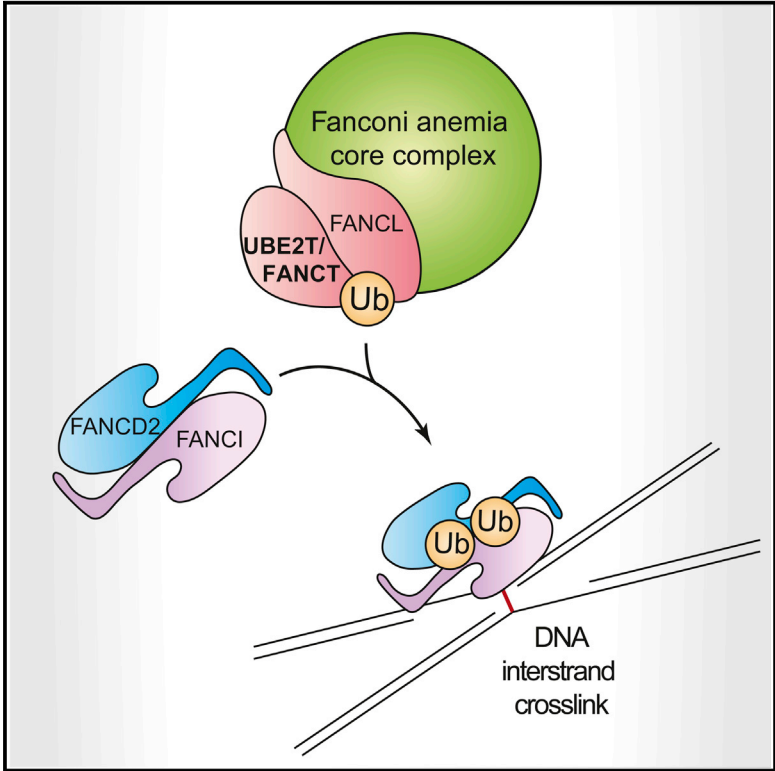


Deficiency of UBE2T, the E2 Ubiquitin Ligase Necessary for FANCD2 and FANCI Ubiquitination, Causes FA-T Subtype of Fanconi Anemia

Graphical Abstract



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In Brief

Fanconi anemia (FA), a rare bone marrow failure and cancer predisposition syndrome, results from pathogenic mutations in genes encoding proteins participating in DNA interstrand crosslink repair. Rickman et al. identify an individual with FA with biallelic *UBE2T* mutations, meriting the alias of *FANCT* for *UBE2T*.

Highlights

- Deficiency of UBE2T causes Fanconi anemia
- *FANCT* becomes an alias for *UBE2T*
- Biallelic *UBE2T* mutations arise due to *Alu*-mediated recombination
- UBE2T is the primary E2 of the FA pathway required for FANCD2 and FANCI activation

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Deficiency of UBE2T, the E2 Ubiquitin Ligase Necessary for FANCD2 and FANCI Ubiquitination, Causes FA-T Subtype of Fanconi Anemia

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SUMMARY

Fanconi anemia (FA) is a rare bone marrow failure and cancer predisposition syndrome resulting from pathogenic mutations in genes encoding proteins participating in the repair of DNA interstrand crosslinks (ICLs). Mutations in 17 genes (*FANCA-FANCS*) have been identified in FA patients, defining 17 complementation groups. Here, we describe an individual presenting with typical FA features who is deficient for the ubiquitin-conjugating enzyme (E2), UBE2T. UBE2T is known to interact with FANCL, the E3 ubiquitin-ligase component of the multiprotein FA core complex, and is necessary for the monoubiquitination of FANCD2 and FANCI. Proband fibroblasts do not display FANCD2 and FANCI monoubiquitination, do not form FANCD2 foci following treatment with mitomycin C, and are hypersensitive to crosslinking agents. These cellular defects are complemented by expression of wild-type *UBE2T*, demonstrating that deficiency of the protein UBE2T can lead to Fanconi anemia. *UBE2T* gene gains an alias of *FANCT*.

INTRODUCTION

Fanconi anemia (FA) is a rare disorder characterized by developmental abnormalities, bone marrow failure in the first decade of life, predisposition to solid tumors and leukemia, and cellular hypersensitivity to crosslinking agents (Auerbach, 2009). FA results from biallelic mutations in genes encoding proteins important for the repair of DNA interstrand crosslinks (ICLs). Patient mutations have been identified in 17 FA genes

(*FANCA-FANCS*), whereas a minority of patients have unknown causative gene mutations (Kottemann and Smogorzewska, 2013; Sawyer et al., 2015; Wang and Smogorzewska, 2015).

