., Hirsch, B. and Auerbach, A.D. (2001) Somatic mosaicism in Fanconi anemia: evidence of genotypic reversion in lymphohematopoietic stem cells. Proc Natl Acad Sci U S A, 98, 2532-2537.

Grompe, M. and van de Vrugt, H. (2007) The Fanconi family adds a fraternal twin. Dev Cell, 12, 661-662.

Gross, M., Hanenberg, H., Lobitz, S., Friedl, R., Herterich, S., Dietrich, R., Gruhn, B., Schindler, D. and Hoehn, H. (2002) Reverse mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction. Cytogenet Genome Res, 98, 126-135.

Heinrich, M.C., Hoatlin, M.E., Zigler, A.J., Silvey, K.V., Bakke, A.C., Keeble, W.W., Zhi, Y., Reifsteck, C.A., Grompe, M., Brown, M.G., Magenis, R.E., Olson, S.B. and Bagby, G.C. (1998) DNA cross-linker-induced G2/M arrest in group C Fanconi anemia lymphoblasts reflects normal checkpoint function. Blood, 91, 275-287.

Hirschhorn, R. (2003) In vivo reversion to normal of inherited mutations in humans. J Med Genet, 40, 721-728.

Hoehn, H., Kalb, R., Neveling, K., Friedl, R., Bechtold, A., Herterich, S., Sun, Y., Gruhn, B., Hanenberg, H., Schindler, D. (2007) Revertant Mosaicism in Fanconi Anemia: Natural Gene Therapy at Work. Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet, Basel, Karger, vol 15, pp 149-172.

Holden, S.T., Cox, J.J., Kesterton, I., Thomas, N.S., Carr, C. and Woods, C.G. (2006) Fanconi anaemia complementation group B presenting as X linked VACTERL with hydrocephalus syndrome. J Med Genet, 43, 750-754.

Howlett, N.G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., De Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G., Ikeda, H., Fox, E.A. and D'Andrea, A.D. (2002) Biallelic inactivation of BRCA2 in Fanconi anemia. Science, 297, 606-609.

Hussain, S., Wilson, J.B., Blom, E., Thompson, L.H., Sung, P., Gordon, S.M., Kupfer, G.M., Joenje, H., Mathew, C.G. and Jones, N.J. (2006) Tetratricopeptide-motif-mediated interaction of FANCG with recombination proteins XRCC3 and BRCA2. DNA Repair (Amst), 5, 629-640.

Hussain, S., Wilson, J.B., Medhurst, A.L., Hejna, J., Witt, E., Ananth, S., Davies, A., Masson, J.Y., Moses, R., West, S.C., de Winter, J.P., Ashworth, A., Jones, N.J. and Mathew, C.G. (2004) Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways. Hum Mol Genet, 13, 1241-1248.

Hussain, S., Witt, E., Huber, P.A., Medhurst, A.L., Ashworth, A. and Mathew, C.G. (2003) Direct interaction of the Fanconi anaemia protein FANCG with BRCA2/FANCD1. Hum Mol Genet, 12, 2503-2510.

Joenje, H., Arwert, F., Eriksson, A.W., de Koning, H. and Oostra, A.B. (1981) Oxygen-dependence of chromosomal aberrations in Fanconi's anaemia. Nature, 290, 142-143.

Joenje, H., Lo ten Foe, J.R., Oostra, A.B., van Berkel, C.G., Rooimans, M.A., Schroeder-Kurth,

T., Wegner, R.D., Gille, J.J., Buchwald, M. and Arwert, F. (1995) Classification of Fanconi anemia patients by complementation analysis: evidence for a fifth genetic subtype. Blood, 86, 2156-2160.

Kalb, R., Duerr, M., Wagner, M., Herterich, S., Gross, M., Digweed, M., Joenje, H., Hoehn, H. and Schindler, D. (2004) Lack of sensitivity of primary Fanconi's anemia fibroblasts to UV and ionizing radiation. Radiat Res, 161, 318-325.

Kalb, R., Neveling, K., Herterich, S., Schindler, D. (2007a) Fanconi Anemia Genes: Structure, Mutations, and Genotype-Phenotype Correlations. Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet, Basel, Karger, vol 15, pp 39-58.

Kalb, R., Neveling, K., Hoehn, H., Schneider, H., Linka, Y., Batish, S.D., Hunt, C., Berwick, M., Callen, E., Surralles, J., Casado, J.A., Bueren, J., Dasi, A., Soulier, J., Gluckman, E., Zwaan, C.M., van Spaendonk, R., Pals, G., de Winter, J.P., Joenje, H., Grompe, M., Auerbach, A.D., Hanenberg, H. and Schindler, D. (2007b) Hypomorphic mutations in the gene encoding a key Fanconi anemia protein, FANCD2, sustain a significant group of FA-D2 patients with severe phenotype. Am J Hum Genet, 80, 895-910.

Kalb, R., Neveling, K., Nanda, I., Schindler, D., Hoehn, H. (2006) Fanconi Anemia: Causes and Consequences of Genetic Instability. Volff JN (ed): Genome and Disease. Genome Dynamics, Basel, Karger, vol 1, pp 218-42.

Kim, H., Chen, J. and Yu, X. (2007) Ubiquitin-binding protein RAP80 mediates BRCA1-dependent DNA damage response. Science, 316, 1202-1205.

Kowal, P., Gurtan, A.M., Stuckert, P., D'Andrea, A.D. and Ellenberger, T. (2007) Structural determinants of human FANCF protein that function in the assembly of a DNA damage signaling complex. J Biol Chem, 282, 2047-2055.

Kubbies, M., Schindler, D., Hoehn, H., Schinzel, A. and Rabinovitch, P.S. (1985) Endogenous blockage and delay of the chromosome cycle despite normal recruitment and growth phase explain poor proliferation and frequent edomitosis in Fanconi anemia cells. Am J Hum Genet, 37, 1022-1030.

