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Identification of the Fanconi anemia complementation group I gene, *FANCI*

Josephine C. Dorsman [a,‡], Marieke Levitus [a,‡,†], Davy Rockx [a], Martin A. Rooimans [a], Anneke B. Oostra [a], Anneke Haitjema [a], Sietske T. Bakker [a], Jûrgen Steltenpool [a], Dezsö Schuler [b], Sheila Mohan [c], Detlev Schindler [d], Fré Arwert [a], Gerard Pals [a], Christopher G. Mathew [e], Quinten Waisfisz [a], Johan P. de Winter [a] and Hans Joenje [a,*]

[a] *Department of Clinical Genetics, Vrije Universiteit Medical Center, Van der Boechorststraat 7, NL-1081 BT Amsterdam, The Netherlands*
[b] *National Institute of Child Health, Tüzoltó Str. 709, H-1094 Budapest, Hungary*
[c] *Paediatric Haematology and Oncology, Apollo Specialty Hospital, 320 Padma Complex, Anna Salai, Chennai, 600 035, India*
[d] *Department of Human Genetics, University of Würzburg, D-97074 Würzburg, Germany*
[e] *Complex Disease Genetics Group, Department of Medical and Molecular Genetics, King's College London School of Medicine, Guy's Hospital, London SE1 9RT, UK*

Abstract. To identify the gene underlying Fanconi anemia (FA) complementation group I we studied informative FA-I families by a genome-wide linkage analysis, which resulted in 4 candidate regions together encompassing 351 genes. Candidates were selected via bioinformatics and data mining on the basis of their resemblance to other FA genes/proteins acting in the FA pathway, such as: degree of evolutionary conservation, presence of nuclear localization signals and pattern of tissue-dependent expression. We found a candidate, *KIAA1794* on chromosome 15q25-26, to be mutated in 8 affected individuals previously assigned to complementation group I. Western blots of endogenous FANCI indicated that functionally active KIAA1794 protein is lacking in FA-I individuals. Knock-down of *KIAA1794* expression by siRNA in HeLa cells caused excessive chromosomal breakage induced by mitomycin C, a hallmark of FA cells. Furthermore, phenotypic reversion of a patient-derived cell line was associated with a secondary genetic alteration at the *KIAA1794* locus. These data add up to two conclusions. First, *KIAA1794* is a FA gene. Second, this gene is identical to *FANCI*, since the patient cell lines found mutated in this study included the reference cell line for group I, EUFA592.

Keywords: Data mining, FANCI, Fanconi anemia, gene identification, positional cloning

1. Introduction

Fanconi anemia (FA) is a genetically heterogeneous chromosomal instability disorder with both autosomal and X-linked recessive inheritance and characterized by developmental abnormalities, retarded growth, bone marrow failure, and a high risk of cancer [6,8, 13]. Cells from FA patients are hypersensitive to cross-linking agents such as mitomycin C (MMC) and cisplatin. Thirteen complementation groups are currently distinguished, all of which – except group I – have been linked to distinct disease genes [8,12,13,16]. All FA proteins are supposed to function in the FA pathway of genomic maintenance. Most of these proteins assemble into a multiprotein core complex which is required for modification of FANCD2 by monoubiquitination [2]. FANCJ/BRIP1, FANCD1/BRCA2 and FANCN/PALB2 are supposed to act downstream of this modification step, because FANCD2 ubiquitination appears normal in cells lacking these proteins. Patient cell lines of complementation group I (FA-I cells) are deficient in FANCD2 ubiquitination and are characterized by a defect in the association of FANCD2 with chromatin [7,8]. Here we report the identification of the gene that causes FA in complementation group I patients.

‡These authors contributed equally to this work.
†Present address: Department of Clinical Chemistry, Vrije Universiteit Medical Center, PO Box 7057, NL-1007 MB Amsterdam, The Netherlands.
*Corresponding author. E-mail: h.joenje@vumc.nl.