DNA ICLs are deleterious DNA lesions that covalently link DNA strands blocking transcription and replication. Compounds such as mitomycin C (MMC), diepoxybutane (DEB), cisplatin, and nitrogen mustards can generate ICLs, whereas naturally occurring biological metabolites such as aldehydes are suspected to cause endogenous lesions (Garaycochea et al., 2012; Hira et al., 2013; Langevin et al., 2011; Ridpath et al., 2007). Diagnosis of FA is made based on chromosomal breakage assays using crosslinking agents DEB or MMC (Auerbach, 1993). FA patient cells show increased breakage levels when challenged with crosslinking agents compared to normal cells.

During DNA replication, ICLs cause replication fork stalling and activation of the FA pathway (Garcia-Higuera et al., 2001; Knipscheer et al., 2009). Removal of ICLs is a multistep process requiring activation of the FA core complex composed of eight FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) and their interacting factors. A key step in ICL repair is the core-complex-mediated monoubiquitination of FANCD2 and FANCI (Dorsman et al., 2007; Garcia-Higuera et al., 2001; Sims et al., 2007; Smogorzewska et al., 2007; Timmers et al., 2001). Ubiquitin transfer requires the activity of an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin-ligating enzyme (reviewed in Hershko and Ciechanover, 1998). The FANCL subunit of the FA core complex is the E3 ubiquitin-ligase that monoubiquitinates FANCD2 and FANCI (Meetei et al., 2003). UBE2T has been identified as the E2 ubiquitin-conjugating enzyme, and its interaction with FANCL is required for monoubiquitination of FANCD2 and FANCI (Alpi et al., 2008; Hodson et al., 2014; Longerich et al., 2009; Machida et al., 2006; Rajendra et al., 2014; Sato et al., 2012). A second E2, UBE2W, has been

described to interact with FANCL and ubiquitinate FANCD2 *in vitro*, but the physiological importance of this interaction has not been explored in mammalian cells (Alpi et al., 2008; Rajendra et al., 2014; Zhang et al., 2011).

Activated FANCD2 and FANCI form the ID2 complex that localizes to chromatin and is required for coordinating repair at the crosslink (Garcia-Higuera et al., 2001; Knipscheer et al., 2009; Smogorzewska et al., 2007). Processing of the ICL encompasses nucleolytic unhooking of the crosslink that is dependent on FANCP/SLX4 and ERCC4/FANCG/XPF, translesion synthesis bypass of the unhooked ICL on one strand, and double-strand break (DSB) repair by homologous recombination (HR) on the other strand (Kim et al., 2011, 2013; Klein Douwel et al., 2014; Long et al., 2011; Niedernhofer et al., 2004; Tischkowitz et al., 2007; Xia et al., 2007).

Here, we describe an individual enrolled in the International Fanconi Anemia Registry (IFAR) presenting with typical FA features and deficiency of the ubiquitin-conjugating enzyme (E2), UBE2T. Sanger sequencing of genomic DNA revealed a large paternal deletion and maternal duplication resulting from *Alu*-mediated recombination. In the absence of UBE2T expression, the proband cells are defective for FANCD2 and FANCI monoubiquitination, do not form FANCD2 foci following treatment with MMC, and are hypersensitive to crosslinking agents. These cellular defects are complemented by expression of wild-type *UBE2T*, demonstrating that deficiency of the protein UBE2T can cause FA.

RESULTS

Cellular Phenotype of FA Cell Line of Unknown Complementation Group

The subject presented at birth with bilateral radial aplasia, absent thumbs, microcephaly, micrognathia, café au lait spots, absent left kidney (Table S1), and elevated chromosomal breakage in peripheral blood samples treated with DEB. Peripheral blood samples tested over the years displayed decreasing chromosomal breakage levels and increasing evidence of somatic mosaicism in the hematopoietic compartment, a phenomenon seen in a small subset of FA patients (Table S2) (Gregory et al., 2001; Lo Ten Foe et al., 1997; Waisfisz et al., 1999). The subject has not developed bone marrow failure at the age of 16.