Kugler, S., Hahnewald, R., Garrido, M. and Reiss, J. (2007) Long-term rescue of a lethal inherited disease by adeno-associated virus-mediated gene transfer in a mouse model of molybdenum-cofactor deficiency. Am J Hum Genet, 80, 291-297.

Kumaresan, K.R., Sridharan, D.M., McMahon, L.W. and Lambert, M.W. (2007) Deficiency in Incisions Produced by XPF at the Site of a DNA Interstrand Cross-Link in Fanconi Anemia Cells. Biochemistry.

Kutler, D.I., Singh, B., Satagopan, J., Batish, S.D., Berwick, M., Giampietro, P.F., Hanenberg, H. and Auerbach, A.D. (2003) A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood, 101, 1249-1256.

Kwee, M.L., van der Kleij, J.M., van Essen, A.J., Begeer, J.H., Joenje, H., Arwert, F. and ten Kate, L.P. (1997) An atypical case of Fanconi anemia in elderly sibs. Am J Med Genet, 68, 362-366.

Leveille, F., Blom, E., Medhurst, A.L., Bier, P., Laghmani el, H., Johnson, M., Rooimans, M.A., Sobeck, A., Waisfisz, Q., Arwert, F., Patel, K.J., Hoatlin, M.E., Joenje, H. and de Winter, J.P. (2004) The Fanconi anemia gene product FANCF is a flexible adaptor protein. J Biol Chem, 279, 39421-39430.

Leveille, F., Ferrer, M., Medhurst, A.L., Laghmani el, H., Rooimans, M.A., Bier, P., Steltenpool, J., Titus, T.A., Postlethwait, J.H., Hoatlin, M.E., Joenje, H. and de Winter, J.P. (2006) The nuclear accumulation of the Fanconi anemia protein FANCE depends on FANCC. DNA Repair (Amst), 5, 556-565.

Levitt, N.C. and Hickson, I.D. (2002) Caretaker tumour suppressor genes that defend genome integrity. Trends Mol Med, 8, 179-186.

Levitus, M., Waisfisz, Q., Godthelp, B.C., de Vries, Y., Hussain, S., Wiegant, W.W., Elghalbzouri-Maghrani, E., Steltenpool, J., Rooimans, M.A., Pals, G., Arwert, F., Mathew, C.G., Zdzienicka, M.Z., Hiom, K., De Winter, J.P. and Joenje, H. (2005) The DNA helicase BRIP1 is defective

in Fanconi anemia complementation group J. Nat Genet, 37, 934-935.
Levran, O., Attwooll, C., Henry, R.T., Milton, K.L., Neveling, K., Rio, P., Batish, S.D., Kalb, R., Velleuer, E., Barral, S., Ott, J., Petrini, J., Schindler, D., Hanenberg, H. and Auerbach, A.D. (2005a) The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet, 37, 931-933.
Levran, O., Diotti, R., Pujara, K., Batish, S.D., Hanenberg, H. and Auerbach, A.D. (2005b) Spectrum of sequence variations in the FANCA gene: an International Fanconi Anemia Registry (IFAR) study. Hum Mutat, 25, 142-149.
Levran, O., Doggett, N.A. and Auerbach, A.D. (1998) Identification of Alu-mediated deletions in the Fanconi anemia gene FAA. Hum Mutat, 12, 145-152.
Levran, O., Erlich, T., Magdalena, N., Gregory, J.J., Batish, S.D., Verlander, P.C. and Auerbach, A.D. (1997) Sequence variation in the Fanconi anemia gene FAA. Proc Natl Acad Sci U S A, 94, 13051-13056.
Liebetrau, W., Buhner, M. and Hoehn, H. (1995) Prototype sequence clues within the Fanconi anaemia group C gene. J Med Genet, 32, 669-670.
Lightfoot, J., Alon, N., Bosnoyan-Collins, L. and Buchwald, M. (1999) Characterization of regions functional in the nuclear localization of the Fanconi anemia group A protein. Hum Mol Genet, 8, 1007-1015.
Ling, C., Ishiai, M., Ali, A.M., Medhurst, A.L., Neveling, K., Kalb, R., Yan, Z., Xue, Y., Oostra, A.B., Auerbach, A.D., Hoatlin, M.E., Schindler, D., Joenje, H., de Winter, J.P., Takata, M., Meetei, A.R. and Wang, W. (2007) FAAP100 is essential for activation of the Fanconi anemia-associated DNA damage response pathway. Embo J, 26, 2104-2114.
Liu, N., Lamerdin, J.E., Tucker, J.D., Zhou, Z.Q., Walter, C.A., Albala, J.S., Busch, D.B. and Thompson, L.H. (1997) The human XRCC9 gene corrects chromosomal instability and mutagen sensitivities in CHO UV40 cells. Proc Natl Acad Sci U S A, 94, 9232-9237.
Lo Ten Foe, J.R., Kwee, M.L., Rooimans, M.A., Oostra, A.B., Veerman, A.J., van Weel, M., Pauli, R.M., Shahidi, N.T., Dokal, I., Roberts, I., Altay, C., Gluckman, E., Gibson, R.A., Mathew, C.G., Arwert, F. and Joenje, H. (1997) Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. Eur J Hum Genet, 5, 137-148.
Machida, Y.J., Machida, Y., Chen, Y., Gurtan, A.M., Kupfer, G.M., D'Andrea, A.D. and Dutta, A. (2006) UBE2T is the E2 in the Fanconi anemia pathway and undergoes negative autoregulation. Mol Cell, 23, 589-596.
Medhurst, A.L., Laghmani el, H., Steltenpool, J., Ferrer, M., Fontaine, C., de Groot, J., Rooimans, M.A., Scheper, R.J., Meetei, A.R., Wang, W., Joenje, H. and de Winter, J.P. (2006) Evidence for subcomplexes in the Fanconi anemia pathway. Blood, 108, 2072-2080.
Meetei, A.R., de Winter, J.P., Medhurst, A.L., Wallisch, M., Waisfisz, Q., van de Vrugt, H.J., Oostra, A.B., Yan, Z., Ling, C., Bishop, C.E., Hoatlin, M.E., Joenje, H. and Wang, W. (2003a) A novel ubiquitin ligase is deficient in Fanconi anemia. Nat Genet, 35, 165-170.
Meetei, A.R., Levitus, M., Xue, Y., Medhurst, A.L., Zwaan, M., Ling, C., Rooimans, M.A., Bier, P., Hoatlin, M., Pals, G., de Winter, J.P., Wang, W. and Joenje, H. (2004) X-linked inheritance of Fanconi anemia complementation group B. Nat Genet, 36, 1219-1224.
Meetei, A.R., Medhurst, A.L., Ling, C., Xue, Y., Singh, T.R., Bier, P., Steltenpool, J., Stone, S., Dokal, I., Mathew, C.G., Hoatlin, M., Joenje, H., de Winter, J.P. and Wang, W. (2005) A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. Nat Genet, 37, 958-963.
Meetei, A.R., Sechi, S., Wallisch, M., Yang, D., Young, M.K., Joenje, H., Hoatlin, M.E. and Wang, W. (2003b) A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. Mol Cell Biol, 23, 3417-3426.
Montes de Oca, R., Andreassen, P.R., Margossian, S.P., Gregory, R.C., Taniguchi, T., Wang, X., Houghtaling, S., Grompe, M. and D'Andrea, A.D. (2005) Regulated interaction of the Fanconi anemia protein, FANCD2, with chromatin. Blood, 105, 1003-1009.
Morgan, N.V., Tipping, A.J., Joenje, H. and Mathew, C.G. (1999) High frequency of large intragenic deletions in the Fanconi anemia group A gene. Am J Hum Genet, 65, 1330-1341.

