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

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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] Mono-ubiquitination 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

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.

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,
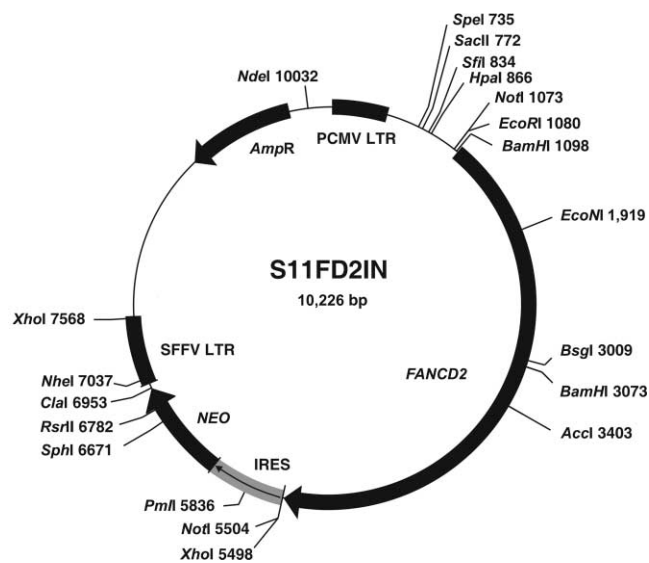
Figure 1. Circular map of vector S11FD2IN. The retroviral-expression vector S11FD2IN contains a bicistronic construct of the full-length *FANCD2* cDNA ("*FANCD2*") and the neomycin resistance gene (*NEO*). Translation of the latter is ensured by an IRES. Also shown are the long terminal repeats (LTR), 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.

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.*

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;
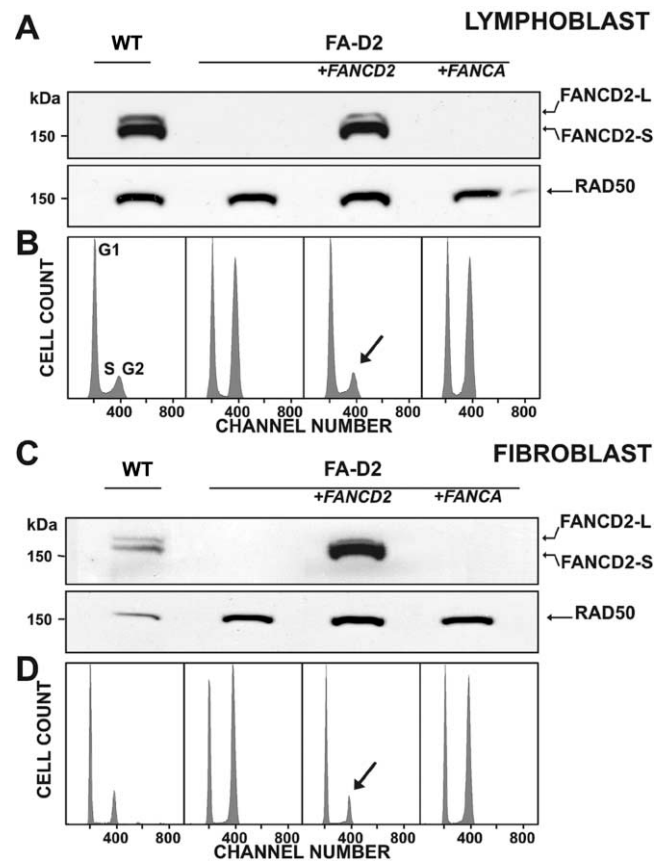
Figure 2. Delineation of FA-D2. *A,* Assignment to group FA-D2 on the basis of the absence of either FANCD2 band on immunoblots after exposure of the patients' cells to MMC, here shown for an LCL from patient 6 (*lane 2*). Transduction with *FANCD2* cDNA with use of S11FD2IN restores both isoforms of 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.

$P = .001$) (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 groups (median age for FA-D2 patients was 10 years and

Table 6. Microsatellite Primers

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

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, type, length, and orientation of the *Alu* and the duplicated

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.

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 c.3803G→A in patient 6 and siblings 26 and 27) (tables 7

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.