Fibroblasts derived from the subject (RA2627) are hypersensitive to crosslinking agents MMC and DEB in survival assays (Figures 1A and 1B). Chromosomal breakage levels are elevated in RA2627 fibroblasts treated with DEB as compared to BJ wild-type fibroblast, although slightly lower than FA-A patient cells (RA3087; Figures 1C and 1D). RA2627 cells are deficient for FANCD2 monoubiquitination (Figure 1E) whereas the lymphoblastoid cell line (LCL; RA2946) derived from blood that showed mosaicism, displays normal FANCD2 monoubiquitination (Figure 1F). By immunofluorescence, FANCD2 foci were not observed in RA2627 cells following 24-hr treatment with MMC (Figure 1G). These data demonstrate that the subject's fibroblasts display deficiency of FA pathway activation, consistent with the deficiency of the FA core complex, one of the associated proteins, or the ID2 complex, whereas the subject's

LCLs are phenotypically reverted consistent with mosaicism observed in subject's blood.

Whole-Exome Sequencing and High-Resolution Array Comparative Genomic Hybridization

High-resolution array comparative genomic hybridization (aCGH) of genomic DNA from the subject's fibroblasts and peripheral blood samples did not detect deletions or duplications in the known FA genes (Table S3). Whole-exome sequencing (WES) of DNA derived from proband LCLs and parental peripheral blood samples was performed. Analysis of WES data revealed a single *FANCA* mutation, c.2574C > G/p.Ser858Arg, previously described in FA (Wijker et al., 1999). Normal levels of *FANCA* were detected by western blot in RA2627 cells (Figure 2A), and overexpression of wild-type *FANCA* in RA2627 failed to rescue the monoubiquitination defect of FANCD2 and FANCI, excluding *FANCA* as a causative gene in this cell line (Figures 2B and 2C). WES analysis identified no other mutations in reported FA genes.

Identification of Biallelic *UBE2T* Mutations in the Subject

RNA sequencing (RNA-seq) was performed on RA2627 fibroblasts to assess altered transcript levels that might indicate functionally significant gene mutations not captured by WES. Compared to a non-FA patient cell line (RA3380), a marked reduction in *UBE2T* was detected, but not for any of the known FA genes (Figure 3A). Decreased *UBE2T* transcript levels were confirmed by qRT-PCR (Figure 3B), and UBE2T protein was undetectable in RA2627 fibroblast lysates (Figure 3C). However, UBE2T transcript and protein were present at near normal levels in proband LCLs (RA2946; Figures 3B and 3D), supporting the presence of a genetic reversion in the hematopoietic compartment.

Sanger sequencing of genomic DNA and cDNA from proband primary fibroblasts, parental peripheral blood, and LCLs revealed compound heterozygous mutations in *UBE2T* (Figures 3E, S1, and S2; see also Supplemental Experimental Procedures). The paternally derived deletion, g.202332626_202341295 del, appears to have resulted from recombination of two *AluYa5* repeats within the *UBE2T* gene (Figure S1). This deletion is expected to be a null allele, as it results in the loss of a majority of the gene, including the start codon.

The maternal duplication, g.202332626_202341295 dup, also appears to be mediated by *Alu* recombination. The maternally derived mutation consists of a large duplication of the genomic region between the two *AluYa5* repeats (Figures 3E and S2). Cloning of proband cDNA revealed a transcript containing the hypothesized duplication c.-64_468 dup (dupEx2_6; Figures 3E, S2D, and S2E). In this transcript, exon 6 is spliced to the duplicated exon 2. Inclusion of the noncoding region from exon 2 results in a frameshift and a premature stop codon. The c.-64_468 dup transcript can be detected at very low frequency in RA2627 cells and is likely degraded by nonsense-mediated decay due to the premature stop codon. If any transcript is translated, it may produce a residual amount of the predicted protein p.A157Cfs*7 (Figures 3E and S2). The maternally derived mutation was absent in the RA2946 LCLs. This indicates that