Neveling, K., Bechtold, A., Hoehn, H. (2007a) Genetic instability syndromes with progeroid features. Z Gerontol Geriat, 40, 339-348.

Neveling, K., Kalb, R., Florl, A.R., Herterich, S., Friedl, R., Hoehn, H., Hader, C., Hartmann, F.H., Nanda, I., Steinlein, C., Schmid, M., Tönnies, H., Hurst, CD., Knowles, M.A., Hanenberg, H., Schulz, W.A., Schindler, D. (2007b) Disruption of the FA/BRCA pathway in bladder cancer. Cytogenet Genome Res, 118:166-176.

Neveling, K., Kalb, R., Schindler, D. (2007c) Cancer in Fanconi Anemia and Fanconi Anemia Genes in Cancer. In Schindler D, H.H. (ed.), Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Karger, Basel, Vol. 15, pp. 59-78.

Niedernhofer, L.J., Lalai, A.S. and Hoeijmakers, J.H. (2005) Fanconi anemia (cross)linked to DNA repair. Cell, 123, 1191-1198.

Nijman, S.M., Huang, T.T., Dirac, A.M., Brummelkamp, T.R., Kerkhoven, R.M., D'Andrea, A.D. and Bernards, R. (2005) The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. Mol Cell, 17, 331-339.

Nookala, R.K., Hussain, S. and Pellegrini, L. (2007) Insights into Fanconi Anaemia from the structure of human FANCE. Nucleic Acids Res, 35, 1638-1648.

Offit, K., Levran, O., Mullaney, B., Mah, K., Nafa, K., Batish, S.D., Diotti, R., Schneider, H., Deffenbaugh, A., Scholl, T., Proud, V.K., Robson, M., Norton, L., Ellis, N., Hanenberg, H. and Auerbach, A.D. (2003) Shared genetic susceptibility to breast cancer, brain tumors, and Fanconi anemia. J Natl Cancer Inst, 95, 1548-1551.

Pace, P., Johnson, M., Tan, W.M., Mosedale, G., Sng, C., Hoatlin, M., de Winter, J., Joenje, H., Gergely, F. and Patel, K.J. (2002) FANCE: the link between Fanconi anaemia complex assembly and activity. Embo J, 21, 3414-3423.

Pagano, G., Degan, P., d'Ischia, M., Kelly, F.J., Nobili, B., Pallardo, F.V., Youssoufian, H. and Zatterale, A. (2005) Oxidative stress as a multiple effector in Fanconi anaemia clinical phenotype. Eur J Haematol, 75, 93-100.

Park, W.H., Margossian, S., Horwitz, A.A., Simons, A.M., D'Andrea, A.D. and Parvin, J.D. (2005) Direct DNA binding activity of the Fanconi anemia D2 protein. J Biol Chem, 280, 23593-23598.

Patel, K.J. and Joenje, H. (2007) Fanconi anemia and DNA replication repair. DNA Repair (Amst), 6, 885-890.

Peng, M., Litman, R., Xie, J., Sharma, S., Brosh, R.M., Jr. and Cantor, S.B. (2007) The FANCJ/MutLalpha interaction is required for correction of the cross-link response in FA-J cells. Embo J, 26, 3238-3249.

Popp, H., Kalb, R., Fischer, A., Lobitz, S., Kokemohr, I., Hanenberg, H. and Schindler, D. (2003) Screening Fanconi anemia lymphoid cell lines of non-A, C, D2, E, F, G subtypes for defects in BRCA2/FANCD1. Cytogenet Genome Res, 103, 54-57.

Rahman, N., Seal, S., Thompson, D., Kelly, P., Renwick, A., Elliott, A., Reid, S., Spanova, K., Barfoot, R., Chagtai, T., Jayatilake, H., McGuffog, L., Hanks, S., Evans, D.G., Eccles, D., Easton, D.F. and Stratton, M.R. (2007) PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat Genet, 39, 165-167.

Reid, S., Renwick, A., Seal, S., Baskcomb, L., Barfoot, R., Jayatilake, H., Pritchard-Jones, K., Stratton, M.R., Ridolfi-Luthy, A. and Rahman, N. (2005) Biallelic BRCA2 mutations are associated with multiple malignancies in childhood including familial Wilms tumour. J Med Genet, 42, 147-151.

