HHS Public Access

Author manuscript

Cell Rep. Author manuscript; available in PMC 2016 July 07.

Published in final edited form as:

Cell Rep. 2015 July 7; 12(1): 35–41. doi:10.1016/j.celrep.2015.06.014.

Deficiency of UBE2T, the E2 ubiquitin ligase necessary for FANCD2 and FANCI ubiquitination, causes FA-T subtype of Fanconi anemia

Kimberly A. Rickman[1], **Francis P. Lach**[1], **Avinash Abhyankar**[2], **Frank X. Donovan**[3], **Erica M. Sanborn**[1], **Jennifer A. Kennedy**[1], **Carrie Sougnez**[4], **Stacey B. Gabriel**[4], **Olivier Elemento**[5], **Settara C. Chandrasekharappa**[3], **Detlev Schindler**[6], **Arleen D. Auerbach**[7], and **Agata Smogorzewska**[1]

[1]Laboratory of Genome Maintenance, The Rockefeller University, New York, NY 10065, USA

[2]New York Genome Center, New York, NY 10013, USA

[3]Cancer Genetics and Comparative Genomics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA

[4]Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

[5]Institute for Computational Biomedicine, Department of Physiology and Biophysics, Weill Cornell Medical College, New York, NY 10021, USA

[6]Institute for Human Genetics, Biozentrum, Universität Würzburg, D-97074 Würzburg, Germany

[7]Human Genetics and Hematology, The Rockefeller University, New York, NY10065, USA

Abstract

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

Corresponding author: Agata Smogorzewska: asmogorzewska@rockefeller.edu.

The content of this study is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Accession Numbers

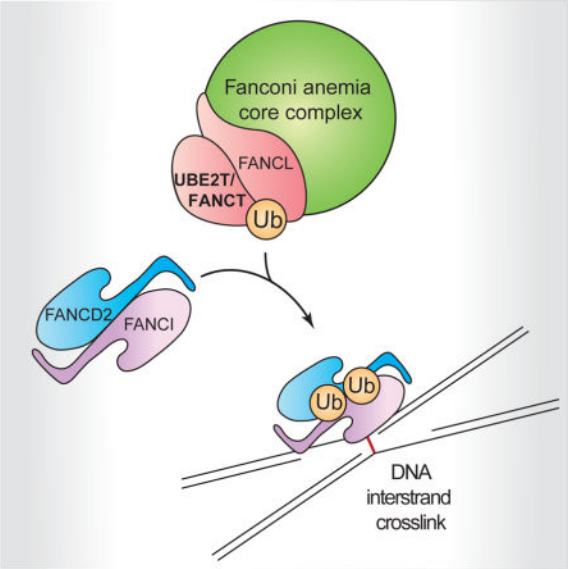
Genomic coordinates were mapped to GRCh38/hg38 human assembly. cDNA coordinates were mapped against RefSeq: NM_014176.3.

Authorship:

KAR, FPL, AS, designed, performed, and interpreted data. FXD and SCC performed aCGH and RNA sequencing. OE performed analysis of RNA-seq data. CS and SG led the WES team at the Broad Institute. AA performed bioinformatic analysis of the exome data. AS is the PI and ES and JK are genetic counselors of the International Fanconi Anemia Registry. ADA directed clinical testing and enrolled the family in the study. DS excluded number of FA genes as being mutated in the subject. KAR and AS wrote manuscript with input from other authors.

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*.

Graphical Abstract



Keywords

Fanconi anemia; UBE2T; FANCL; FANCI; FANCD2; FANCT; ubiquitination; bone marrow failure; somatic mosaicism

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*) while 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, while naturally occurring biological metabolites such as aldehydes are suspected to cause endogenous lesions (Garaycoechea 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/FANCQ/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; Kim et al., 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.

Experimental Procedures

Study Subject/Cell lines

DNA samples and cell lines were derived from subjects enrolled in the International Fanconi anemia Registry (IFAR) after obtaining informed written consent. The Institutional Review Board of The Rockefeller University, New York, NY, USA, 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 manufacture's protocol (Mirus). For details see Extended 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 3–4 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), FANCI (antibody raised in-house, #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 1:1000 in blocking buffer. Cells were washed and incubated with Alexa Fluor 488 secondary antibody. Cells were washed and coverslips were embedded with DAPI Fluoromount-G (SouthernBiotech).