2. Materials and methods

2.1. Patients, cell lines, and controls

The 4 FA-I cell lines, EUFA592, EUFA816, BD952 and EUFA961, which were all hypersensitive to growth inhibition by mitomycin C, have previously been assigned to complementation group I [7]. Following the same methods [7] patients EUFA695 and EUFA1399 were subsequently classified as FA-I based on the lack of complementation after hybridization with FA-I lymphoblasts; hybrids were checked for ploidy to exclude lack of complementation due to loss of complementing chromosomes (unpublished results). Patients and families analyzed in this study are summarized in Fig. 1. Clinical features of the patients are summarized in Table 1. Control DNA samples were isolated from blood samples obtained from The Netherlands Blood Transfusion Service; the donors were healthy and unselected for ethnic background. This study was carried out after approval by the Institutional Review Board (VU University Medical Center) and consent was obtained from the subjects involved.

2.2. Genome-wide scan

The genome-wide scan for genetic linkage was performed using the Applied Biosystems microsatellite polymorphism linkage mapping kit MD10 and the Weber 6B Screening set, in accordance with the manufacturer's protocols and performed with the GeneAmp PCR system 9700 (Applied Biosystems). Combination

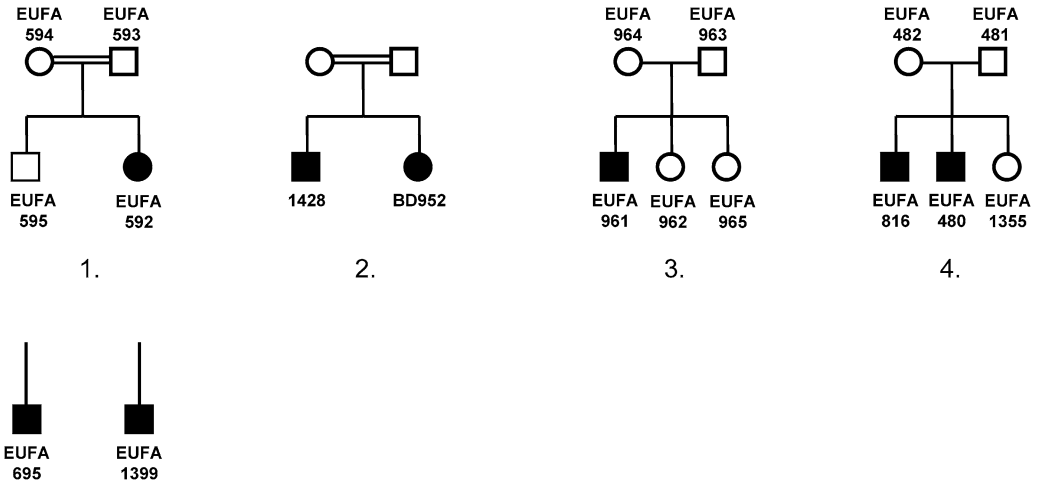

Fig. 1. FA-I families and patients analyzed in this study. Filled-in symbols are the affected individuals. Families 1–4 were used to delineate the candidate regions for *FANCI* by homozygosity mapping (patients EUFA592, BD952, and 1428 in consanguineous families 1 and 2) and linkage analysis (families 3 and 4). DNA samples from both parents of individuals BD952 and EUFA695, and from the father of EUFA1399 were not available.

Table 1
Clinical features of the FA-I individuals studied

Patient ID	Retarded growth	Thumb/radius anomalies	Kidney/heart anomalies	Onset marrow failure (yr)	Age at death (yr)	Comments
EUFA592	Yes	Yes	Yes	2.5	6.5	Consanguineous
BD952	Yes	No	No	7.3	23	Consanguineous
1428	Yes	No	No	7.3	15	Consanguineous
EUFA695					12	No details known[a]
EUFA816	Yes	No	No	6	12	Died after transplant
EUFA480	Yes	No	No	4.8		Age 24, transplanted at age 8.5 yr
EUFA961	Yes	Yes	Yes	8	21	Died after transplant
EUFA1399	Yes	Yes	Yes	1		Age 30.5 yr

[a]Clinical details for this patient, including cause of death, could not be filed. Lymphoblasts were hypersensitive to mitomycin C and used for cell fusion analysis leading to classification as FA-I.

of both sets, each of which is composed of approximately 400 markers (10 cM apart, on average), results in an average marker spacing of 5 cM. Samples were analyzed on ABI 3730 DNA Analyzer (Applied Biosystems). Genomic DNA was isolated from whole blood or lymphoblastoid cell lines from patients and family members, using a Qiagen Blood mini kit (Qiagen). The genomic DNA of patient EUFA480 was isolated from hair follicles using a 2 h incubation with proteinase K and a Qiagen Blood mini kit. Due to the lack of sufficient DNA whole genome amplification was carried out on DNA from patients EUFA480 and 1428, using the GenomiPhi DNA amplification kit (Amersham Biosciences).