Reid, S., Schindler, D., Hanenberg, H., Barker, K., Hanks, S., Kalb, R., Neveling, K., Kelly, P., Seal, S., Freund, M., Wurm, M., Batish, S.D., Lach, F.P., Yetgin, S., Neitzel, H., Ariffin, H., Tischkowitz, M., Mathew, C.G., Auerbach, A.D., Rahman, N (2007). Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet 39,162-164. Epub Dec 31.

Rosenberg, P.S., Socie, G., Alter, B.P. and Gluckman, E. (2005) Risk of head and neck squamous cell cancer and death in patients with Fanconi anemia who did and did not receive transplants. Blood, 105, 67-73.

Sasaki, M.S. and Tonomura, A. (1973) A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res, 33, 1829-1836.

Schindler D, Friedl R., Gavvovidis I, Kalb R, Neveling K, et al (2007a) Applications of cell cycle testing in Fanconi anemia. In Schindler D, H.H. (ed.), Fanconi Anemia. A paradigmatic disease for the understanding of cancer and aging. Karger, Basel, Vol. 15, pp. 110-130.

Schindler, D. and Hoehn, H. (1988) Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. Am J Hum Genet, 43, 429-435.

Schindler, D. and Neveling, K. Fanconi Anemia. In: Schwab M (ed) Enzyclopedia of Cancer. Springer, in press.

Seal, S., Thompson, D., Renwick, A., Elliott, A., Kelly, P., Barfoot, R., Chagtai, T., Jayatilake, H., Ahmed, M., Spanova, K., North, B., McGuffog, L., Evans, D.G., Eccles, D., Easton, D.F., Stratton, M.R. and Rahman, N. (2006) Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. Nat Genet, 38, 1239-1241.

Seyschab, H., Sun, Y., Friedl, R., Schindler, D. and Hoehn, H. (1993) G2 phase cell cycle disturbance as a manifestation of genetic cell damage. Hum Genet, 92, 61-68.

Shivji, M.K. and Venkitaraman, A.R. (2004) DNA recombination, chromosomal stability and carcinogenesis: insights into the role of BRCA2. DNA Repair (Amst), 3, 835-843.

Sims, A.E., Spiteri, E., Sims, R.J. 3rd, Arita, A.G., Lach, F.P., Landers, T., Wurm, M., Freund, M., Neveling, K., Hanenberg, H., Auerbach, A.D., Huang, TT (2007). FANCI is a second monoubiquitinated member of the Fanconi anemia pathway. Nat Struct Mol Biol 14, 564-567. Epub 2007 Apr 25.

Sobhian, B., Shao, G., Lilli, D.R., Culhane, A.C., Moreau, L.A., Xia, B., Livingston, D.M. and Greenberg, R.A. (2007) RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. Science, 316, 1198-1202.

Strathdee, C.A., Duncan, A.M. and Buchwald, M. (1992) Evidence for at least four Fanconi anaemia genes including FACC on chromosome 9. Nat Genet, 1, 196-198.

Taniguchi, T. and D'Andrea, A.D. (2002) The Fanconi anemia protein, FANCE, promotes the nuclear accumulation of FANCC. Blood, 100, 2457-2462.

Tarsounas, M., Davies, D. and West, S.C. (2003) BRCA2-dependent and independent formation of RAD51 nuclear foci. Oncogene, 22, 1115-1123.

Timmers, C., Taniguchi, T., Hejna, J., Reifsteck, C., Lucas, L., Bruun, D., Thayer, M., Cox, B., Olson, S., D'Andrea, A.D., Moses, R. and Grompe, M. (2001) Positional cloning of a novel Fanconi anemia gene, FANCD2. Mol Cell, 7, 241-248.

Titus, T.A., Selvig, D.R., Qin, B., Wilson, C., Starks, A.M., Roe, B.A. and Postlethwait, J.H. (2006) The Fanconi anemia gene network is conserved from zebrafish to human. Gene, 371, 211-223.

Wagner, J.E., Tolar, J., Levran, O., Scholl, T., Deffenbaugh, A., Satagopan, J., Ben-Porat, L., Mah, K., Batish, S.D., Kutler, D.I., MacMillan, M.L., Hanenberg, H. and Auerbach, A.D. (2004) Germline mutations in BRCA2: shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. Blood, 103, 3226-3229.

Waisfisz, Q., Morgan, N.V., Savino, M., de Winter, J.P., van Berkel, C.G., Hoatlin, M.E., Ianzano, L., Gibson, R.A., Arwert, F., Savoia, A., Mathew, C.G., Pronk, J.C. and Joenje, H. (1999a) Spontaneous functional correction of homozygous fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. Nat Genet, 22, 379-383.

Waisfisz, Q., Saar, K., Morgan, N.V., Altay, C., Leegwater, P.A., de Winter, J.P., Komatsu, K., Evans, G.R., Wegner, R.D., Reis, A., Joenje, H., Arwert, F., Mathew, C.G., Pronk, J.C. and Digweed, M. (1999b) The Fanconi anemia group E gene, FANCE, maps to chromosome 6p. Am J Hum Genet, 64, 1400-1405.

Wajnrajch, M.P., Gertner, J.M., Huma, Z., Popovic, J., Lin, K., Verlander, P.C., Batish, S.D., Giampietro, P.F., Davis, J.G., New, M.I. and Auerbach, A.D. (2001) Evaluation of growth and hormonal status in patients referred to the International Fanconi Anemia Registry. Pediatrics, 107, 744-754.

Wang, B., Matsuoka, S., Ballif, B.A., Zhang, D., Smogorzewska, A., Gygi, S.P. and Elledge, S.J.

(2007) Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. Science, 316, 1194-1198.

Wang, W. (2007) Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nat Rev Genet, 8, 735-748.