Next-generation sequencing

Indexed RNA sequencing (RNA-seq) libraries were constructed using TruSeq RNA Sample Prep Kit version 2 (Illumina). Each library was sequenced in pair-end mode using 1 lane of Illumina HiSeq2000 flowcell 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). Whole exome sequencing was performed as described in Extended Experimental Procedures.

PCR, reverse transcription, and RT qPCR 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 messenger RNA 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 Extended Experimental Procedures.

Results

Cellular phenotype of Fanconi anemia 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 diexpoxybutane (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 (Figure 1A–B). 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) (Figure 1C–D). RA2627 cells are deficient for FANCD2 monoubiquitination (Figure 1E) while the lymphoblastoid cell line (LCLs) (RA2946) derived from blood that showed mosaicism, display normal FANCD2 monoubiquitination (Figure 1F). By immunofluorescence, FANCD2 foci were not observed in RA2627 cells following 24-hour 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, while 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 (Figure 2B–C). 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 RT-qPCR (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) (Figure 3B, D) 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* (Figure 3E, S1, S2, also see Extended Experimental Procedures). The paternally derived deletion, g. 202332626_202341295del, 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_202341295dup, 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 (Figure 3E, Figure S2). Cloning of proband cDNA revealed a transcript containing the hypothesized duplication c.-64_468dup (dupEx2_6) (Figure 3E, Figure S2D and E). 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_468dup 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 (Figure 3E and S2). The maternally derived mutation was absent in the RA2946 LCLs. This indicates that 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 (Figure 4A-C). 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 Fanconi anemia-T subtype.

Discussion

In this study we have identified a Fanconi anemia 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 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, while the maternally derived duplication results in very low levels of the c.-64_468dup *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., 2014; Longerich et al., 2009; 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 FAND2 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 interstrand crosslinks. Deficiency of UBE2T causes Fanconi anemia-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*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank the subject and family for their participation in this study. We would like to acknowledge Richard Harris and Stella Davies for providing clinical data and patient care and Minoru Takata for sharing unpublished data. The work was supported by the Starr Center Consortium grant (AS and SG), the Burroughs Welcome Fund Career Award for Medical Scientists (AS), the Doris Duke Charitable Foundation Clinical Scientist Development Award (AS), and the Vilcek Foundation (ADA). The International Fanconi Anemia Registry is supported by the Rockefeller University, NIH RO1 HL120922 (AS) and grant # 8 UL1 TR000043 from the National Center for Advancing Translational Sciences, NIH Clinical and Translational Science Award program. FXD and SCC acknowledge the support from the Intramural Research Program of the National Human Genome Research Institute, NIH. KAR was supported by a Medical Scientist Training Program grant from the National Institute of General Medical Sciences of the NIH under award number: T32GM007739 to the Weill Cornell/Rockefeller/Sloan-Kettering Tri-Institutional MD-PhD Program.

References

Alpi AF, Pace PE, Babu MM, Patel KJ. Mechanistic insight into site-restricted monoubiquitination of FANCD2 by Ube2t, FANCL, and FANCI. Molecular cell. 2008; 32:767–777. [PubMed: 19111657]

Auerbach AD. Fanconi anemia diagnosis and the diepoxybutane (DEB) test. Experimental hematology. 1993; 21:731–733. [PubMed: 8500573]

Auerbach AD. Fanconi anemia and its diagnosis. Mutation research. 2009; 668:4–10. [PubMed: 19622403]

Deininger P. Alu elements: know the SINEs. Genome biology. 2011; 12:236. [PubMed: 22204421]

Dorsman JC, Levitus M, Rockx D, Rooimans MA, Oostra AB, Haitjema A, Bakker ST, Steltenpool J, Schuler D, Mohan S, et al. Identification of the Fanconi anemia complementation group I gene, FANCI. Cellular oncology: the official journal of the International Society for Cellular Oncology. 2007; 29:211–218. [PubMed: 17452773]