2.3. Determination of candidate regions

As a first step, the initial genome-wide genetic linkage analysis with the patients EUFA592 (and parents) and BD952 from consanguineous families 1 and 2 (Fig. 1) and the parents and affected individuals of the multiplex family 4 yielded candidate regions on chromosome 2, 4, 6, 7, 8, 15, 16, 17 and 18. In the next step, these regions were further analyzed using patient 1428 from family 2, patient EUFA1355 from family 4, and all individuals of family 3. This resulted in the identification of 4 candidate regions. The following potential candidate known genes were sequenced: the kinase DBF4/ASK (on chromosome 7q21.3) for its role in replication initiation and S-phase progression; the putative E2 ubiquitin conjugating enzyme FLJ11011 (on chromosome 8q21.11) for its interaction with FANCD2 in Drosophila (FlyGrid); the aprataxin like HIT domain containing hydrolase LOC390637 (on chromosome 15q26.1) for its putative role in DNA repair; the RING finger Nse1 (on chromosome 16p12.1), for its role in DNA damage response as part of the SMC5/6 complex; the RING finger RNF40 (on chromosome 16p11.2), for its putative function as E3 ubiquitin ligase; and the vitamin K epoxide reductase complex subunit 1 (VKORC1 on chromosome 16p11.2), for its presence in a cDNA expression library-'complemented' FA-I cell line.

In DBF4/ASK and VKORC1 heterozygous polymorphisms were found in the consanguineous patient EUFA592, which narrowed down the candidate region on chromosome 16. Polymorphisms described in LOC390637 were found to be homozygous in both consanguineous patients BD952 and EUFA592, strengthening the idea that this was a candidate region.

Because of the degree of consanguinity in the parents (first cousins) relatively large candidate regions were to be expected in the single consanguineous patients. Relatively large regions in at least one of the consanguineous patients that were compatible with linkage in the additional families and family members helped to define the candidate regions. Thus the regions on chromosomes 7q, 15q, 16q and 17q were identified as best candidate regions for further analysis.

2.4. Bioinformatics and data mining

The positions of DNA markers were identified via NCBI map viewer (option STS; http://www.ncbi.nlm.nih.gov/mapview/maps) followed by gene identification in the relevant regions (option Gene). In these regions, first known genes were selected and excluded (see Section 2.3). For the genes with unknown function, the following strategy was used. Proteins were first selected for which mouse proteins exist with a 50–85% identity with the human amino acid sequence (http://www.ncbi.nlm.nih.gov/sutils/blink.cgi). Pseudogenes were not further analysed. This yielded 11 proteins, 3 of which were excluded based on unlikely properties. The remaining 8 proteins (KIAA1794/NP_060663, C15Orf42/NP_689472, NP_064597.1, NP_859058.1, NP 001013679.2, NP 073581.1, XP 933746.1, and XP 934096) were subjected to a WoLFPSort and NUCDISC search (wolfpsort.org) and those were selected for which the nucleus was the most likely location and which contained at least 1 putative nuclear localization signal (NLS: pat4, pat7 or bipartite): KIAA1794/NP_060663, C15Orf42/NP_689472, NP_064597.1, and NP_859058.1. These 4 genes/proteins – with a focus on the two highest rankers of the pSORT analysis – were compared for (1) degree of evolutionary conservation, (2) orphan status, (3) mRNA expression patterns in normal tissues (e.g. http://bioinfo2.weizmann.ac.il/cgi-bin/genenote/home_page.pl), (4) protein modification motifs (phosphorylation motifs: http://www.cbs.dtu.dk/services/NetPhos/) and (5) protein motifs/domains (http://smart.embl-heidelberg.de/). KIAA1794 is an orphan protein displaying a similar conservation as FANCD2 (human *versus* mouse: ~75%; both genes are present in Drosophila). It showed an expected expression pattern for an FA gene (low and ubiquitous, but relatively prevalent in bone marrow and thymus, which is the same pattern found for FANCM) and contained 3 ATM/ATR motifs. Although C15orf42 was less conserved in the mouse than

KIAA1794 (68% *versus* 75%), displayed a higher level of expression than usually found for FA genes and contained 1 ATM/ATR motif, both candidates were sequenced.

