

Biallelic inactivation of *REV7* is associated with Fanconi anemia

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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 FANCD 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.

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*, *FANCL* (also known as *BRIP1* or *BACH1*), *FANCL*, *FANCM*, *FANCN* (also known as *PALB2*), *FANCO* (also known as *RAD51C*), *FANCP* (also known as *SLX4*), *FANCQ* (also known as *XPF* or *ERCC4*), *FANCR* (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.

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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, $0.43 \times 10^9/l$; and platelets, $10 \times 10^9/l$). 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 mitomycin 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 *CDKN1A* (also known as *p21*) transcriptional induction (Figure 1, F and G).

Sequencing of known *FANCD* 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 EGF123 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

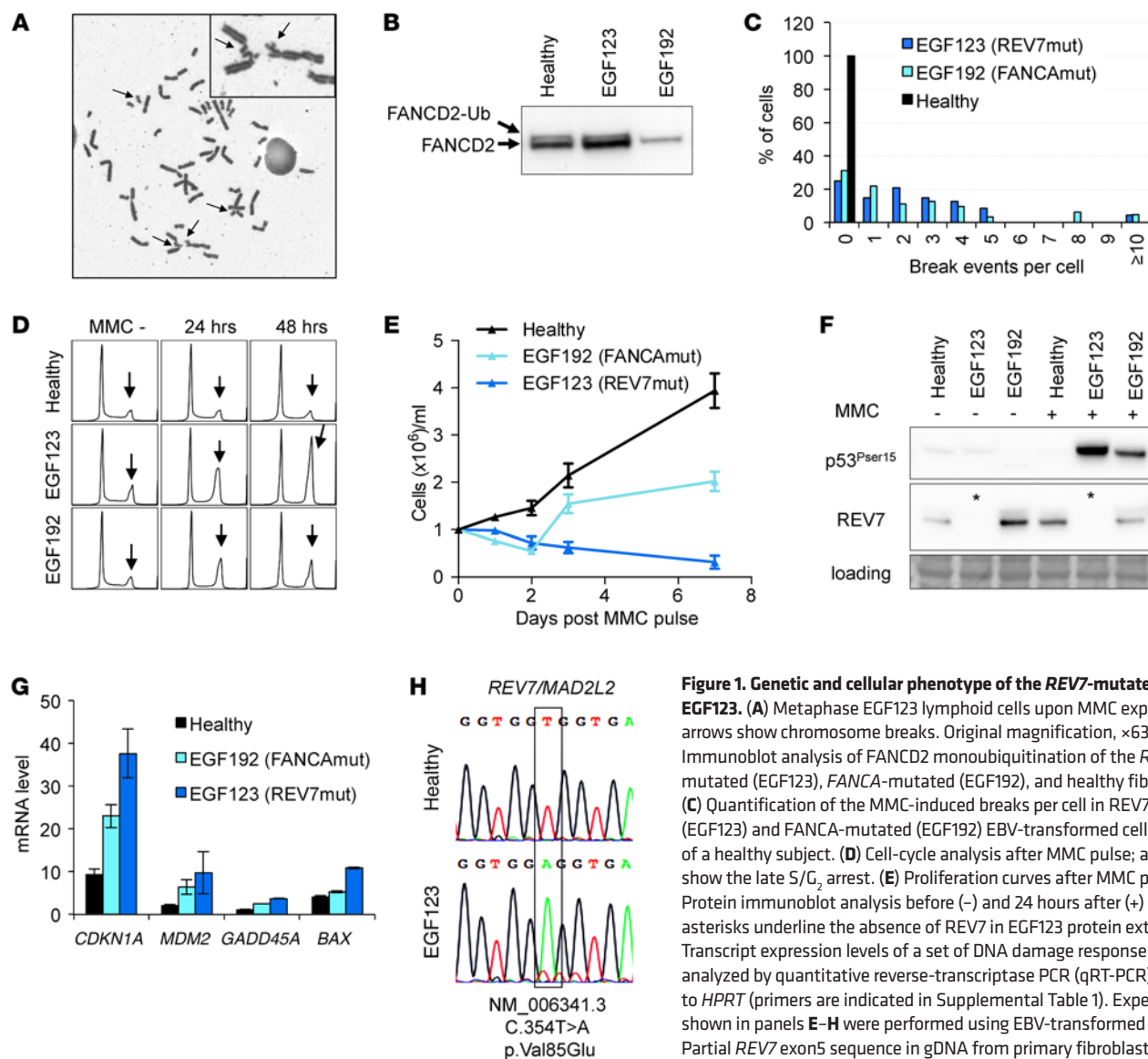


Figure 1; supplemental material available online with this article; doi:10.1172/JCI88010DS1). The c.354 C>T *REV7* is a variant based on a survey of publicly accessible variant databases. The mutation affects an amino acid at a highly conserved position, resulting in an amino acid substitution, p.V85E, 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).

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 Supple-

mental Figure 3). Ser15 phosphorylation of p53 and phosphorylated γ H2ax 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 γ H2AX 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).

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 the *REV7*^{-/-} line, whereas REV7 was readily detectable in the parental cell line (Figure 3A). Consistent with the patient-derived EGF123 cells, FANCD2 monoubiquitination was largely unaffected in *REV7*^{-/-} cells. As expected, *REV7*^{-/-} cells exhibited the hallmark FA phenotypes, including an increase in chromosome breaks and radials upon MMC treatment, cellular hypersensitivity to MMC, and a pronounced G₂/M arrest (Fig-