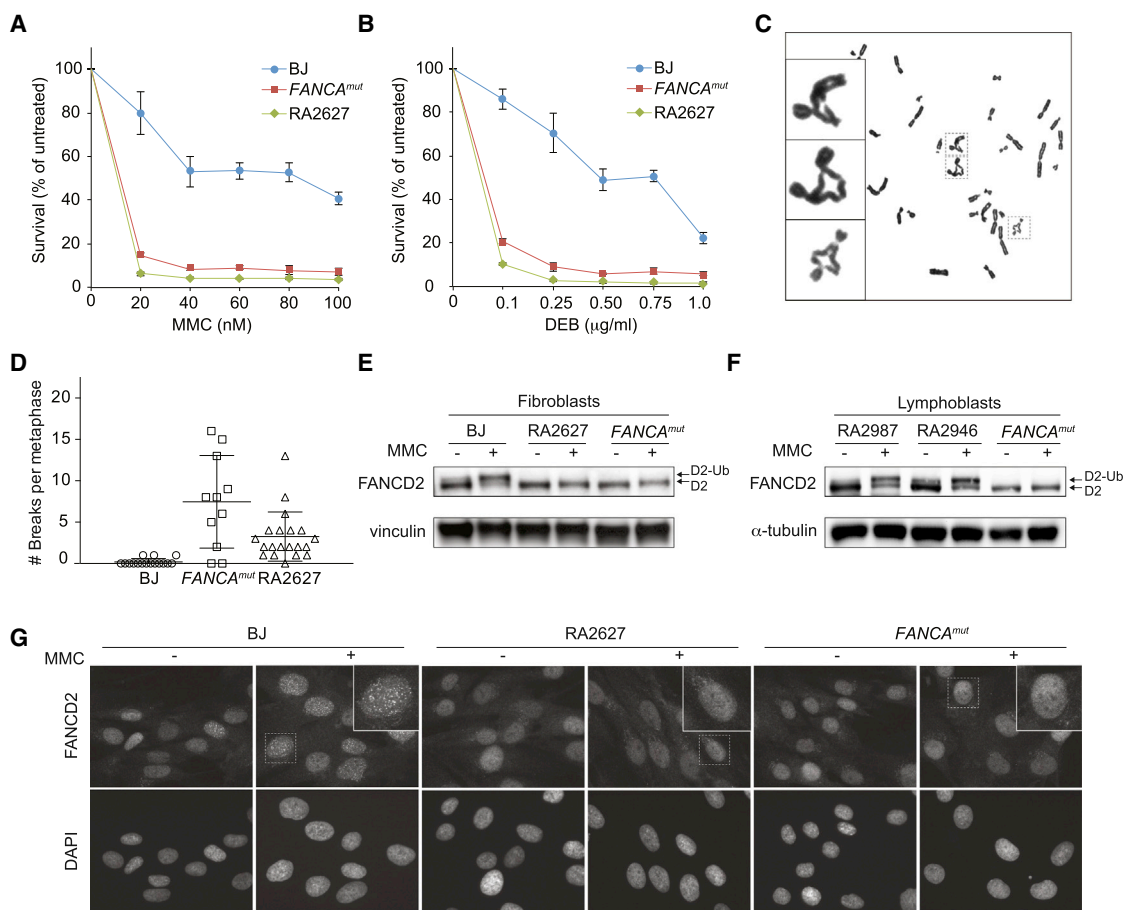


Figure 1. Characterization of Cell Lines from an Individual with FA under Study

(A and B) MMC and DEB cell survival assays of the subject's RA2627 fibroblasts in comparison to *FANCA* mutant and BJ wild-type fibroblasts. Cells were treated in triplicate with increasing concentration of MMC or DEB. Cell numbers were determined after 7 days and normalized to untreated control to give percent survival. Error bars represent SD.

(C) Example of metaphase spread of RA2627 following 0.1 μ g/ml DEB treatment. Inset images emphasize radial chromosomes.

(D) Quantification of chromosome breaks of DEB-treated BJ, *FANCA* mutant, and RA2627 fibroblasts. Mean breaks per cell were 0.19, 7.5, and 3.3, respectively.

(E and F) Western blot with FANCD2 antibody of BJ, RA2627 proband fibroblasts, and *FANCA* mutant fibroblasts or non-FA control RA2987 lymphoblasts, RA2946 proband lymphoblasts, and *FANCA* mutant lymphoblasts. Cells were cultured with or without 1 μ M MMC for 24 hr.

(G) FANCD2 foci formation following treatment with or without 1 μ M MMC for 24 hr.

the expression of wild-type UBE2T in the proband blood may be due to recombination of the maternally derived allele (Figure S2A).

Complementation of RA2627 Cellular Defects by Wild-Type UBE2T Expression

To prove that UBE2T deficiency is the cause of the subject's FA, we introduced wild-type *UBE2T* into RA2627 fibroblasts and assayed for rescue of FA phenotypes. Overexpression of UBE2T rescued cellular hypersensitivity to crosslinking agents MMC, DEB, and cisplatin (Figures 4A–4C). Analysis of cell-cycle distribution following treatment with MMC revealed an accumulation of RA2627 cells in G2 that was rescued by UBE2T overexpression to the levels observed in wild-type BJ cells (Figure 4D). UBE2T expression restored monoubiquitination of FANCD2 and FANCI (Figure 4F) and FANCD2 foci following treatment with

MMC (Figure 4G). These results confirm that deficiency of UBE2T results in FA-T subtype.

DISCUSSION

In this study, we have identified a FA subtype resulting from deficiency of UBE2T. Analysis of RNA-seq data was critical in identifying UBE2T deficiency, stressing that multipronged diagnostic approaches are often necessary in a genetically heterogeneous disease like FA.

