Fanconi anemia (FA) is a recessive genetic disease characterized by congenital abnormalities, chromosome instability, progressive bone marrow failure (BMF), and a strong predisposition to cancer. Twenty FA genes have been identified, and the FANC proteins they encode cooperate in a common pathway that regulates DNA crosslink repair and replication fork stability. We identified a child with severe BMF who harbored biallelic inactivating mutations of the translesion DNA synthesis (TLS) gene REV7 (also known as MAD2L2), which encodes the mutant REV7 protein REV7-V85E. Patient-derived cells demonstrated an extended FA phenotype, which included increased chromosome breaks and G₂/M accumulation upon exposure to DNA crosslinking agents, γH2AX and 53BP1 foci accumulation, and enhanced p53/p21 activation relative to cells derived from healthy patients. Expression of WT REV7 restored normal cellular and functional phenotypes in the patient’s cells, and CRISPR/Cas9 inactivation of REV7 in a non-FA human cell line produced an FA phenotype. Finally, silencing Rev7 in primary hematopoietic cells impaired progenitor function, suggesting that the DNA repair defect underlies the development of BMF in FA. Taken together, our genetic and functional analyses identified REV7 as a previously undescribed FA gene, which we term FANCV.
BRIEF REPORT

Biallelic inactivation of REV7 is associated with Fanconi anemia

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The authors have declared that no conflict of interest exists.

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Introduction

Fanconi anemia (FA) is the most frequent cause of inherited bone marrow failure (IBMF) syndromes (1, 2). Twenty FA genes have been identified, including FANCA, FANCB, FANCC, FANCD1 (also known as BRCA2), FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ (also known as BRIP1 or BACH1), FANCL, FANCN, FANCQ (also known as PALB2), FANCQ (also known as RAD51C), FANCP (also known as SLX4), FANCQ (also known as XPF or ERCC4), FANCQ (also known as RAD51), FANCS (also known as BRCA1), FANCT (also known as UBE2T), and FANCU (also known as XRCC2) (3–5). The products of these genes cooperate in a unique FA/BRCA pathway, regulating the response to physiological stress or exposure to genotoxic agents and maintaining genome integrity (3–5). Most FA patients develop a progressive bone marrow failure (BMF) during childhood due to the depletion or impairment of the hematopoietic stem cell (HSC) pool (1, 2, 6–8).

Here, we identified biallelic mutations in REV7 (also known as MAD2L2) in a child with a classic presentation of FA. Interestingly, REV7 has several cellular functions including translesion DNA synthesis (TLS) (9, 10), mitotic checkpoint regulation (11, 12), and DNA repair pathway choice (13, 14). Which if any of these functions of REV7 is required for suppressing the FA cellular and developmental phenotypes is unknown.

Results and Discussion

A child with a clinical and cellular FA phenotype and a constitutive REV7 mutation. Patient EGF123, an 8-year-old girl, presented with severe BMF involving all 3 lineages (hemoglobin, 8.0 g/dl; neutrophil count, 0.43 × 10^9/l; and platelets, 10 × 10^9/l). She exhibited FA physical signs (short size at less than tenth percentile, microcephaly, and abnormal facial features), a renal tubulopathy, elevated serum α-fetoprotein, and a positive mitomycine C (MMC) chromosome breakage test of blood lymphocytes (15), establishing a definitive diagnosis of FA (Figure 1A). Monoubiquitination of the FANCD2 protein in the patient cells suggested an abnormality downstream or independent of the FA core complex (Figure 1B) (16). Functional analysis of the patient’s skin fibroblasts and EBV-transformed lymphoid cells confirmed the FA phenotype (i.e., a hypersensitivity to the interstrand-crosslinking [ICL] agent MMC), with increased chromosome radials (Figure 1C), arrest at the G2 phase of the cell cycle, and growth inhibition (Figure 1, D and E). Consistent with previous studies with FA cells (7, 17), MMC exposure strongly activated Ser15 phosphorylation of p53 and CDK-NIA (also known as p21) transcriptional induction (Figure 1, F and G).

Sequencing of known FANC genes failed to identify a deleterious nucleotide variant or deletion. Whole exome sequencing (WES) on genomic DNA from the EGF123 proband identified a homozygous REV7 variant, c.354T>A. Sanger resequencing confirmed the homozygous variant in EFG123 skin fibroblast and EBV-transformed cells (Figure 1H). Rare regions of germline homozygosity (which included the REV7 gene) were observed in this patient, consistent with distant consanguinity (Supplemental

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Crispr/Cas9-mediated knockout of REV7 recapitulates an FA phenotype. To confirm that loss of REV7 directly causes the FA phenotype, we used Crispr/Cas9-mediated gene editing to generate a homozygous knockout in U2OS cells (denoted REV7–/–) (Figure 3). Western blotting confirmed the absence of detectable REV7 protein in several tissues, despite normal transcript levels, suggested that protein destabilization may result from the mutation (Figure 1F).

Lentiviral complementation of REV7 restores cellular and functional phenotype in patient cells. To provide additional evidence for the disease causality of the REV7 mutation, we lentivirally reexpressed a WT REV7 cDNA in patient fibroblasts and EBV-transformed cells. Western blot analysis of whole cell lysates revealed that REV7 expression was restored in REV7-transduced cells (Figure 2A). Reexpression of the WT REV7 fully rescued chromosome breakage, cell cycle arrest, and cell proliferation defects (Figure 2, B–D and Supplemental Figure 1). Ser15 phosphorylation of p53 and phosphorylated γH2ax levels was also decreased in the REV7 complemented cells, and transcript analysis revealed a decrease of CDKN1A (Figure 2, A and E). Moreover, EGF123 cells exhibited an excess of γH2AX and 53BP1 foci, suggesting unresolved DNA damage at steady state and upon MMC exposure, and these defects were resolved by REV7 reexpression (Figure 2, A, F, and G, and Supplemental Figure 4).