Wegner, R.D., Henrichs, I., Joenje, H. and Schroeder-Kurth, T. (1996) Fanconi anemia complementation group E: clinical and cytogenetic data of the first patient. Clin Genet, 50, 479-482.

Welch, E.M., Barton, E.R., Zhuo, J., Tomizawa, Y., Friesen, W.J., Trifillis, P., Paushkin, S., Patel, M., Trotta, C.R., Hwang, S., Wilde, R.G., Karp, G., Takasugi, J., Chen, G., Jones, S., Ren, H., Moon, Y.C., Corson, D., Turpoff, A.A., Campbell, J.A., Conn, M.M., Khan, A., Almstead, N.G., Hedrick, J., Mollin, A., Risher, N., Weetall, M., Yeh, S., Branstrom, A.A., Colacino, J.M., Babiak, J., Ju, W.D., Hirawat, S., Northcutt, V.J., Miller, L.L., Spatrick, P., He, F., Kawana, M., Feng, H., Jacobson, A., Peltz, S.W. and Sweeney, H.L. (2007) PTC124 targets genetic disorders caused by nonsense mutations. Nature, 447, 87-91.

Wells, D.J. (2006) Therapeutic restoration of dystrophin expression in Duchenne muscular dystrophy. J Muscle Res Cell Motil, 27, 387-398.

West, S.C. (2003) Molecular views of recombination proteins and their control. Nat Rev Mol Cell Biol, 4, 435-445.

6

Curriculum Vitae

Personal Data

Family name	Neveling
First name	Kornelia
Adress	Steubenstr. 6a, 97074 Würzburg, Germany
Date of birth	03.03.1979
Place of birth	Rheinberg, Germany
Personal status	unmarried
Parents:	Wolfram Neveling Dipl.-komm. Dipl.-Verwaltungswirt Ulla Neveling Dipl.-Verwaltungswirtin
Brother:	Alexander Neveling Dipl. Ing. / Teacher

Education (School)

1985 -1989	Primary School: Gerhard-Tersteegen-Schule, Neukirchen-Vluyn, Germany
1989 -1998	Secondary School: Julius-Stursberg-Gymnasium, Neukirchen-Vluyn, Germany
Juni 1998	High School Graduation (Abitur)

Education (University)

1998 - 2004	Student of Biology, Heinrich-Heine University of Düsseldorf, Germany
09/2000	Intermediate Diploma (Cause of Proposal for „Studienstiftung des Deutschen Volkes“ by the „Diplomprüfungsausschuß Biologie“)
02/2004	Diploma Major: Microbiology, 1. Minor: Genetics, 2. Minor: Informatics
Diploma Thesis:	Der Einfluss der Spleißstellen-Effizienz auf die Erkennung alternativer HIV-1 Exons
Since 05/2004	Ph.D. Student , Department of Human Genetics, Medical School, University of Würzburg, Germany
Ph.D. Thesis:	Molekulargenetische Ursachen und Folgen genetischer Instabilität am Beispiel des FA/BRCA Caretaker Pathways

Previous Activities

19.08.98 – 30.09.98	Student Job at the Company „Eraco Handel GmbH“, Moers, Germany
24.04.01 – 26.06.01	Student Job at the Department of Zoomorphology, Cellbiology and Parasitology, University of Düsseldorf, Germany
18.10.01 – 18.04.02/ 19.04.02 – 18.10.02	Student Job at the Department of Human Genetics and Anthropology, University of Düsseldorf, Germany

Scientific meetings

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, Prague, Czech Republic
09/2005	17th Annual Scientific Fanconi Anemia Research Fund Symposium, Geneva, Switzerland
03/2006	17th Annual Meeting of the German Society of Human Genetics, Heidelberg, Germany
05/2006	19. Tumorzytogenetische Arbeitstagung, Wernigerode (Harz), Germany
10/2006	18th Annual Scientific Fanconi Anemia Research Fund Symposium, North Bethesda, USA
03/2007	18th Annual Meeting of the German Society of Human Genetics, Bonn, Germany
10/2007	19th Annual Scientific Fanconi Anemia Research Fund Symposium, Chicago, USA

Special courses

08/2004	Flow cytometry seminar (BD), Würzburg
03/2006	Flow cytometry course, Würzburg
04/2006	Real-time user meeting, Würzburg

Awards

09/2005	Gene discovery award for the identification of FANCJ, Fanconi Anemia Research Fund, Geneva, Switzerland
10/2005	Ehrenauszeichnung für die Identifizierung von FANCJ, Deutsche Fanconi Anemie Hilfe e.V., Gersfeld, Germany
03/2007	„Vortragspreis des Jahres 2007“ for „The BRCA2-binding protein PALB2 is defective in the tumor prone Fanconi Anemia subtype FA-N“, 18.GfH-Jahrestagung, Bonn, Germany
10/2007	Gene discovery awards for the identification of FANCN and FANCI, Fanconi Anemia Research Fund, Chicago, USA
11/2007	Ehrenauszeichnungen für die Identifizierung von FANCN und FANCI, Deut sche Fanconi Anemie Hilfe e.V., Gersfeld, Germany

Würzburg, December 20th, 2007

7

Publications/Presentations

Publications

Peer-reviewed articles

Levran O, Attwooll C, Henry RT, Milton KL, **Neveling K**, Rio P, Batish SD, Kalb R, Velleuer E, Barral S, Ott J, Petrini J, Schindler D, Hanenberg H, Auerbach AD. The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet. 2005 Sep;37(9):931-3. Epub 2005 Aug 21.

Bechtold A, Friedl R, Kalb R, Gottwald B, **Neveling K**, Gavvovidis I, Herterich S, Schindler D, Hoehn H. Prenatal Exclusion/Confirmation of Fanconi Anemia via Flow Cytometry: A Pilot Study. Fetal Diagn Ther. 2006;21(1):118-24.