Flynn EK, Kamat A, Lach FP, Donovan FX, Kimble DC, Narisu N, Sanborn E, Boulad F, Davies SM, Gillio AP 3rd, et al. Comprehensive analysis of pathogenic deletion variants in Fanconi anemia genes. Human mutation. 2014; 35:1342–1353. [PubMed: 25168418]

Garaycoechea JI, Crossan GP, Langevin F, Daly M, Arends MJ, Patel KJ. Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. Nature. 2012; 489:571–575. [PubMed: 22922648]

Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, Grompe M, D'Andrea AD. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Molecular cell. 2001; 7:249–262. [PubMed: 11239454]

Gregory JJ Jr, Wagner JE, Verlander PC, Levran O, Batish SD, Eide CR, Steffenhagen A, Hirsch B, Auerbach AD. Somatic mosaicism in Fanconi anemia: evidence of genotypic reversion in lymphohematopoietic stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2001; 98:2532–2537. [PubMed: 11226273]

Hershko A, Ciechanover A. The ubiquitin system. Annual review of biochemistry. 1998; 67:425–479.

Hira A, Yabe H, Yoshida K, Okuno Y, Shiraishi Y, Chiba K, Tanaka H, Miyano S, Nakamura J, Kojima S, et al. Variant ALDH2 is associated with accelerated progression of bone marrow failure in Japanese Fanconi anemia patients. Blood. 2013; 122:3206–3209. [PubMed: 24037726]

Hodson C, Purkiss A, Miles JA, Walden H. Structure of the human FANCL RING-Ube2T complex reveals determinants of cognate E3-E2 selection. Structure. 2014; 22:337–344. [PubMed: 24389026]

Kim Y, Lach FP, Desetty R, Hanenberg H, Auerbach AD, Smogorzewska A. Mutations of the SLX4 gene in Fanconi anemia. Nature genetics. 2011; 43:142–146. [PubMed: 21240275]

Kim Y, Spitz GS, Veturi U, Lach FP, Auerbach AD, Smogorzewska A. Regulation of multiple DNA repair pathways by the Fanconi anemia protein SLX4. Blood. 2013; 121:54–63. [PubMed: 23093618]

Klein Douwel D, Boonen RA, Long DT, Szypowska AA, Raschle M, Walter JC, Knipscheer P. XPF-ERCC1 acts in Unhooking DNA interstrand crosslinks in cooperation with FANCD2 and FANCP/SLX4. Molecular cell. 2014; 54:460–471. [PubMed: 24726325]

Knipscheer P, Raschle M, Smogorzewska A, Enoiu M, Ho TV, Scharer OD, Elledge SJ, Walter JC. The Fanconi anemia pathway promotes replication-dependent DNA interstrand cross-link repair. Science. 2009; 326:1698–1701. [PubMed: 19965384]

Kottemann MC, Smogorzewska A. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. Nature. 2013; 493:356–363. [PubMed: 23325218]

Langevin F, Crossan GP, Rosado IV, Arends MJ, Patel KJ. Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice. Nature. 2011; 475:53–58. [PubMed: 21734703]

Lo Ten Foe JR, Kwee ML, Rooimans MA, Oostra AB, Veerman AJ, van Weel M, Pauli RM, Shahidi NT, Dokal I, Roberts I, et al. Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. European journal of human genetics: EJHG. 1997; 5:137–148. [PubMed: 9272737]

Long DT, Raschle M, Joukov V, Walter JC. Mechanism of RAD51-dependent DNA interstrand cross-link repair. Science. 2011; 333:84–87. [PubMed: 21719678]

Longerich S, Kwon Y, Tsai MS, Hlaing AS, Kupfer GM, Sung P. Regulation of FANCD2 and FANCI monoubiquitination by their interaction and by DNA. Nucleic acids research. 2014; 42:5657–5670. [PubMed: 24623813]