We have identified compound heterozygous mutations in *UBE2T*, a large genomic deletion in the paternally derived allele, and a large duplication in the maternally derived allele. Both of the mutations appear to be driven by *Alu*-mediated non-allelic homologous recombination (NAHR). NAHR is a prevalent mechanism in genetic disorders arising from copy-number defects

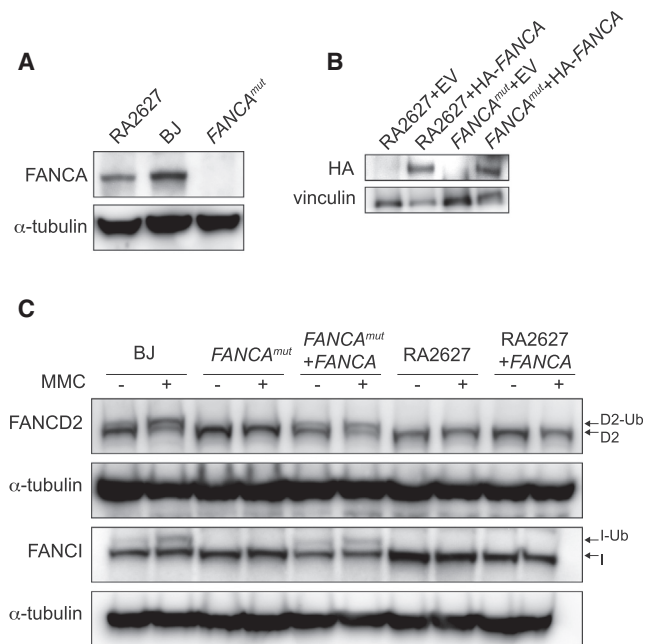


Figure 2. *FANCA* cDNA Fails to Complement RA2627 *FANCD2* and *FANCI* Monoubiquitination Defect

(A) Western blot with *FANCA* antibody of RA2627, BJ, and *FANCA* mutant fibroblasts. (B) Expression of wild-type HA-FLAG-tagged *FANCA* cDNA or empty vector control (EV) in RA2627 and *FANCA* mutant fibroblasts. (C) Western blot with *FANCD2* and *FANCI* antibody of *FANCA*-complemented cells.

due to recurrent intrachromosomal recombination events (reviewed in [Deininger, 2011](#)). Recently, sequencing of *FANCA* deletion variants identified that breakpoints preferentially lie within *Alu* elements and has revealed NAHR as a major mechanism of deletion in *FANCA* ([Flynn et al., 2014](#)).

The subject presented at birth with classic FA features including developmental defects and increased chromosomal breakage. The subject has not yet developed bone marrow failure at the age of 16. Blood counts are likely preserved due to the somatic mosaicism of the hematopoietic compartment. Restoration of *UBE2T* expression is detected in the individual's lymphoblast cells, and we have observed increasing rescue of chromosomal breakage in peripheral blood since birth. The subject's bone marrow remains stable but hypocellular for age (30%–40%), and it is unclear whether mosaicism will continue to improve the bone marrow cellularity.

The paternally derived deletion mutation is expected to not produce protein due to deletion of the majority of the coding region and start codon, whereas the maternally derived duplication results in very low levels of the c.-64_468 dup *UBE2T* transcript. This transcript may theoretically produce a *UBE2T* p.A157Cfs*7 protein, but it is clearly insufficient to fully support FA pathway function as evident by the phenotype of the subject and the cellular defects. The subject's fibroblasts are as sensitive as FA-A patient cells by cell survival assay, and no monoubiquitination of *FANCD2* or *FANCI* is detected. However, breakage levels

are not as elevated as control FA-A fibroblasts, leaving the possibility that residual truncated *UBE2T* may be present and active at a very low level, affecting the breakage phenotype. An alternative explanation could be the presence of another E2 with low levels of activity.

UBE2T has been demonstrated to be the major E2 ubiquitin-conjugating enzyme required for the monoubiquitination of *FANCD2* and *FANCI* ([Alpi et al., 2008](#); [Hodson et al., 2014](#); [Longerich et al., 2009, 2014](#); [Machida et al., 2006](#); [Sato et al., 2012](#)). Efficient and specific *FANCD2* monoubiquitination in vitro by *UBE2T*/*FANCL* requires the presence of *FANCI* and DNA ([Longerich et al., 2014](#); [Sato et al., 2012](#)). In vitro studies have demonstrated that a second E2, *UBE2W*, can also ubiquitinate *FANCD2* ([Alpi et al., 2008](#); [Zhang et al., 2011](#)). In recent work, the in vitro monoubiquitination of *FANCD2* by *UBE2W* is demonstrated to be nonspecific, does not require interaction of ID2 complex, and is not stimulated by DNA ([Rajendra et al., 2014](#)). Additionally, Δ *UBE2W* chicken DT40 cells do not display sensitivity to MMC and display normal levels of MMC-induced monoubiquitination of *FANCD2* ([Rajendra et al., 2014](#)).