Heinrich T, Prowald C, Friedl R, Gottwald B, Kalb R, **Neveling K**, Herterich S, Hoehn H, Schindler D. Exclusion/confirmation of Ataxia-telangiectasia via cell-cycle testing. Eur J Pediatr. 2006 Apr;165(4):250-7. Epub 2006 Jan 13.

Huck K, Hanenberg H, Gudowius S, Fenk R, Kalb R, **Neveling K**, Betz B, Niederacher D, Haas R, Gobel U, Kobbe G, Schindler D. Delayed diagnosis and complications of Fanconi anaemia at advanced age--a paradigm. Br J Haematol. 2006 Apr;133(2):188-97.

Hanenberg H, Huck K, Gudowius S, Gobel U, Kobbe G, Haas R, Kalb R, **Neveling K**, Schindler D. Fanconi anaemia – response to Manoharan. Br J Haematol. 2006 Oct;135(1):139-40. Epub 2006 Aug 22.

Antonio Casado J, Callen E, Jacome A, Rio P, Castella M, Lobitz S, Ferro T, Munoz A, Sevilla J, Cantalejo A, Cela E, Cervera J, Sanchez-Calero J, Badell I, Estella J, Dasi A, Olive T, Jose Ortega J, Rodriguez-Villa A, Tapia M, Molines A, Madero L, Segovia JC, **Neveling K**, Kalb R, Schindler D, Hanenberg H, Surralles J, Bueren JA. A comprehensive strategy for the subtyping of patients with Fanconi anaemia: conclusions from the Spanish Fanconi Anemia Research Network. J Med Genet. 2007 Apr;44(4):241-9. Epub 2006 Nov 14.

Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, Kalb R, **Neveling K**, Kelly P, Seal S, Freund M, Wurm M, Batish SD, Lach FP, Yetgin S, Neitzel H, Ariffin H, Tischkowitz M, Mathew CG, Auerbach AD, Rahman N. Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet. 2007 Feb;39(2):162-4. Epub Dec 31.

Bogliolo M, Lyakhovich A, Callen E, Castella M, Capelli E, Ramirez MJ, Creus A, Marcos R, Kalb R, **Neveling K**, Schindler D, Surralles J. Histone H2AX and Fanconi anemia FANCD2 function in the same pathway to maintain chromosome stability. EMBO J. 2007 Mar 7;26(5):1340-51. Epub 2007 Feb 15.

Ling C, Ishiai M, Ali AM, Medhurst AL, **Neveling K**, Kalb R, Yan Z, Xue Y, Oostra AB, Auerbach AD, Hoatlin ME, Schindler D, Joenje H, de Winter JP, Takata M, Meetei AR, Wang W. FAAP100 is essential for activation of the Fanconi anemia-associated DNA damage response pathway. EMBO J. 2007 Apr 18;26(8):2104-14. Epub 2007 Mar 29.

Kalb R, **Neveling K**, Hoehn H, Schneider H, Linka Y, Batish SD, Hunt C, Berwick M, Callen E, Surralles J, Casado JA, Bueren J, Dasi A, Soulier J, Gluckman E, Zwaan CM, van Spaendonk R, Pals G, de Winter JP, Joenje H, Grompe M, Auerbach AD, Hanenberg H, Schindler D. Hypomorphic Mutations in the Gene Encoding a Key Fanconi Anemia Protein, FANCD2, Sustain a Significant Group of FA-D2 Patients with Severe Phenotype. Am J Hum Genet. 2007 May;80(5):895-910. Epub 2007 Apr 6.

Sims AE, Spiteri E, Sims RJ 3rd, Arita AG, Lach FP, Landers T, Wurm M, Freund M, **Neveling K**, Hanenberg H, Auerbach AD, Huang TT. FANCI is a second monoubiquitinated member of the Fanconi anemia pathway. Nat Struct Mol Biol. 2007;14:564-7. Epub 2007 Apr 25.

Neveling K, Kalb R, Florl AR, Herterich S, Friedl R, Hoehn H, Hader C, Hartmann FH, Nanda I, Steinlein C, Schmid M, Tönnies H, Hurst CD, Knowles MA, Hanenberg H, Schulz WA, Schindler D. Disruption of the FA/BRCA pathway in bladder cancer. Cytogenet Genome Res. 2007 Nov;118:166-76.

Reviews and book articles

Neveling K, Kalb R, Gross M. Mutationsanalyse in den Fanconi-Anämie-Genen. Fanconi Anämie - Ein Handbuch für Eltern, Patienten und ihre Ärzte. Deutsche Fanconi-Anämie-Hilfe e.V. 2005, pp173-82.

Kalb R, **Neveling K**, Nanda I, Schindler D, Hoehn H. Fanconi Anemia: Causes and Consequences of Genetic Instability. Volff JN (ed): Genome and Disease. Genome Dynamics, Basel, Karger, 2006, vol 1, pp 218-42.

Kalb R, **Neveling K**, Herterich S, Schindler D. Fanconi Anemia Genes: Structure, Mutations, and Genotype-Phenotype Correlations. Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet, Basel, Karger, 2007, vol 15, pp 39-58.

Neveling K, Kalb R, Schindler D. Cancer in Fanconi Anemia and Fanconi Anemia Genes in Cancer. Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet, Basel, Karger, 2007, vol 15, pp 59-78.

Schindler D, Friedl R, Gavvovidis I, Kalb R, **Neveling K**, Linka Y, Hanenberg H, Kubbies M, Hoehn H. Applications of Cell Cycle Testing in Fanconi Anemia. Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet, Basel, Karger, 2007, vol 15, pp 110-130.

Bechtold A, Kalb R, **Neveling K**, Friedl R, Gottwald B, Herterich S, Schmugge Liner M, Heilmann C, Hanenberg H, Schindler D. Prenatal Diagnosis of Fanconi Anemia: Functional and Molecular Testing. Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet, Basel, Karger, 2007, vol 15, pp 131-148.

Hoehn H, Kalb R, **Neveling K**, Friedl R, Bechtold A, Herterich S, Sun Y, Gruhn B, Hanenberg H, Schindler D. Revertant Mosaicism in Fanconi Anemia: Natural Gene Therapy at Work. Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet, Basel, Karger, 2007, vol 15, pp 149-172.