Figure 1; supplemental material available online with this article; doi:10.1172/JCI88010DS1). The c.354 C>T REV7 is a variant based on a survey of publicly accessible variant databases. The mutation affects an amino acid at a highly conserved position, resulting in an amino acid substitution, p.V85E, predicted to be pathogenic by different tools. The substitution was located in the highly conserved HORMA domain of the protein (Supplemental Figure 2), known to mediate the REV7 interaction with REV1 and REV3 (10). Moreover, the absence of detectable levels of REV7 protein in several tissues, despite normal transcript levels, suggested that protein destabilization may result from the mutation (Figure 1F).

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FA cells are hypersensitive to DNA ICL agents and exhibit DNA repair and replication fork defects. How a deficiency in REV7 contributes to these cellular phenotypes remains unknown. Previous studies have indicated that FA proteins are required for normal hematopoiesis and hematopoietic progenitor cell (HPC) survival (6–8, 18). To evaluate whether REV7 deficiency can affect hematopoiesis, we silenced this gene in mouse HPCs. Rev7 knockdown impaired the ability of HPCs to form in vitro CFU in short-term methylcellulose culture, similar to the defect observed in Fancg–/– HPCs (Figure 3G). In addition, colonies had a decreased proportion of c-Kit+/progenitor cells and an increased proportion of CD11b+/differentiated cells (Figure 3H). Together, these data suggest that the Rev7 defect directly alters HPCs, leading to increased lineage engagement, consistent with a DNA damage–mediated mechanism of BMF.

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nuclei, indicating accumulation of DNA damage (31). The Rev7-deficient mice, therefore, have a very similar defect in development, embryonic lethality, infertility, and DNA damage accumulation, which is also observed in other Fanc–/– mouse models (32).

In conclusion, these human genetic and functional data establish REV7 as a new bona fide FA gene, FANCV.

Methods

Further details can be found in the Supplemental Methods.

Samples and cell lines. This study was based on a cohort of 268 consecutive FA patients referred for medical diagnosis at the French Reference Center for Constitutional Bone Marrow Failure, Saint-Louis and Robert Debré Hospitals. All patients had an FA diagnosis based on FA tests, including the chromosomal breakage test. Primary fibroblasts were established and cells were analyzed at early passage. Genetic analyses of the FANC gene mutations were performed in fibroblast genomic DNA. EBV-transformed cell lines were produced for functional analyses (7).

Exome sequencing. All sequencing data were deposited in ArrayExpress (E-MTAB-4817).

CRISPR-Cas9 knockout. Guide RNA sequences were cloned into the pSpCas9(BB)-2A-GFP (PX458) vector, a gift from Feng Zhang (Addgene plasmid no. 48138; Massachusetts Institute of Technology, Cambridge, Massachusetts, USA.). The genomic sequence targeted for anaphase promoting complex/cyclosome (APC/C) by the activation of CDH1 (11, 12). Accordingly, a deficiency in REV7 could account for the mitotic defects and cytokinesis failure observed in FA cells (24–26). Third, REV7 participates in the cellular choice of DNA repair pathways during DNA double-strand break repair. In this role, REV7 functions downstream of 53BP1 and RIF1 and inhibits the 5’ DNA end resection, thereby promoting nonhomologous end joining (NHEJ) and suppressing homologous recombination (HR) (13, 14). A deficiency in REV7 could therefore account, at least in part, for the dysregulated levels of NHEJ repair and HR repair observed in FA cells (27, 28).

Previous work in chicken DT40 cells had demonstrated that inactivation of Rev7 confers a cell hypersensitivity to ICL agents, suggesting that REV7 may be an FA gene (29). Interestingly, 2 Rev7 deficient mouse models have been generated, Rev7–/– and Rev7C70R mice (30, 31). In one model, Rev7–/– mice exhibited growth retardation and a partial embryonic lethal phenotype, and those mice that survived to adulthood were infertile and showed germ cell aplasia in the testes and ovaries (30). In the second model, a missense mutation in Rev7C70R disrupted Pol ζ assembly, thereby impairing mouse development and the repair of genotoxic agent–induced DNA lesions (31). Rev7C70R mutant cells also showed decreased proliferation, increased apoptosis, and arrest in the S phase with extensive γH2AX foci in
CRISPR-Cas9 disruption in REV7 was GAGGCTTTGTCGTGAG-GG. U2OS cells were transduced using Lipofectamine 2000 (Invitrogen catalyst catalog 11668). Twenty-four hours after transfection, GFP - cells were selected and single cells were seeded using a BD FACsAria II cell sorter. Single cells were grown for approximately 3 weeks. Putative REV7 knockouts were identified using Western blotting. For reconstitution, WT REV7 cDNA was cloned into the pBabe-puro retroviral vector.

Statistics. Results were evaluated by performing unpaired 2-tailed \( t \) tests using GraphPad Prism version 5.0 (software GraphPad Software). Results are presented as mean ± SEM.

Study approval. Informed consent for medical diagnosis and research was obtained from the patients and their relatives. The study was approved by the Institutional Review Board of Institut Universitaire d’Hématologie (IUH) at Saint-Louis Hospital (project IUH2012-12-078). All experiments in mice were performed in accordance with a protocol approved by the Committee on the Ethics of Animal Experiments Paris-Nord (C2EA-121), project No. 2014-IUH013.

Author contributions
DB, JMP, CC, AR, RC, CDE, WC, and SG performed experiments. All authors contributed to the research. DB, JMP, ADD, and JS designed the study and wrote the manuscript. All authors approved the final manuscript.

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