Longerich S, San Filippo J, Liu D, Sung P. FANCI binds branched DNA and is monoubiquitinated by UBE2T-FANCL. The Journal of biological chemistry. 2009; 284:23182–23186. [PubMed: 19589784]

Machida YJ, Machida Y, Chen Y, Gurtan AM, Kupfer GM, D'Andrea AD, Dutta A. UBE2T is the E2 in the Fanconi anemia pathway and undergoes negative autoregulation. Molecular cell. 2006; 23:589–596. [PubMed: 16916645]

Meetei AR, de Winter JP, Medhurst AL, Wallisch M, Waisfisz Q, van de Vrugt HJ, Oostra AB, Yan Z, Ling C, Bishop CE, et al. A novel ubiquitin ligase is deficient in Fanconi anemia. Nature genetics. 2003; 35:165–170. [PubMed: 12973351]

Niedernhofer LJ, Odijk H, Budzowska M, van Drunen E, Maas A, Theil AF, de Wit J, Jaspers NG, Beverloo HB, Hoeijmakers JH, et al. The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks. Molecular and cellular biology. 2004; 24:5776–5787. [PubMed: 15199134]

Rajendra E, Oestergaard VH, Langevin F, Wang M, Dornan GL, Patel KJ, Passmore LA. The genetic and biochemical basis of FANCD2 monoubiquitination. Molecular cell. 2014; 54:858–869. [PubMed: 24905007]

Ridpath JR, Nakamura A, Tano K, Luke AM, Sonoda E, Arakawa H, Buerstedde JM, Gillespie DA, Sale JE, Yamazoe M, et al. Cells deficient in the FANC/BRCA pathway are hypersensitive to plasma levels of formaldehyde. Cancer research. 2007; 67:11117–11122. [PubMed: 18056434]

Sato K, Toda K, Ishiai M, Takata M, Kurumizaka H. DNA robustly stimulates FANCD2 monoubiquitylation in the complex with FANCI. Nucleic acids research. 2012; 40:4553–4561. [PubMed: 22287633]

Sawyer SL, Tian L, Kahkonen M, Schwartzentruber J, Kircher M, Majewski J, Dyment DA, Innes AM, et al. University of Washington Centre for Mendelian, G., Consortium, F.C. Biallelic

mutations in BRCA1 cause a new Fanconi anemia subtype. Cancer discovery. 2015; 5:135–142. [PubMed: 25472942]

Sims AE, Spiteri E, Sims RJ 3rd, Arita AG, Lach FP, Landers T, Wurm M, Freund M, Neveling K, Hanenberg H, et al. FANCI is a second monoubiquitinated member of the Fanconi anemia pathway. Nature structural & molecular biology. 2007; 14:564–567.

Smogorzewska A, Matsuoka S, Vinciguerra P, McDonald ER 3rd, Hurov KE, Luo J, Ballif BA, Gygi SP, Hofmann K, D'Andrea AD, et al. Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. Cell. 2007; 129:289–301. [PubMed: 17412408]

Timmers C, Taniguchi T, Hejna J, Reifsteck C, Lucas L, Bruun D, Thayer M, Cox B, Olson S, D'Andrea AD, et al. Positional cloning of a novel Fanconi anemia gene, FANCD2. Molecular cell. 2001; 7:241–248. [PubMed: 11239453]

Tischkowitz M, Xia B, Sabbaghian N, Reis-Filho JS, Hamel N, Li G, van Beers EH, Li L, Khalil T, Quenneville LA, et al. Analysis of PALB2/FANCN-associated breast cancer families. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:6788–6793. [PubMed: 17420451]

Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature protocols. 2012; 7:562–578.