Our identification of biallelic *UBE2T* mutations and *UBE2T* deficiency in an individual with FA corroborates that *UBE2T* is the primary E2 of the FA pathway required for the activation of the ID2 complex and repair of DNA ICLs. Deficiency of *UBE2T* causes FA-T disease subtype, and *FANCT* will be the alias for *UBE2T*. Future evaluation of FA patients with unknown gene mutations should include complementation studies of *UBE2T*.

EXPERIMENTAL PROCEDURES

Study Subject/Cell Lines

DNA samples and cell lines were derived from subjects enrolled in the IFAR after obtaining informed written consent. The Institutional Review Board of The Rockefeller University approved these studies.

Cell Culture and Viral Transfection/Transduction

Human cell lines were transformed and/or immortalized using standard protocols. cDNAs were delivered using retroviral transduction after packaging in HEK293T cells according to manufacturer's protocol (Mirus). For details, see the [Supplemental Experimental Procedures](#).

Cell-Cycle, Chromosomal Breakage, and Cell-Survival Analyses

Analysis of cell-cycle and chromosomal breakage following treatment with DNA-damaging agents was performed as described ([Kim et al., 2011](#)). For cell survival assays, cells were seeded overnight and treated next day with DNA-damaging agents. Cells were grown for 3 to 4 days, passaged at appropriate ratios, and counted once nearly confluent.

Western Blot and Antibodies

Whole-cell extracts were prepared by lysing cell pellets in Laemmli sample buffer (Bio-Rad) followed by sonication. Samples were boiled and separated on 4%–12% or 3%–8% gradient gels (Invitrogen) by SDS-PAGE. Immunoblotting was performed using the following antibodies: *FANCD2* (Novus NB100-182); HA (Covance MMS-101R); *UBE2T* (Abcam EPR9446); and *FANCI* (antibody raised in-house; no. 589).

Immunofluorescence

Cells were fixed in 3.7% formaldehyde and permeabilized with 0.5% Triton in PBS, blocked in 5% (v/v) FBS in PBS, and incubated with antibodies 1:1,000 in blocking buffer. Cells were washed and incubated with Alexa 488 secondary antibody. Cells were washed, and coverslips were embedded with DAPI Fluoromount-G (SouthernBiotech).