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 homozygous in consanguineous families. Three unrelated pa-
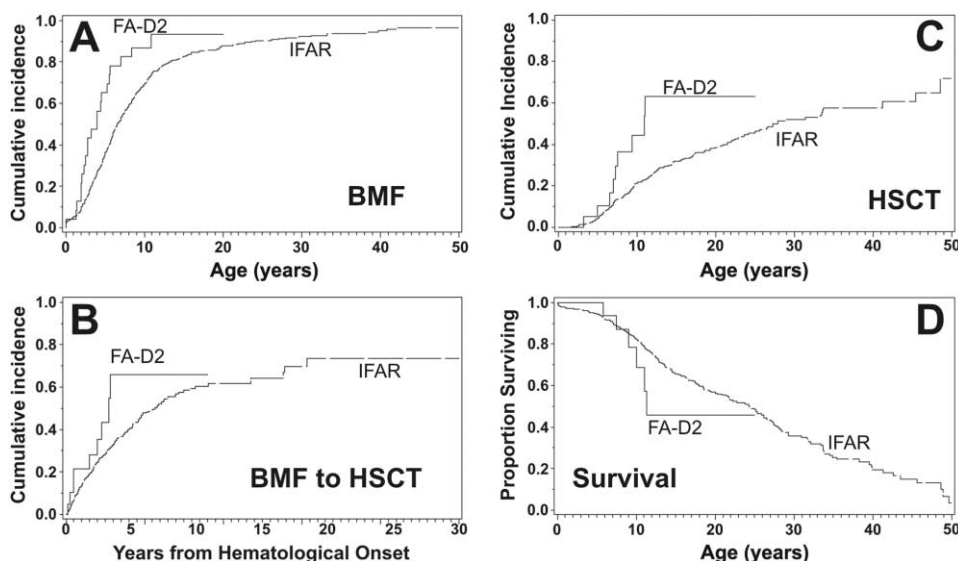
Figure 3. Clinical course of 23 fully informative, nonmosaic FA-D2 patients in this study. *A,* The cumulative incidence of BMF of the FA-D2 patients in the present study (FA-D2) precedes that of all patients with FA in the IFAR[42] (*P* = .001). *B,* The period from BMF to HSCT, which was shorter in 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.

tients 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 consan-

guineous 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 Turkish patient (patient 10). The Czech patient (patient
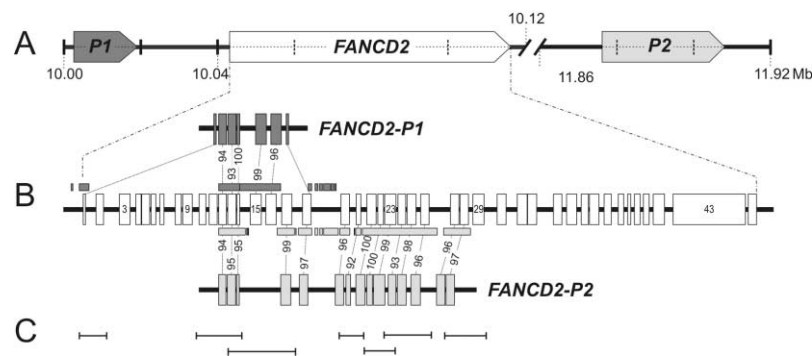
Figure 4. Topography of *FANCD2,* its pseudogenes, and the superamplicons. *A,* The two pseudogenes—*FANCD2-P1* and *FANCD2-P2*—located upstream and downstream, respectively, of the functional *FANCD2* gene. All three have the same orientation. The scale denotes Mb on chromosome 3. *B, FANCD2* exons 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.