2.5. Amplification of KIAA1794/FANCI sequences

Primer sequences for amplification of FANCI cDNA and genomic DNA and PCR conditions are available upon request. Total RNA was isolated from lymphoblasts EUFA592, BD952, EUFA816, EUFA961 and EUFA1399 using the High Pure RNA Isolation Kit (Roche Applied Science), from which cDNA was prepared using iScript^{TM} cDNA Synthesis Kit (BioRad Laboratories). The PCR reactions for amplification of *FANCI* were performed on cDNA using Platinum Taq polymerase (Invitrogen) and sequenced as described below.

2.6. Sequencing of KIAA1794/FANCI

PCR products were processed using a SAP/EXO treatment (Amersham Biosciences) according to the manufacturer's instructions. Sequencing reactions were prepared using specific primers and Big Dye terminator cycle sequencing kit (Applied Biosystems). Samples were analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems).

2.7. Western blot

Whole-cell extracts of lymphoblastoid cells were prepared in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl and 1% NP40 supplemented with protease and phosphatase inhibitors). Extracts of siRNA treated HeLa cells were prepared in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40 and 0.1% SDS supplemented with protease and phosphatase inhibitors). Equivalents of 600,000 lymphoblastoid cells (56–84 μg protein) or 150,000 HeLa cells (29–54 μg protein) were loaded on a 3–8% Tris-Acetate NuPAGE gradient gel (Invitrogen) and proteins were separated at 150 V during 3.5 h, according to the manufacturer's protocol. Proteins were transferred to Immobilon-P Transfer membranes (Millipore) and the membranes were blocked with 5% dry milk in TBST (10 mM Tris HCL pH 8.0, 150 mM NaCl, 0.05% Tween-20). The membranes were incubated with a rabbit polyclonal antiserum against KIAA1794/FLJ10719 amino acids 450–500 (Bethyl laboratories; A300-213A). After washing with TBST, the membranes were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulins (DAKO). Proteins were visualized with ECL plus Western blotting detection system (Amersham Biosciences). FANCD2 was visualized by using mouse monoclonal antibody FL-17 (Santa Cruz Biotechnology) and peroxidase-conjugated goat anti-mouse immunoglobulins (DAKO).

2.8. siRNA experiments and chromosomal breakage test

HeLa cells were transfected with ON-TARGETplus SMARTpool siRNA oligos (Dharmacon) against KIAA1794 (L-022320-01) or FAAP24 (L-016958-00) as described in [11]. ON-TARGETplus siCONTROL (D-001810-10) was used as a non targeting pool of control oligos. Metaphases of transfected HeLa cells were evaluated for chromatid-type aberrations, essentially as described in [5]. At the same time as the metaphase spreads were made, cells were harvested for KIAA1794 and FANCD2 western blot analysis.

3. Results and discussion

A two-step genome-wide linkage approach involving 4 genetically informative families, including two first-cousin marriages (Fig. 1 and Table 1), resulted in 4 candidate regions that were compatible with linkage and were considered most likely to harbour the *FANCI* gene: on chromosome 7q between markers D7S2204 and D7S820 (5.6 Mb, 8.6 cM, 12 genes), on 15q between D15S653 and D15S652 (7.1 Mb, 10.5 cM, 79 genes), on 16q between VKORC1 and D16S3105 (14.4 Mb, 1.5 cM, 102 genes) and on 17q between D17S1290 and D17S2059 (12.3 Mb, 15.3 cM, 158 genes), together encompassing 39.4 Mb of genomic DNA and 351 genes.

We identified in candidate regions 6 known genes connected with DNA repair/chromatin and with cellular roles compatible with the FA cellular phenotype. After excluding those genes by DNA sequencing we selected novel genes via data mining and bioinformatics incorporating known features of already identified FA proteins. Most human FA genes typically encode orphan proteins, whose mouse orthologs displayed a 50 to ∼80% amino acid identity. We first selected genes according to evolutionary conservation, which resulted in 8 candidates. We next selected on the basis of predicted nuclear localization. This selection resulted in 4 candidates: with the two high-

est ranking being KIAA1794/NP_060663 and C15orf 42/NP_689472, both on chromosome 15. For further details of the analysis, see Section 2.