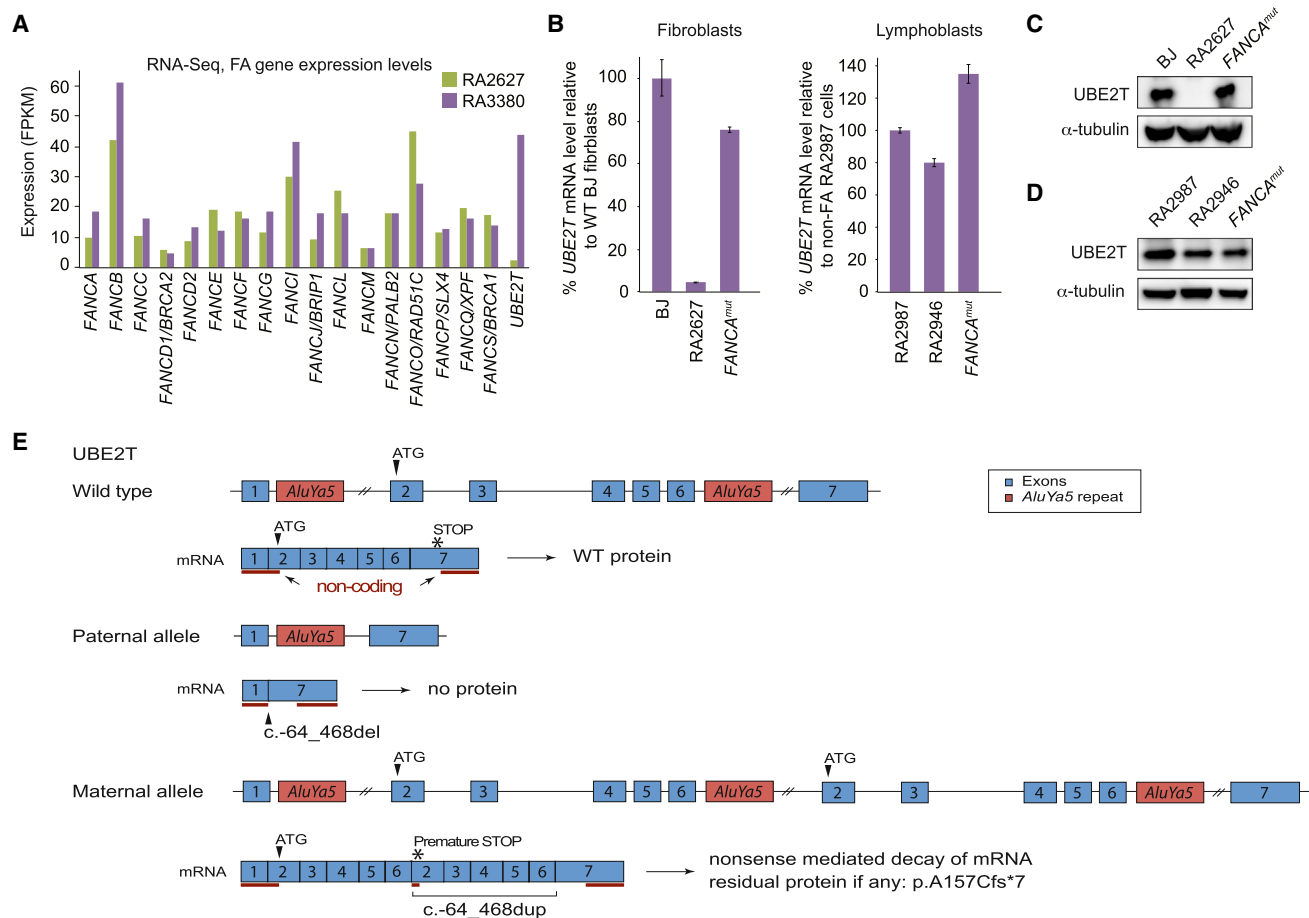


Figure 3. UBE2T Is Deficient in RA2627

(A) Comparison of normalized RNA-seq expression, fragments per kilobase of transcripts per million reads (FPKM), of known FA genes and *UBE2T* for RA2627 and non-FA control RA3380 primary cells. FPKM values are normalized to reflect transcript abundance and account for the variability in length of different gene transcripts.

(B) qRT-PCR of *UBE2T* expression levels in RA2627 fibroblasts (left) and RA2946 lymphoblasts (right) in comparison to wild-type control and *FANCA* mutant cells. Error bars represent SE of three replicates.

(C and D) Western blot with *UBE2T* antibody.

(E) Schematic of the paternally derived deletion and maternally derived duplication resulting from *Alu-Alu*-mediated non-allelic homologous recombination (NAHR) of *AluYa5* repeats present in IVS1 and IVS6 of the *UBE2T* gene. For the paternal allele, recombination resulted in the loss of the sequence between the *AluYa5* repeats and one *AluYa5* repeat (8,670 bp; see also Figure S1). For the maternal allele, recombination resulted in the duplication of the sequence between the *AluYa5* repeats and one *AluYa5* (see also Figure S2).

Next-Generation Sequencing

Indexed RNA-seq libraries were constructed using TruSeq RNA Sample Prep Kit version 2 (Illumina). Each library was sequenced in pair-end mode using one lane of Illumina HiSeq2000 flow cell to generate 2 × 100 bp reads. Raw reads were aligned to the human genome (hg19) using TopHat with default parameters. Cufflinks with GC and upper quartile normalization was then used to calculate normalized expression levels, fragments per kilobase of transcripts per million reads (FPKM) (Trapnell et al., 2012). WES was performed as described in the Supplemental Experimental Procedures.

PCR, Reverse Transcription, and qRT-PCR Performed to Identify *UBE2T* Mutations in Proband

PCR reactions were performed using *Taq* DNA Polymerase (QIAGEN), Phusion High-Fidelity PCR Master Mix with GC buffer (Thermo Scientific), and PCR SuperMix High Fidelity (Invitrogen) according to manufacturer's protocols, and primers are listed in Table S4. Total mRNA was extracted using RNeasy

plus kit (QIAGEN). Superscript III reverse transcriptase followed by Platinum SYBR Green SuperMix-UDG (Invitrogen) was used according to manufacturer's protocol and normalized against GAPDH. For details, see the Supplemental Experimental Procedures.