Waisfisz Q, Morgan NV, Savino M, de Winter JP, van Berkel CG, Hoatlin ME, Ianzano L, Gibson RA, Arwert F, Savoia A, et al. Spontaneous functional correction of homozygous fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. Nature genetics. 1999; 22:379–383. [PubMed: 10431244]

Wang AT, Smogorzewska A. SnapShot: Fanconi anemia and associated proteins. Cell. 2015; 160:354–354. e351. [PubMed: 25594185]

Wijker M, Morgan NV, Herterich S, van Berkel CG, Tipping AJ, Gross HJ, Gille JJ, Pals G, Savino M, Altay C, et al. Heterogeneous spectrum of mutations in the Fanconi anaemia group A gene. European journal of human genetics: EJHG. 1999; 7:52–59. [PubMed: 10094191]

Xia B, Dorsman JC, Ameziane N, de Vries Y, Rooimans MA, Sheng Q, Pals G, Errami A, Gluckman E, Llera J, et al. Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. Nature genetics. 2007; 39:159–161. [PubMed: 17200672]

Zhang Y, Zhou X, Zhao L, Li C, Zhu H, Xu L, Shan L, Liao X, Guo Z, Huang P. UBE2W interacts with FANCL and regulates the monoubiquitination of Fanconi anemia protein FANCD2. Molecules and cells. 2011; 31:113–122. [PubMed: 21229326]