Neveling K, Bechtold A, Hoehn H. Genetic instability syndromes with progeroid features. Z Gerontol Geriat. 2007 Nov;40:339-48.

Schindler D and **Neveling K**. Fanconi Anemia. In: Schwab M (ed) Enzyclopedia of Cancer. Springer, in press.

Presentations

Oral

Kalb R, **Neveling K**, Velleuer E, Sobeck A, Schneider H, Lobitz S, Rele I, Auerbach AD, Hanenberg H, Schindler D. Examination of Candidate Genes in Unclassified Fanconi Anemia Cell Line. 16th Annual Fanconi Anemia Research Fund Scientific Symposium, 2004, Boston, USA.

Heinrich T, Friedl R, Sobeck A, Kalb R, **Neveling K**, Gottwald B, Herterich S, Hoehn H, Schindler D. Exclusion/confirmation of Ataxia telangiectasia via cell cycle testing. 16th Annual Meeting of the German Society of Human Genetics, 2005, Halle, Germany.

Bechtold A, Friedl R, Kalb R, Gottwald B, **Neveling K**, Gavvovidis I, Herterich S, Schindler D, Hoehn H. Prenatal exclusion/confirmation of Fanconi anemia. 16th Annual Meeting of the German Society of Human Genetics, 2005, Halle, Germany.

Neveling K, Gottwald B, Kalb R, Herterich S, Schaal H, Hoehn H, Schindler D. Usage of a TT 5' splice site in Fanconi anemia. ICGEB-ESF Workshop, 2005, Trieste, Italy.

Kalb R, **Neveling K**, Hoehn H, Vervenne r, de Winter J, Surralles J, Callen E, Auerbach AD, Joenje H, Hanenberg H, Schindler D. Mutation analysis of 29 FA patients assigned to complementation group FA-D2. 17th Annual Fanconi Anemia Research Fund Scientific Symposium, 2005, Geneva, Switzerland.

Levran O, Atwoll C, Henry RT, Milton KL, **Neveling K**, Rio P, Batish SD, Kalb R, Barral S, Ott J, Petrini J, Schindler D, Hanenberg H, Auerbach AD. A BRCA1-interacting Protein is Mutated in Fanconi Anemia FA-J. 17th Annual Fanconi Anemia Research Fund Scientific Symposium, 2005, Geneva, Switzerland.

Attwoll C, Levran O, Henry RT, Milton KL, **Neveling K**, Rio P, Batish SD, Kalb R, Schindler D, Hanenberg H, Petrini J, Auerbach AD. Fanconi Anemia FA-J Cells are Mutated in a BRCA1-interacting Protein and Show Comprised DNA Repair. 17th Annual Fanconi Anemia Research Fund Scientific Symposium, 2005, Geneva, Switzerland.

Hanenberg H, Lobitz S, Schneider H, Kalb R, Friedl R, Gottwald B, **Neveling K**, Velleuer E, Furlan S, Rio P, Linka Y, Huck K, Batish SD, Levran O, Auerbach AD, Schindler D. Sensitivity and Specificity of Retroviral Complementation Group Assignment in Primary T Cells and Fibroblasts from Patients with Fanconi Anemia. 17th Annual Fanconi Anemia Research Fund Scientific Symposium, 2005, Geneva, Switzerland.

Ling C, Meetei AR, Xue Y, Medhurst AL, Singh TR, **Neveling K**, Auerbach AD, Schindler D, Hoatlin ME, Joenje H, de Winter JP, Wang W. The role of the Fanconi Anemia Core Complex in DNA Damage Response. 17th Annual Fanconi Anemia Research Fund Scientific Symposium, 2005, Geneva, Switzerland.

Kalb R, **Neveling K**, Hoehn H, Schindler D. DNA damage signaling through the Fanconi/BRCA pathway. 9th Joint Meeting; Signal Transduction: Receptors, Mediatorsand Genes, 2005, Weimar, Germany.

Neveling K, Kalb R, Hader C, Schulz WA, Hanenberg H, Baumer A, Hoehn H, Schindler D. Proficiency testing of the FA/BRCA pathway via FANCD2 immunoblotting. 17th Annual Meeting of the German Society of Human Genetics, 2006, Heidelberg, Germany.

Kalb R, **Neveling K**, Rio P, Hanenberg H, Schindler D. Revertant mosaicism in Fanconi anemia: a 5 bp deletion is compensated by an in cis splice site mutation in a patient of subtype FA-L. 17th Annual Meeting of the German Society of Human Genetics, 2006, Heidelberg, Germany.

Neveling K, Kalb R, Florl A, Hader C, Schulz WA, Hanenberg H, Baumer A, Hoehn H, Schindler D. Hypermethylierung des Fanconi Anämie Gens FANCF in Blasentumoren. 19. Tumorzytogenetische Arbeitstagung, 2006, Wernigerode (Harz)

Kalb R, **Neveling K**, Hoehn H, Schmugge M, Heilmann C, Hanenberg H, Schindler D. Prenatal Diagnosis in FA-D2 Families. 18th Annual Fanconi Anemia Research Fund Scientific Symposium, 2006, North Bethesda, USA.

Rahman N, Schindler D, Hanenberg H, Tischkowitz M, Hanks S, Barker K, Kelly P, Seal S, Batish SD, Lach FP, Kalb R, **Neveling K**, Mathew CG, Auerbach AD, Reid S. Biallelic Mutations in PALB2 cause Fanconi Anemia and Predispose to Childhood Cancer. 18th Annual Fanconi Anemia Research Fund Scientific Symposium, 2006, North Bethesda, USA.

Neveling K, Kalb R, Reid S, Neitzel H, Hanenberg H, Auerbach AD, Rahman N, Schindler D. The BRCA2-binding protein PALB2 is defective in the tumor prone Fanconi anemia subtype FA-N. 18th Annual meeting of the German Society of Human Genetics, 2007, Bonn, Germany.