Sequence analysis of *KIAA1794* in 8 FA individuals assigned to complementation group FA-I revealed mutations in all these affected individuals (Table 2). *KIAA1794*, which is localized to 15q25-26, has 38 exons with a translation start in exon 2, encoding a 1328 amino acid (146 kDa) protein with 3 predicted nuclear localization and 3 predicted ATM/ATR phosphorylation motifs.

As expected, mutations appeared homozygous in the patients from consanguineous marriages. In the affected individual from the first family (EUFA592), the reference case for complementation group I, a homozygous mutation c.2T>C was found, which eliminates the translation initiation site of the gene; the unaffected sib was homozygous for the normal allele. This mutation was absent in a panel of 96 healthy controls. Patients BD952 and 1428 from the second family were homozygous for two missense variants, c.164C>T (in exon 4) and c.3854G>A (in exon 36), resulting in a Proline to Leucine substitution at aa position 55 and an Arginine to Glutamine substitution at aa position 1285, respectively (Table 2). We tested 96 healthy individuals for the occurrence of these variants and found c.164C>T heterozygously present in 9 individuals, indicating that this variant must be a polymorphism. Variant c.3854G>A, which was not detected in the control panel nor in any public database, creates an additional putative ATM/ATR phosphorylation motif, which might disturb the protein's proper regulatory response. In addition, the affected amino acid Arg1285 is conserved in mammals, chicken, zebrafish, Drosophila, and Arabidopsis orthologs of KIAA1794. Although c.3854G>A therefore seems compatible with a pathogenic mutation, definite proof for its pathogenicity would require more detailed functional studies.

Additional mutations encountered in the remaining affected individuals included three (partial) exon deletions, one inserted exon, three protein truncations, and one amino acid substitution (Table 2). None of these mutations was encountered in the panel of 96 healthy controls. As some of these alterations predicted changes in protein expression, we used western blotting to visualize the protein. A band of approximately 150 kDa was detected in extracts from wild type cells and FA-I individual BD952, but this band was completely absent in EUFA592, EUFA695, and EUFA816 (Fig. 2a). These data indicate that FA-I individuals lack functionally active KIAA1794 protein.

Cell lines derived from FA-I individuals are deficient in FANCD2 ubiquitination [7,8] and are hypersensitive to cross-linking agents, such as mitomycin C. To verify that deficiency of KIAA1794 causes a FA-

Table 2
Sequence variants at the *KIAA1794* locus in FA-I patients[a]

		Maternal allele		Paternal allele	
Patient ID	Origin	DNA	Effect	DNA	Effect
EUFA592[b]	Turkey	c.2T>C	p.Met1?	c.2T>C	p.Met1?
BD952[b]	India	c.164C>T[a]	p.Pro55Leu	c.164C>T	p.Pro55Leu
		c.3854G>A	p.Arg1285Gln	c.3854G>A	p.Arg1285Gln
EUFA695	USA	c.3006+3A>G[c]	p.Arg964_Gln1002del	c.1264G>C	p.Gly422Arg
EUFA816	Hungary	c.3853C>T	p.Arg1285X	c.3350-88A>G[d]	p.Glu1117fs
EUFA961	Austria	c.3437_3455del[e]	p.Glu1117fs	c.2572C>T[f]	p.His858_Arg879del
EUFA1399	Germany	c.3895C>T	p.Arg1299X	c.3895C>T[g]	p.Arg1299X

[a]Description of variants refers to isoform 3 of KIAA1794 (Q9NVI1-3; http://www.expasy.org/uniprot). Mutations were found by cDNA sequencing, followed by sequencing of the genomic DNA. Variant c.164C>T was observed in 9/96 healthy controls (Dutch blood donors) and was therefore considered a polymorphism. The other sequence variants were considered possibly pathogenic, since none was observed in the controls nor in the public databases that list common polymorphisms.

[b]Consanguineous marriages (parents are first cousins).

[c]Changes splice donor site score from 0.92 to 0.43, which results in in-frame deletion of exon 27 (http://www.fruitfly.org/cgi-bin/seq_tools/splice.html).

[d]Generates a new splice donor site resulting in an additional exon (see Fig. 2e).

[e]This 19-base pair deletion leads to skipping of the entire exon 32.