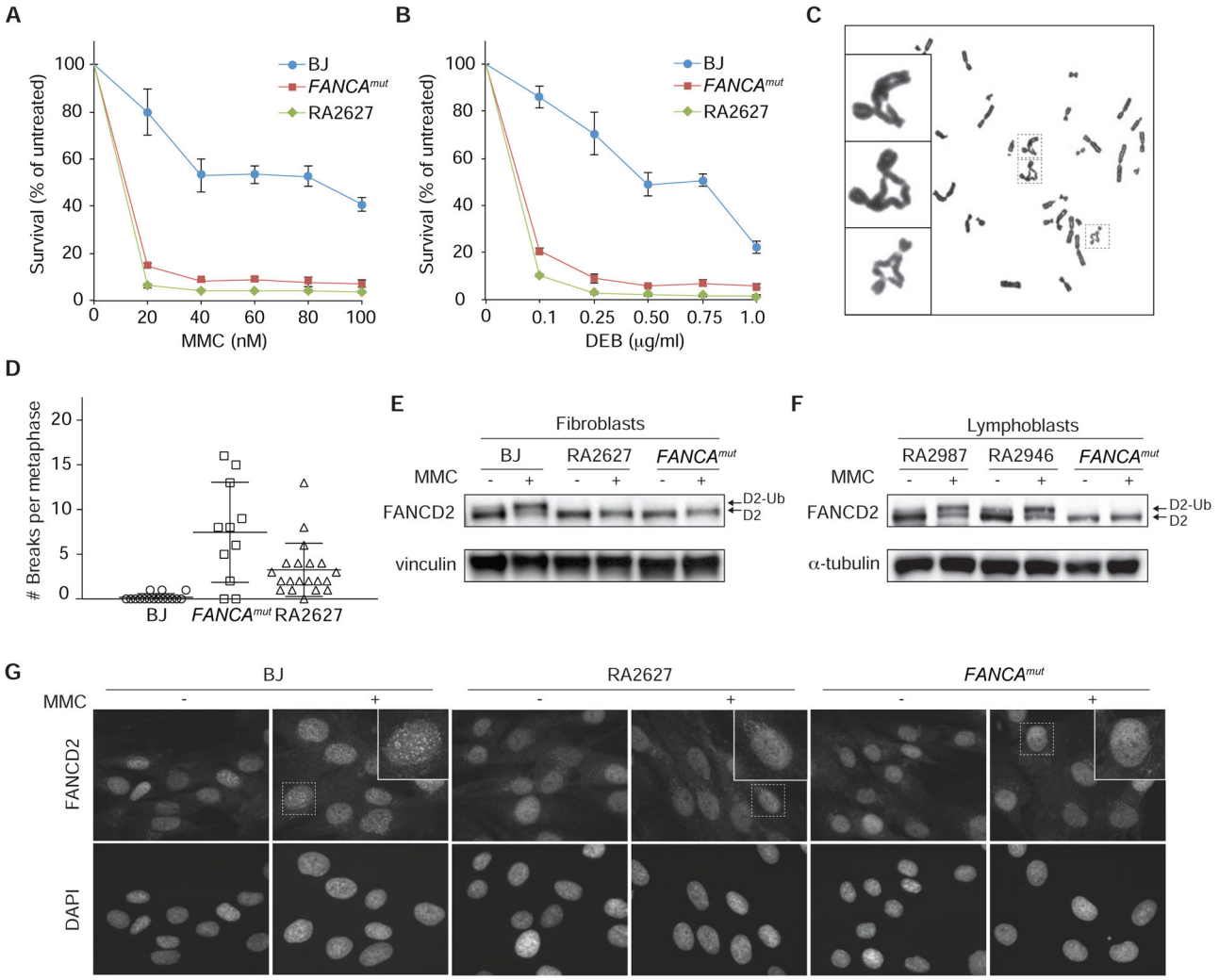


Figure 1. Characterization of cell lines from an individual with FA under study

(a,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, s.d. (c) Example of metaphase spread of RA2627 following 0.1ug/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,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 1μM MMC for 24h. (g) FANCD2 foci formation following treatment with or without 1μM MMC for 24h.

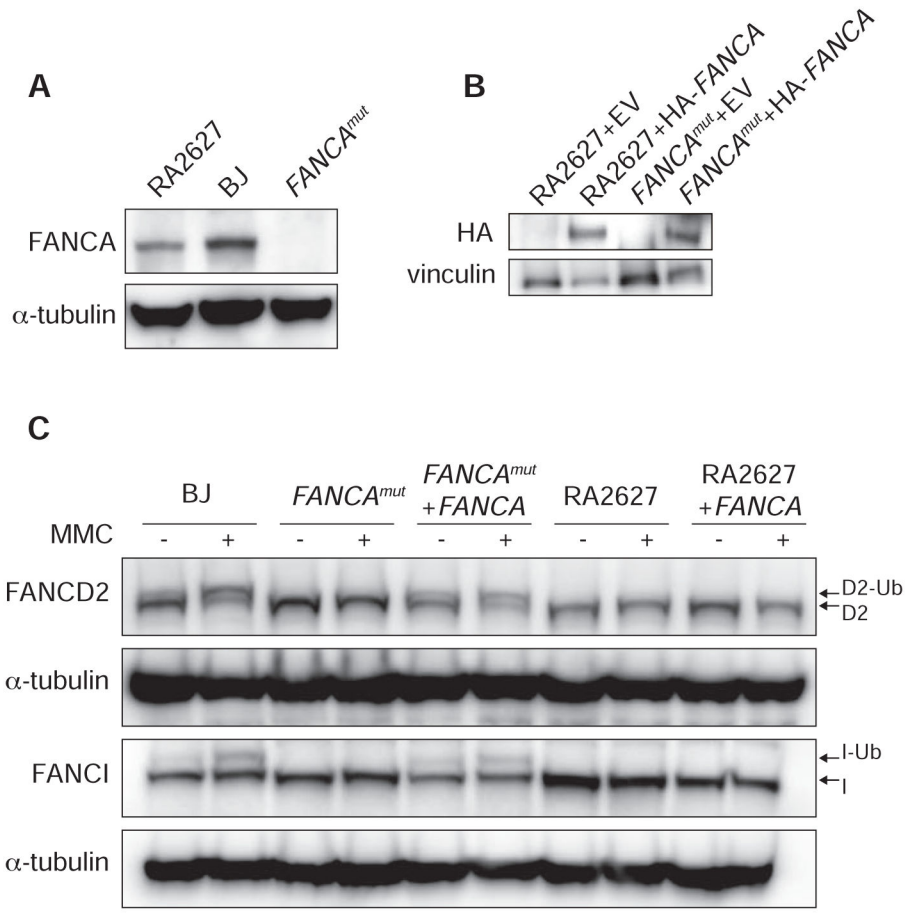


Figure 2. *FANCA* cDNA fails to complement RA2627 FANCD2 and FANCI monoubiquitination defect

(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.