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 mu-

tation, 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-blood cells. Two patients (3 and 25) with the c.1948-
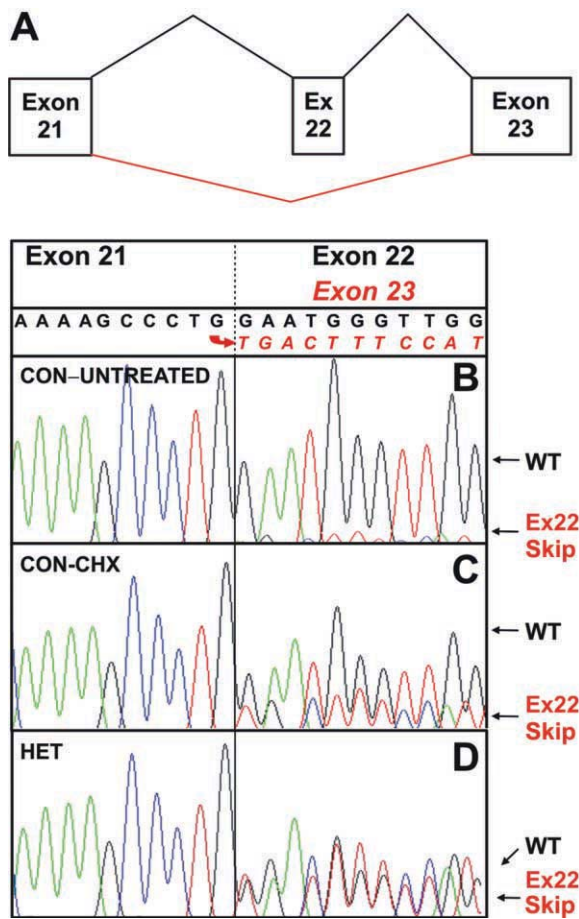
Figure 5. Exon 22 splicing. *A,* Schematic depiction of the splicing patterns resulting from exon 22 retention or skipping. *B,* cDNA sequencing in an LCL from a normal control (CON), showing predominance of exon 22 sequence following that of exon 21 but also low levels of underlying sequence readable 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.

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 lacked G2-phase arrest, whereas the nonmosaic patients

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**gtgtggag	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.

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 levels of residual FANCD2 were used to examine its char-
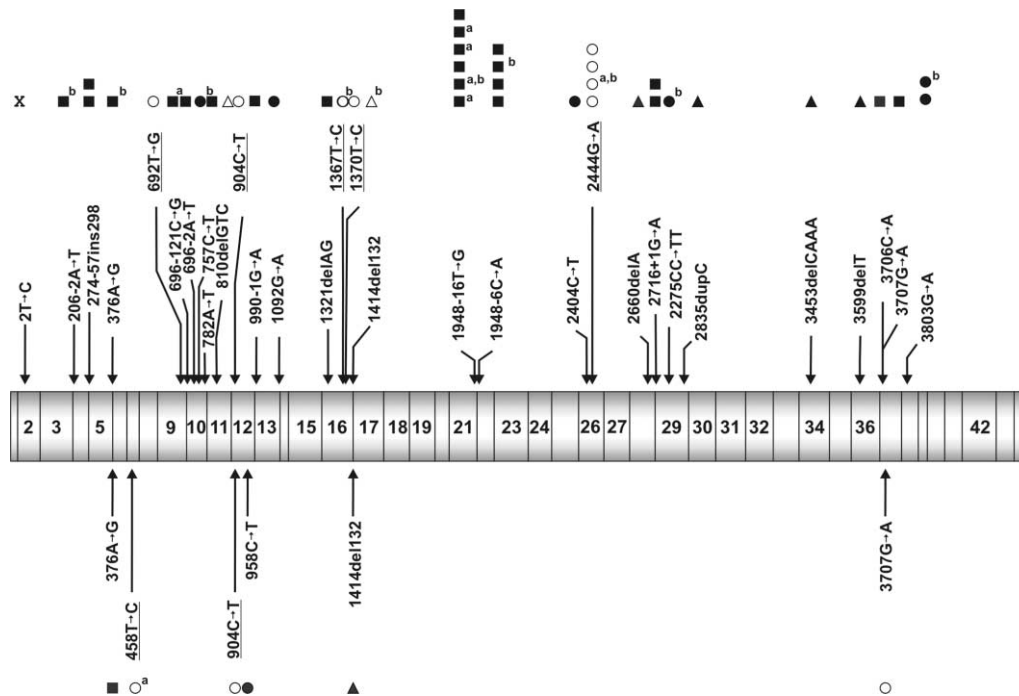
Figure 6. Positions and identity of mutations detected in *FANCD2*. Mutations identified in the present study are shown above, mutations reported elsewhere[24,45] are indicated below the schematic display of *FANCD2* cDNA. Blackened squares (■) represent mutations resulting in aberrant splicing patterns, blackened circles (•) represent nonsense mutations, unblackened circles (○) represent missense mutations, blackened triangles (▲) represent frameshift deletions or duplications, and unblackened triangles (△) 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.
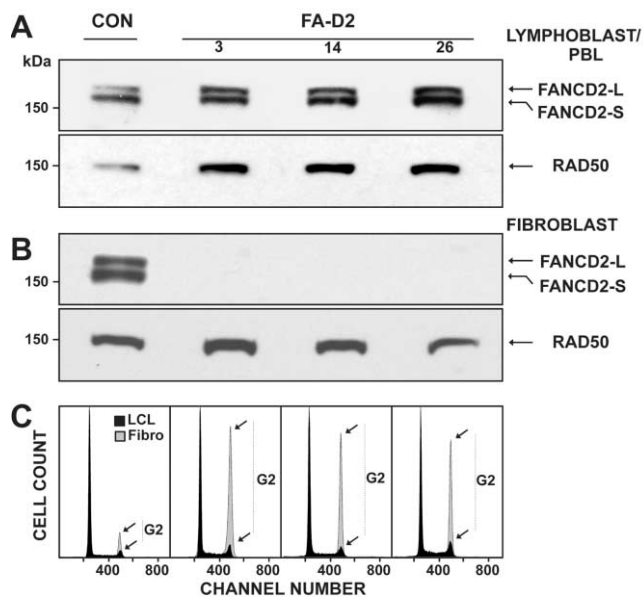
Figure 7. Reverse mosaicism. Blood-derived cells from FA-D2 patients with reverse mosaicism of the hematopoietic system (patients 3 and 26, LCLs; patient 14, stimulated PBL; *panel A, lanes 2, 3,* and *4*) reveal both FANCD2 bands at levels similar to a random normal control (*lane 1*) after exposure 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.