[f]Creates splice donor site in exon 24 leading to an in-frame deletion of base pairs 2571 to 2636 from the cDNA.

[g] The maternal mutation appeared homozygous in the patient, but hemizygosity cannot be excluded, since DNA from the father was not available for analysis.

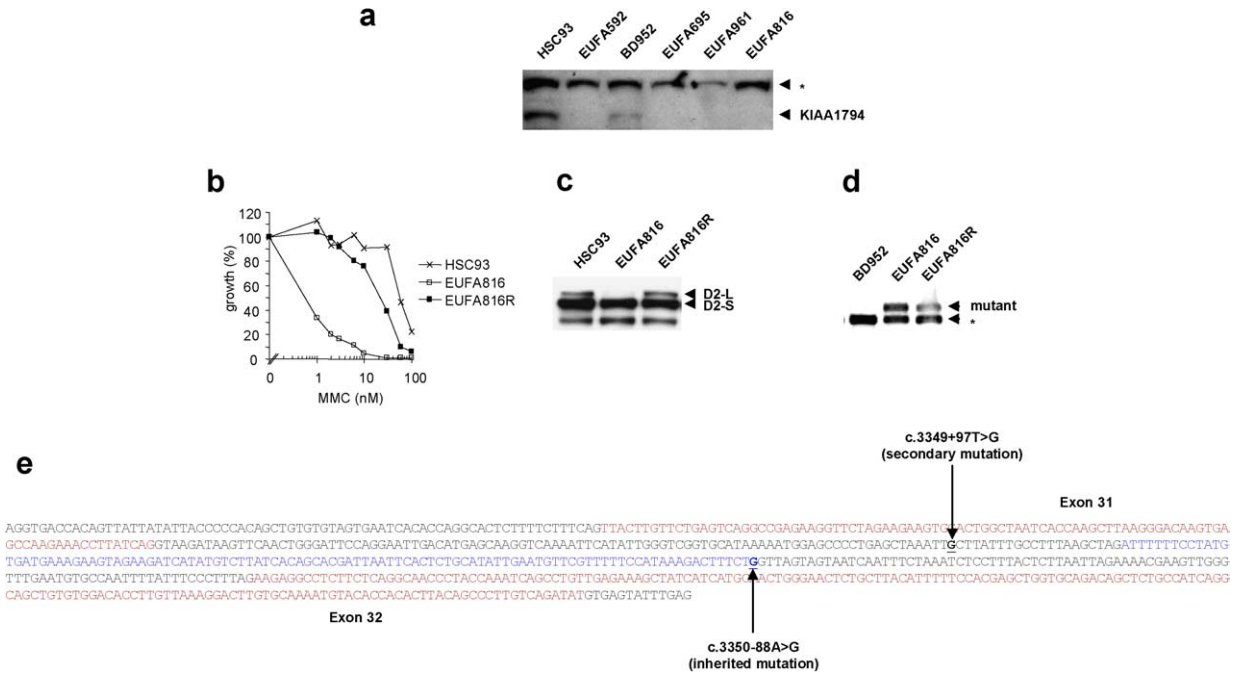

Fig. 2. Consequences of genomic sequence alterations at the *KIAA1794* locus for the encoded protein and cellular phenotype. (a) Western blot showing absence of full-length KIAA1794 protein in extracts from lymphoblasts derived from FA-I individuals, except for individual BD952 carrying a missense mutation. HSC93, wild type lymphoblasts. The upper arrow head points to an aspecific band, which may serve as a loading control. According to molecular weight markers, the KIAA1794 protein band is at approximately 150 kDa. (b) Compensatory sequence alteration in *KIAA1794* is associated with phenotypic reversion of FA-I lymphoblasts. A subline of lymphoblasts derived from patient EUFA816 was phenotypically reverted to mitomycin C (MMC) resistance (EUFA816R), as shown by MMC-induced growth inhibition curves; HSC93, wild type lymphoblasts. (c) Monoubiquitination of FANCD2 (formation of D2-L) is absent in EUFA816 lymphoblasts, but restored in the reverted cells. D2-S, non-ubiquitinated form of FANCD2. (d) Amplification of base pairs 3019 to 3765 of the KIAA1794 cDNA, encompassing exons 31 and 32, generated an extra (larger) fragment in EUFA816, which appeared weaker in the reverted cells, EUFA816R. Patient BD952 (who carries no mutations in the amplified region) served as a control. The lower band in EUFA816 cells (marked with asterisk) represents the other allele containing the premature stop mutation c.3853C>T in exon 37 (Table 2), whereas in the EUFA816R cells the lower band represents a mixture of the premature stop-containing allele and the wild type allele, due to an acquired secondary sequence alteration. (e) Genomic sequence showing exon 31 and 32 (red) and the additional exon (blue) resulting from the pathogenic mutation c.3350-88A>G (lower arrow) which creates a splice donor site with a score of 0.85 according to http://www.fruitfly.org/cgi-bin/seq_tools/splice.html. In EUFA816R cells a second mutation was observed (c.3349+97T>G, upper arrow), which reduces the splice acceptor score for the additional exon from 0.45 to 0.33, allowing the normal splicing to take place with a probability that appears sufficient to correct the cellular phenotype.