Yan Z, Ling C, Lin T, **Neveling K**, Schindler D, Joenje H, de Winter J, Wang W. A Novel Component of Fanconi Anemia Core Complex is Essential for Activation of the FA-associated DNA Repair Pathway. 19. Annual Fanconi Anemia Research Fund Scientific Symposium, 2007, Chicago, USA.

Poster

Neveling K, Kalb R, Schaal H, Hoehn H, Schindler D. Usage of a TT 5' splice site in the Fanconi anemia gene FANCC. 16th Annual meeting of the German Society of Human Genetics, 2005, Halle, Germany.

Kalb R, **Neveling K**, Herterich S, Hoehn H, Schindler D. Abundance of splice site mutations in FANCD2. 16th Annual meeting of the German Society of Human Genetics, 2005, Halle, Germany.

Kalb R, **Neveling K**, Hoehn H, Surralles J, de Winter J, Joenje H, Auerbach AD, Hanenberg H, Schindler D. FANCD2 is a major FA gene with high prevalence of splicing mutations. HUGO 2005, Kyoto, Japan.

Neveling K, Florl A, Hader C, Herterich S, Hoehn H, Hanenberg H, Knowles M, Baumer A, Schulz WA, Schindler D, Kalb R. Hypermethylation of the Fanconi anemia gene FANC-F in bladder carcinoma. European Journal of Human Genetics 2005, vol. 13, supp 1, Prague, Czech Republic.

Kalb R, **Neveling K**, Hoehn H, Vervenne R, Velleuer E, de Winter J, Surralles J, Callen E, Auerbach AD, Joenje H, Hanenberg H, Schindler D. Predominance of splicing mutations in the central Fanconi anemia gene FANCD2. European Journal of Human Genetics 2005, vol. 13, supp 1,

Prague, Czech Republic.

Neveling K, Kalb R, Florl A, Hader C, Herterich S, Hoehn H, Hanenberg H, Knowles M, Baumer A, Schulz WA, Schindler D. Fanconi Anemia Mutation Screen in Bladder Carcinoma. 17th Annual Fanconi Anemia Research Fund Scientific Symposium, 2005, Geneva, Switzerland.

Müller AS, **Neveling K**, Schindler D, Neitzel H, Tönnies H. Fanconi anemia with a high rate of spontaneous and MMC-induced chromosomal translocations – diagnostic screening strategy. 17th Annual meeting of the German Society of Human Genetics, 2006, Heidelberg, Germany.

Neveling K, Kalb R, Hoehn H, Rio P, Hanenberg H, Schindler D. Case Report of a consanguineous FA-J Familiy. 18th Annual Fanconi Anemia Research Fund Scientific Symposium, 2006, North Bethesda, USA.

Rio P, Casado JA, **Neveling K**, Kalb R, Jacome A, Castella M, Hanenberg H, Dasi A, Surralles J, Schindler D, Bueren JA. Studying the Role of BRIP1 and Interacting Proteins in FA-J Spanish Patients. 18th Annual Fanconi Anemia Research Fund Scientific Symposium, 2006, North Bethesda, USA.

Castella M, Callen E, Casado JA, Jacome A, Kalb R, **Neveling K**, Schindler D, Hanenberg H, Niederacher D, Gutiérrez-Enriquez S, Diez O, Lach F, Auerbach AD, Vervenne R, Pals G, Bueren JA, Surralles J. Molecular Characterization of Four Exceptional Fanconi Anemia Patients from Spain. 18th Annual Fanconi Anemia Research Fund Scientific Symposium, 2006, North Bethesda, USA.

Ling C, Ciccia A, Li Y, Yan Z, Xue Y, Laghmani E, Ameziane N, Kalb R, **Neveling K**, Schindler D, Joenje H, de Winter JP, West SC, Wang W. Studies of New Components of the Fanconi Anemia Core Complex. 18th Annual Fanconi Anemia Research Fund Scientific Symposium, 2006, North Bethesda, USA.

Casado JA, Jacome A, Rio P, Castella M, Segovia JC, Schindler D, **Neveling K**, Kalb R, Hanenberg H, Surralles J, Bueren JA. A comprehensive Strategy for the Genetic Subtyping of Fanconi Anemia Patients. 18th Annual Fanconi Anemia Research Fund Scientific Symposium, 2006, North Bethesda, USA.

Neveling K, Kalb R, Castella M, Rio P, Bueren J, Surralles J, Hanenberg H, Auerbach AD, Hoehn H, Schindler D. Genetic alterations of FANCE, a rare Fanconi anemia gene with multiple functions. 18th Annual meeting of the German Society of Human Genetics, 2007, Bonn, Germany.
Kalb R, Neveling K, Friedl R, Gottwald B, Endt D, Herterich S, Hanenberg H, Hoehn H, Schindler D. The Fanconi anemia gene family: structure, function, mutations and their consequences. 18th Annual meeting of the German Society of Human Genetics, 2007, Bonn, Germany.

Bechtold A, Kalb R, **Neveling K**, Friedl R, Gottwald B, Herterich S, Hanenberg H. Revertant mosaicism in Fanconi anemia: natural gene therapy at work. 18th Annual meeting of the German Society of Human Genetics, 2007, Bonn, Germany.

Neveling K, Kalb R, Castella M, Casado JA, Bueren J, Surralles J, Hanenberg H, Auerbach AD, Höhn H, Schindler D. Unusual Pattern of Genetic Alterations in the Rare FANCE Gene. 19th Annual Fanconi Anemia Research Fund Scientific Symposium, 2007, Chicago, USA.

Schindler D, **Neveling K**, Kalb R, Herterich S, Hanenberg H, Höhn H. Hematopoetic Mosaicism in FA: More Questions than Answers. 19th Annual Fanconi Anemia Research Fund Scientific Symposium, 2007, Chicago, USA.