acteristics on overexposed blots. The intensity of the FANCD2-L band increased as a function of the concentration of the DNA crosslinking agent (fig. 8C) 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 age of hematological onset (i.e., BMF) in our patients with
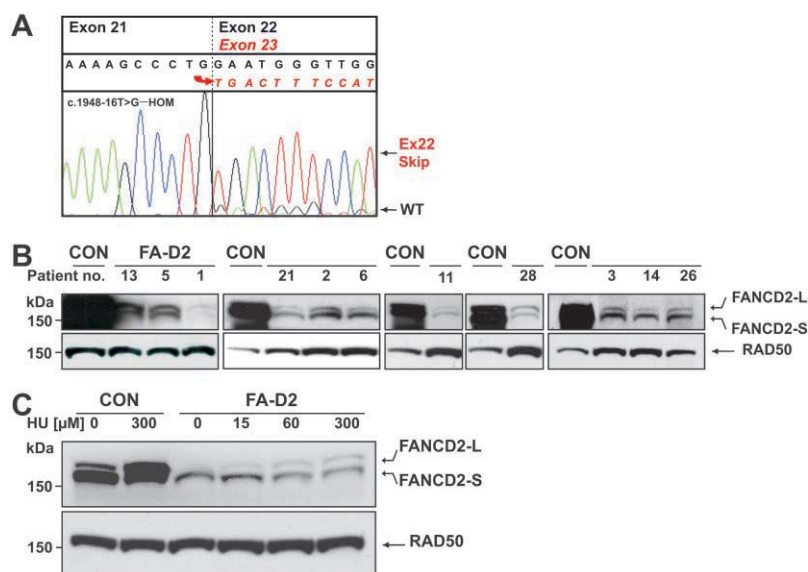
Figure 8. Residual FANCD2 protein. *A,* Exon 23 sequence following that of exon 21 (exon 22 exclusion, aberrant splicing), which prevails in cDNA from homozygotes for the splice-acceptor mutation in intron 21, c.1948-16T→G, but, at low level, underlying sequence is readable as exon 22 (exon inclusion). 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.

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]).

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)

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