like cellular phenotype, we knocked down its expression in HeLa cells. As shown in Fig. 3a and 3b, transfection with gene-specific oligonucleotides reduced KIAA1794 protein levels, which was associated with loss of FANCD2 monoubiquitination and increased chromosomal instability. The extent of spontaneous and MMC-induced chromosomal breakage caused by KIAA1794 knock-down was essentially similar to that observed with the gene-specific knockdown of FAAP24, a recently discovered FA protein core complex component [1].

In EUFA816 the maternal allele contained a premature stop (c.3853C>T) in exon 37, whereas the paternal allele carried a mutation (c.3350-88A>G) in intron 31 resulting in abnormal splicing, introducing an aberrant exon in the transcript. From this individual a lymphoblastoid subline had been obtained that was phenotypically reverted to MMC resistance, while these cells had regained their capacity to monoubiquitinate FANCD2 (Fig. 2b and c). Phenotypic reversion has previously been noted in mosaic patients from various complementation groups [3,4,9,10,14,15]. In such cases reversion invariably was associated with a secondary genetic alteration at the disease locus that resulted in restoration of a functional gene product. When investigating cDNA-amplified fragments from MMC-sensitive EUFA816 cells we noted that amplification of the sequence encompassing exons 31 and 32 generated an additional larger fragment, which appeared weaker in the reverted cells (Fig. 2d). Sequenc-