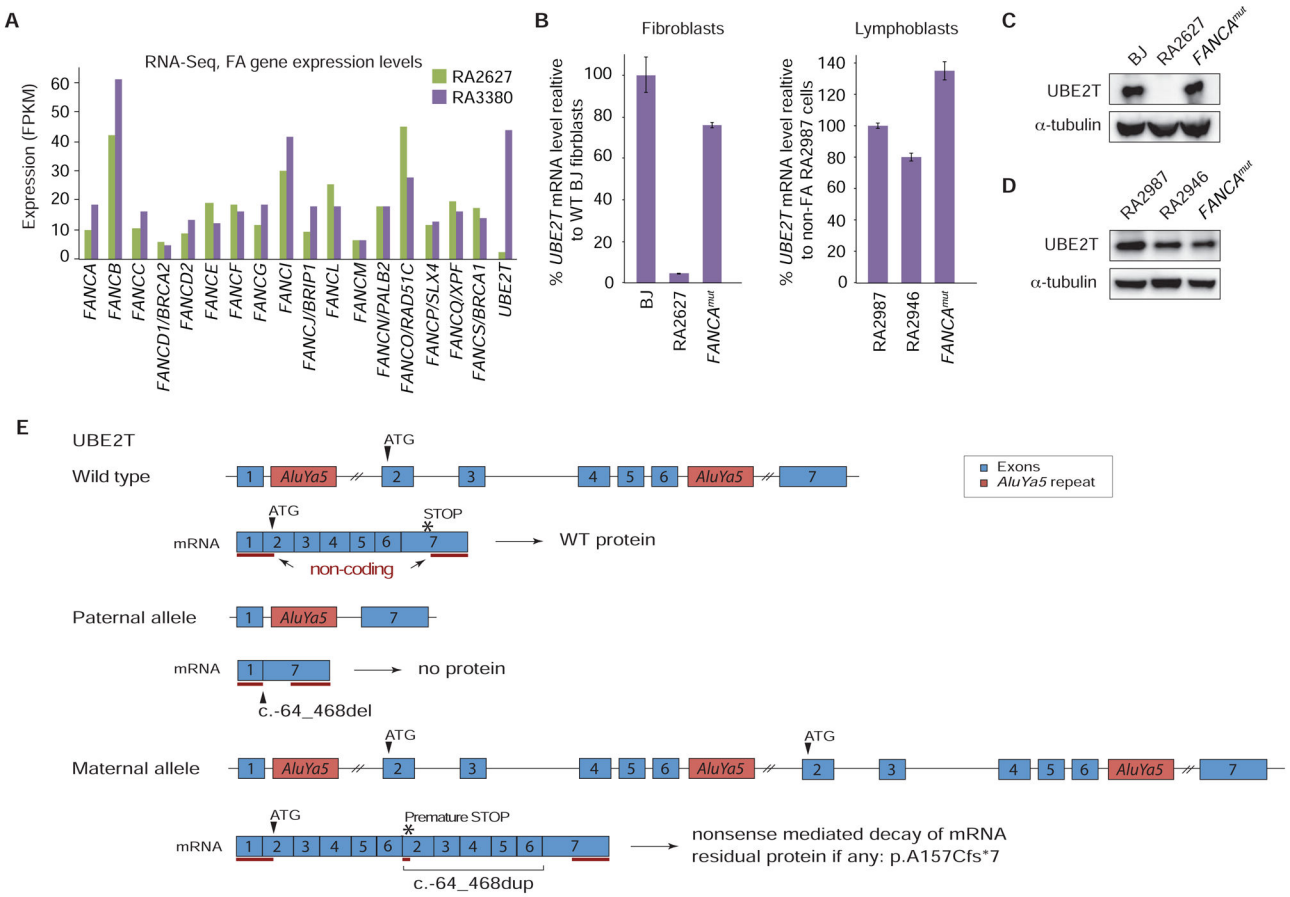


Figure 3. UBE2T is deficient in RA2627

(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) RT-quantitative 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 standard error of three replicates. (c,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 intervening sequence and one *AluYa5* repeat (8,670bp) (see also Figure S1). For the maternal allele, recombination resulted in the insertion of another *AluYa5* repeat and duplication of the sequence between the *Alu* repeats (see also Figure S2).