ACCESSION NUMBERS

Genomic coordinates were mapped to GRCh38/hg38 human assembly. cDNA coordinates were mapped against RefSeq: NM_014176.3.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.06.014>.

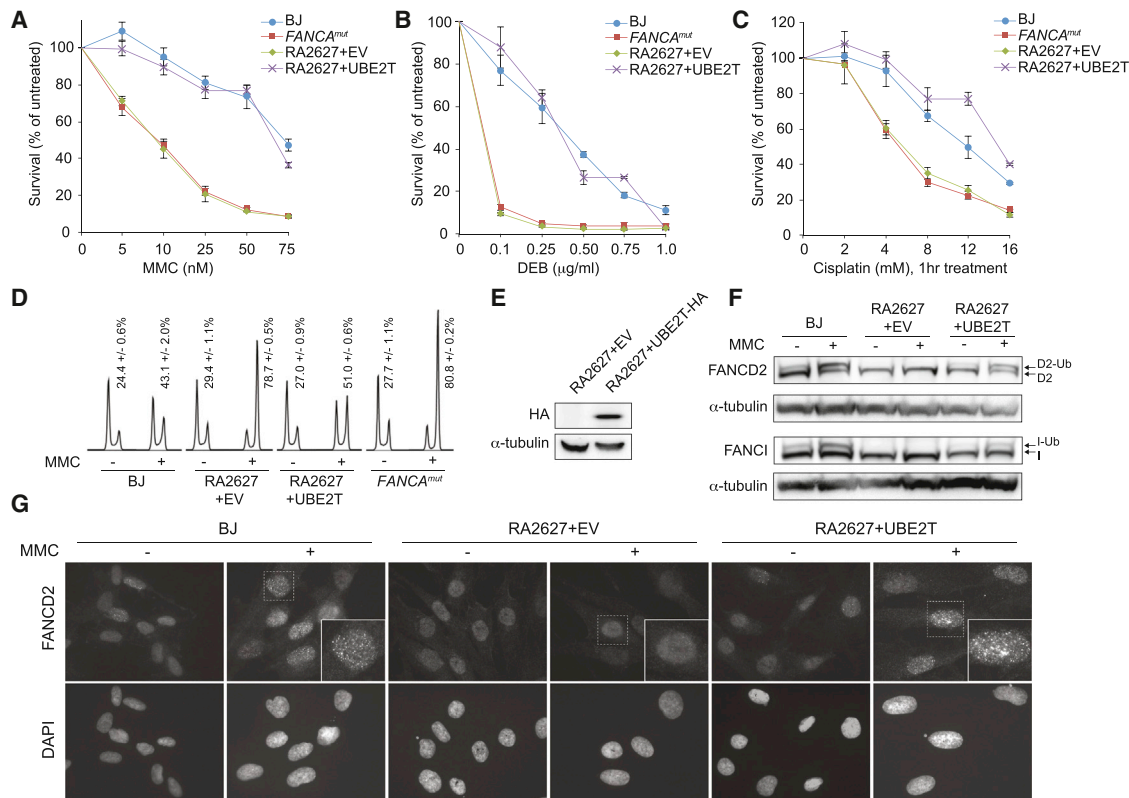


Figure 4. UBE2T cDNA Complements RA2627 Hypersensitivity to Crosslinking Agents and Monoubiquitination of FANCD2 and FANCI

(A–C) Complementation of MMC, DEB, and cisplatin sensitivity of proband fibroblasts (RA2627). Error bars represent SD. (D) Complementation of cell-cycle defect after 45 nM MMC treatment. Cells were treated with drug and cultured for 48 hr before analysis. (E) Expression of wild-type HA-FLAG-tagged *UBE2T* cDNA or empty vector control (EV) in RA2627 fibroblasts used in experiments (A)–(G). (F) Western blot analysis with FANCD2 and FANCI antibody of *UBE2T*-complemented cells with or without 24 hr 1 μM MMC treatment. (G) FANCD2 foci formation of complemented cells following treatment with or without 1 μM MMC for 24 hr.

AUTHOR CONTRIBUTIONS

K.A.R., F.P.L., and A.S. designed, performed, and interpreted experiments. F.X.D. and S.C.C. performed aCGH and RNA-seq. O.E. performed analysis of RNA-seq data. C.S. and S.B.G. led the WES team at the Broad Institute. A.A. performed bioinformatic analysis of the exome data. A.S. is the PI, and E.M.S. and J.A.K. are genetic counselors of the IFAR. A.D.A. directed clinical testing and enrolled the family in the study. D.S. excluded number of FA genes as being mutated in the subject. K.A.R. and A.S. wrote manuscript with input from other authors.

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