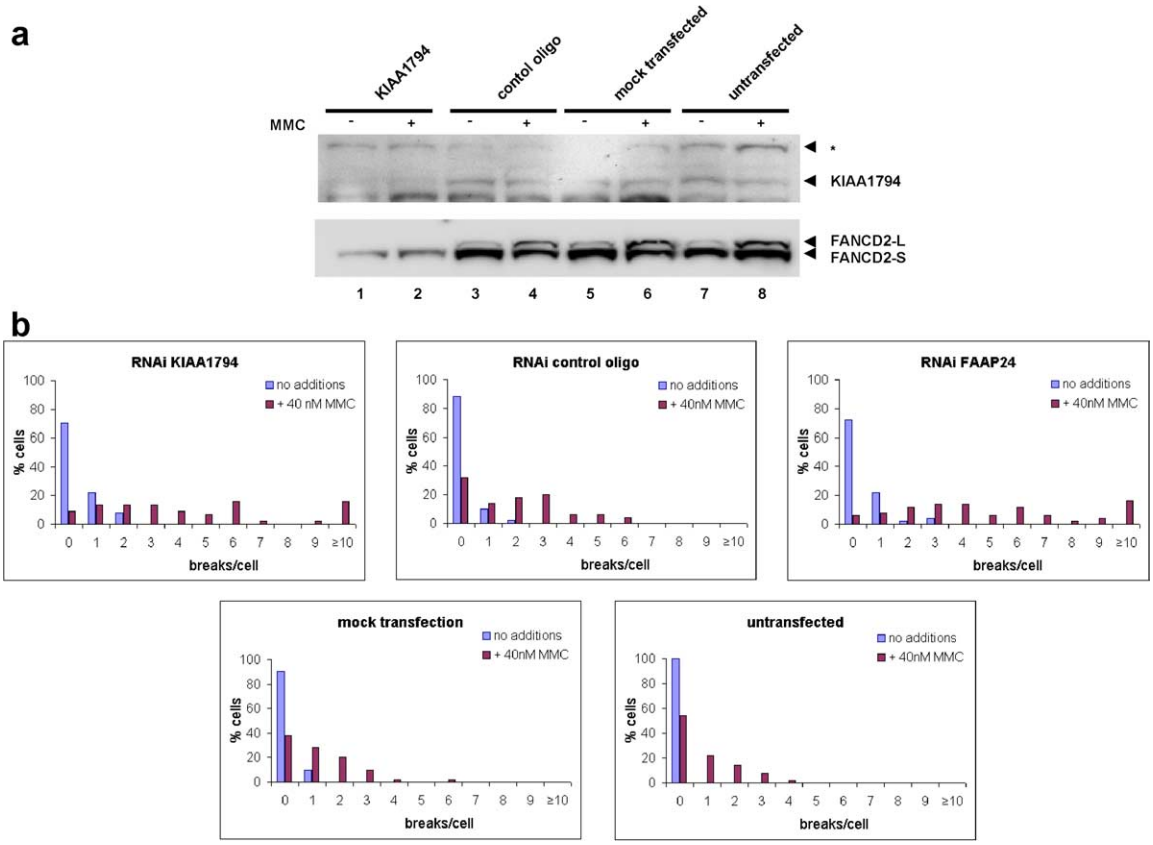

Fig. 3. Effect of KIAA1794 knockdown on FANCD2 ubiquitination and chromosomal instability in HeLa cells. (a) Effect of KIAA1794-specific siRNA oligo transfection (lane 1 and 2) on KIAA1794 protein levels and FANCD2 monoubiquitination (lanes 3–8 are controls, as indicated). Cells were treated with MMC (+) or not treated (−). FANCD2 was visualized to assess its mono-ubiquitinated isoform, FANCD2-L, which is increased upon MMC treatment. FANCD2-L was not detected in lanes 1 and 2, not even after extended exposure times. (b) Chromosomal breakage analysis in the corresponding HeLa cell cultures, with or without exposure to MMC (40 nM, 24 h). Knockdown of FAAP24, a known functional component of the FA core complex was included as a positive control [1]. The increases in MMC-induced chromosomal breakage, as caused by KIAA1794-specific oligo transfection and by FAAP24-specific oligo transfection, were equally significant (compared with control oligo-transfected cells; $p < 0.0001$, 2-sample χ^2 test).

ing of the cDNA from the reverted cells showed partial restoration of normal splicing. Furthermore, by sequencing genomic DNA we found an additional mutation in intron 31 (c.3349+97T>G), which reduced the splice acceptor score for the aberrant exon (Fig. 2e). This indicated that phenotypic reversion was associated with a secondary DNA alteration at the locus under study, implicating this locus as the disease gene for this individual. Based on this evidence, taken together with the mutational data presented for the other FA-I individuals including the reference FA-I case, the absence of KIAA1794 protein in cell lines derived from them, and the FA-like cellular phenotype induced in HeLa cells upon siRNA-mediated knock-down of KIAA1794 expression, we conclude that *KIAA1794* is the disease-causing gene in FA complementation group I, *FANCI.*

A striking feature of FA-I cells is their apparent deficiency in the association of FANCD2 with chromatin [8]. FANCI possesses several SQD/SQE motifs for ATM- or ATR-induced phosphorylation in its C-terminal domain, a feature that suggests a role in a DNA damage response. Interestingly, the splice site mutation in patient EUFA695 results in an in-frame deletion of exon 27, encoding one of the SQE motifs, while the missense mutation in BD952 creates an additional SQD motif. FANCI could thus be a signal-regulated localizer of FANCD2. Further characterization of FANCI's molecular function may reveal how this protein is involved in the recruitment of FANCD2 to chromatin sites where the FA pathway acts to maintain chromosomal stability.

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Competing interests statement

The authors declare that they have no competing financial interests.

Note added in proof

A paper has been published (A. Smogorzewska et al., Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair, *Cell*, April 5, 2007, E-publication ahead of print) in which KIAA1794 is claimed to be identical with FANCI, based on a pathogenic mutation found in only one FA cell line, BD952. However, this conclusion is premature, since mutations were not demonstrated in the reference cell line for complementation group I, EUFA592. The present report is therefore the first to unequivocally demonstrate that KIAA1794 is identical with FANCI.

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