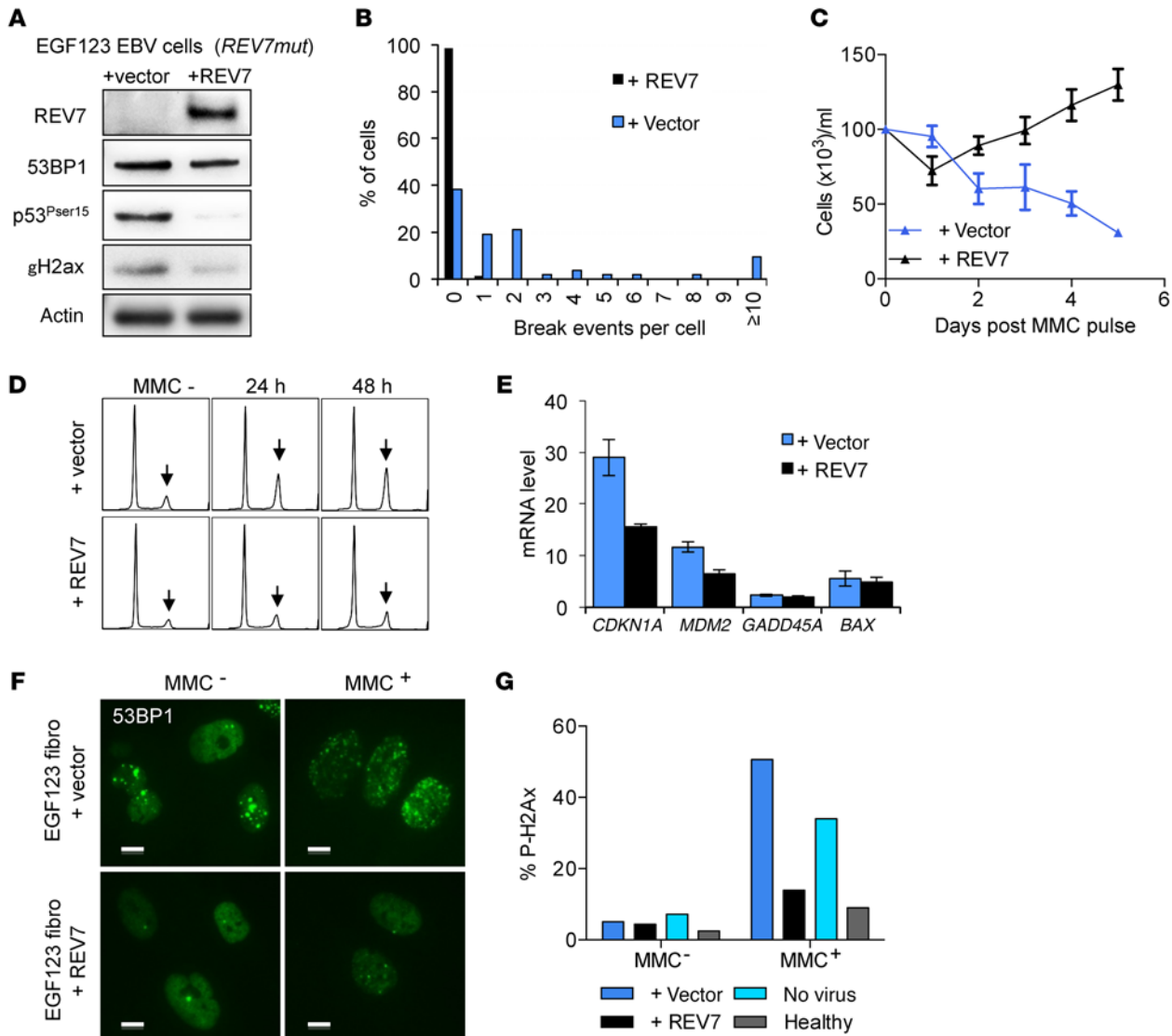


Figure 2. Correction of the extended phenotype of the EGF123 cells by WT REV7 expression. (A) Protein immunoblot analysis 24 hours after MMC pulse of EBV-transformed cells from the patient transduced with REV7 or the empty vector. (B) Quantification of MMC-induced breaks per cell. (C) Proliferation curves after MMC pulse. (D) Cell-cycle analysis after MMC pulse; arrows show late S/G₂ arrests. (E) Transcript expression levels of a set of DNA damage response genes analyzed by qRT-PCR relative to HPRT. (F) 53BP1 immunofluorescence analysis of EGF123 fibroblasts transduced with REV7 or empty vector, with or without MMC exposure. Scale bars: 10 μm. (G) Percentage of γH2AX-positive cells by flow cytometry analysis with or without MMC exposure. Experiments shown in panels B–F were performed using EBV-transformed cells.

ure 3, C–F). All of these phenotypes were corrected by retroviral transduction with WT REV7 cDNA (Figure 3, B–F).

REV7 deficiency directly impairs hematopoietic cells. 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.

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 demonstrated that the REV7 protein has several normal cellular functions. First, REV7 is a subunit of the DNA polymerase ζ complex (POL ζ), a TLS error-prone polymerase complex containing REV1, REV7, REV3, and 2 additional accessory subunits, POLD2 and POLD3 (9, 10, 19). POL ζ acts downstream of the FA core proteins in the repair of DNA ICL lesions (3–5, 20). In this role, REV7 acts as an adaptor protein between REV1 and REV3, the catalytic subunit of the POL ζ complex. A deficiency in REV7 could account for the well-known hypomutability of FA cells, resulting from a defect in TLS repair (20–23). Second, REV7 plays a role in mitosis by preventing premature activation of the