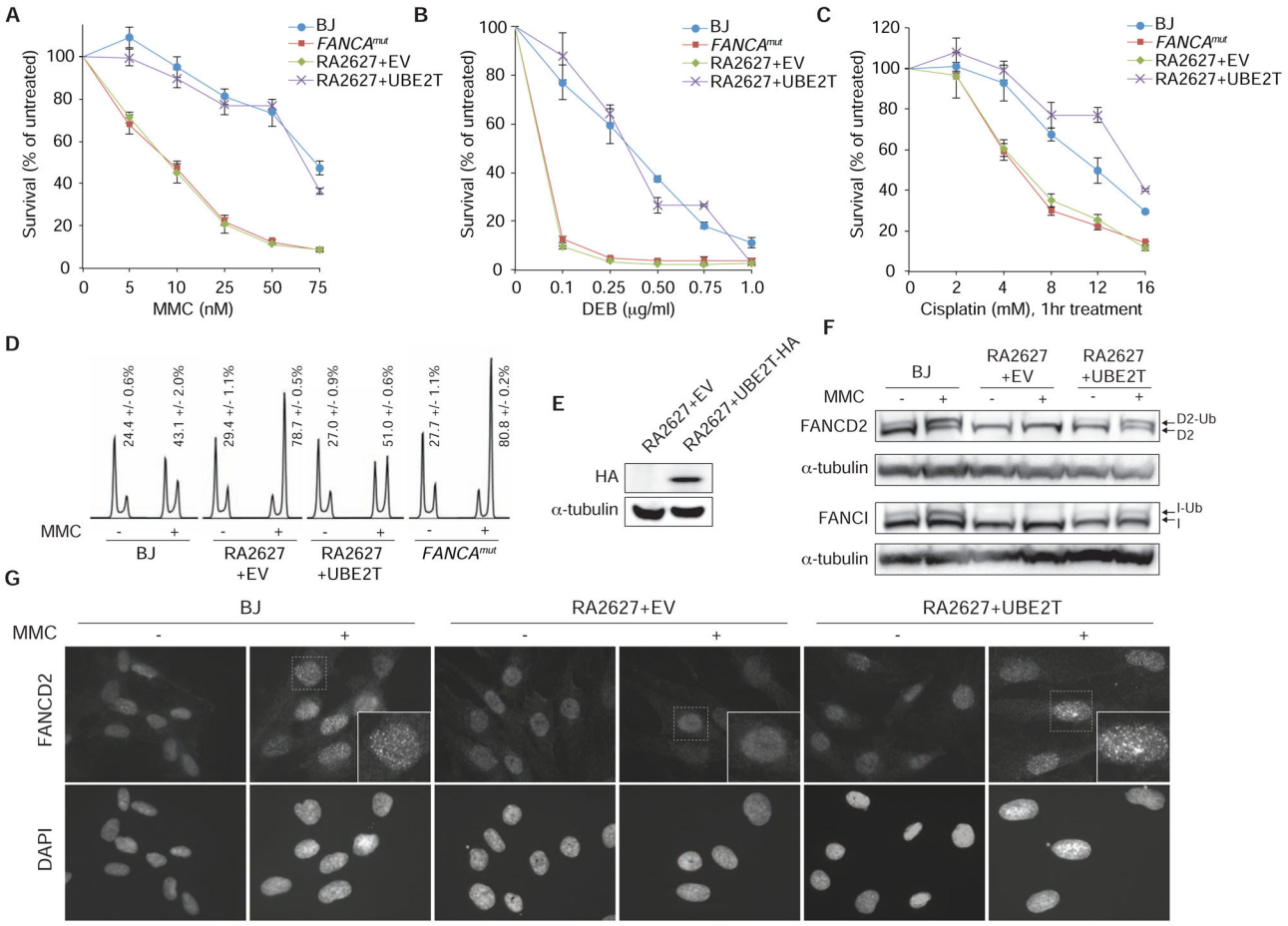


Figure 4. UBE2T cDNA complements RA2627 hypersensitivity to crosslinking agents and monoubiquitination of FANCD2 and FANCI

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