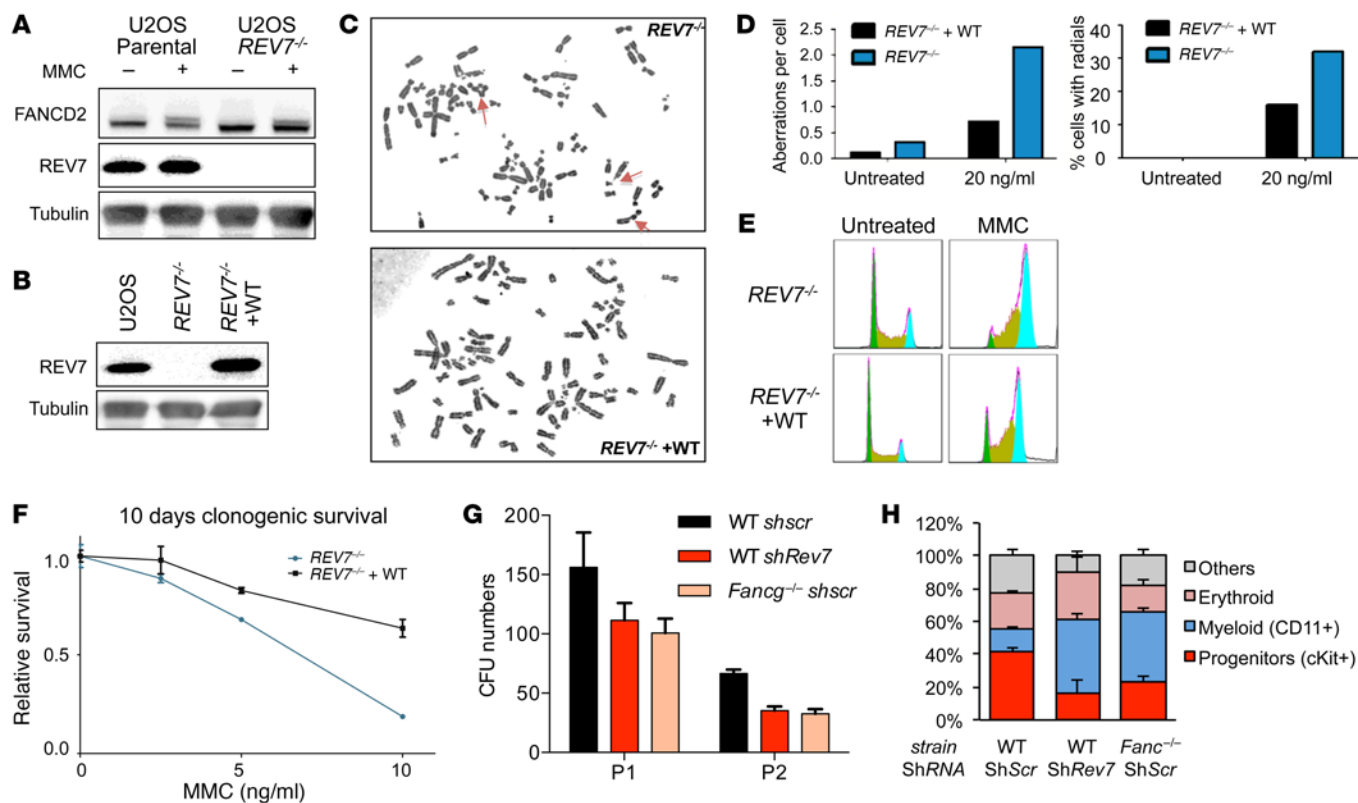


Figure 3. CRISPR/Cas9 knockout of *REV7* recapitulates the FA phenotype. (A) Western blot of whole cell lysates shows no detectable REV7 protein in the *REV7*^{-/-} line. FANCD2 is still efficiently monoubiquitylated in response to 100 ng/ml MMC. (B) Western blot showing reconstitution of WT REV7 expression in *REV7*^{-/-} cells. (C–D) Increase in chromosome aberrations (left graph) and radials (right) after 48-hour treatment with 20 ng/ml MMC as compared with cells complemented with WT REV7 cDNA. Original magnification, $\times 1,000$. (E) Increase in G₂/M arrest with and without MMC treatment (20 ng/ml). (F) Impaired clonogenic capacity of *REV7*^{-/-} cells over 10-day treatment with MMC. (G) REV7 is required for normal hematopoiesis; CFU scoring of bone marrow Lin⁻ cells after lentiviral silencing using *REV7* or scramble (scr) shRNAs. *Fancg*^{-/-} cells are used as FA cell control. CFUs were numbered after 7 days on methylcellulose; 2 passages (P1 and P2) were performed. (H) Cell population percentages in CFUs after 7 days of culture.

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 *Rev7*^{C70R} 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 *Rev7*^{C70R} disrupted Pol ζ assembly, thereby impairing mouse development and the repair of genotoxic agent-induced DNA lesions (31). *Rev7*^{C70R} mutant cells also showed decreased proliferation, increased apoptosis, and arrest in the S phase with extensive γ H2AX foci in

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 *Fancg*^{-/-} 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

CRISPR-Cas9 disruption in *REV7* was GAGGTCTTGTCGTGTGAG-CG. U2OS cells were transfected using Lipofectamine 2000 (Invitrogen 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 GraphPadPrism 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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