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# Mutations in the gene encoding the E2 conjugating enzyme UBE2T cause Fanconi Anemia

--Manuscript Draft--

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| **Abstract:** | Fanconi anemia (FA) is a rare genetic disorder characterized by genome instability, increased cancer susceptibility, progressive bone marrow failure (BMF) and various developmental abnormalities resulting from the defective FA pathway. FA is caused by mutations in genes that mediate repair processes of interstrand crosslinks and/or DNA adducts generated by endogenous aldehydes. The UBE2T E2 ubiquitin conjugating enzyme acts in FANCD2/FANCI monoubiquitination, a critical event in the pathway. Here we identified two unrelated FA individuals each harboring biallelic mutations in UBE2T. They both produced a defective UBE2T protein with the same missense alteration p.Gln2Glu that abolished FANCD2 monoubiquitination and interaction with FANCL. We suggest this FA complementation group be named FA-T. |

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W have accepted all changes we made and deleted line numbers. We also have checked the figures and supplement materials.

Our point-by-point response to the comments is described in the separate file.

We are looking forward to hearing from you.

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# Mutations in the gene encoding the E2 conjugating enzyme UBE2T cause Fanconi Anemia

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**Abstract**

Fanconi anemia (FA) is a rare genetic disorder characterized by genome instability, increased cancer susceptibility, progressive bone marrow failure (BMF) and various developmental abnormalities resulting from the defective FA pathway. FA is caused by mutations in genes that mediate repair processes of interstrand crosslinks and/or DNA adducts generated by endogenous aldehydes. The UBE2T E2 ubiquitin conjugating enzyme acts in FANCD2/FANCI monoubiquitination, a critical event in the pathway. Here we identified two unrelated FA individuals each harboring biallelic mutations in *UBE2T*. They both produced a defective UBE2T protein with the same missense alteration p.Gln2Glu that abolished FANCD2 monoubiquitination and interaction with FANCL. We suggest this FA complementation group be named FA-T.

## Introduction

Fanconi anemia (FA) is a rare genetic disease characterized by genome instability, cancer predisposition, progressive bone marrow failure (BMF), various developmental abnormalities that often include radial ray anomalies, short stature, and visceral malformations[1]. FA cells are hypersensitive to DNA interstrand crosslink damage (ICL) and various types of damage due to endogenous aldehydes[2-5]. FA is caused by mutations in any one of sixteen genes that together comprise the FA pathway. These genes include *FANCA* (MIM 617139), *FANCB* (MIM 300515), *FANCC* (MIM 613899), *FANCD1 (BRCA2)* (MIM 600185), *FANCD2* (MIM 613984), *FANCE* (MIM 613976), *FANCF* (MIM 603467), *FANCG (XRCC9)* (MIM 600901), *FANCI* (MIM 6*11360)*, *FANCJ (BRIP1)* (MIM 614082), *FANCL (PHF9)* (MIM 614083), *FANCN (PALB2)* (MIM 610832), *FANCO* (*RAD51C*) (MIM 613390), *FANCP* (*SLX4*) (MIM 613951), *FANCQ* (*XPF*) (MIM 615272), and *FANCS (BRCA1)* (MIM 113705). A recent study indicated that biallelic mutations in FA-related *FANCM* (MIM 609644) do not cause an FA phenotype in humans[6], raising a concern whether this nomenclature is appropriate or not. In the upstream part of the pathway, the FA core E3 ligase complex consisting of eight gene products and other associated proteins monoubiquitinates FANCD2 and FANCI, resulting in chromatin accumulation/focus formation of FANCD2 that probably recognizes stalled replication forks upon ICL or aldehyde damage. This is the critical event that regulates recruitment of structure-specific nucleases and subsequent incision/unhooking of fork-blocking lesions, mobilizing the downstream repair pathway components[2,3]. *UBE2T* (MIM 610538) encodes an E2 ubiquitin conjugating enzyme (EC: 6.3.2.19) which has been implicated in this monoubiquitination reaction both *in vivo* [7-9] and i*n vitro* [10-13].

We previously analyzed the *ALDH2* genotypes in 64 Japanese FA individuals with the approval by the Research Ethics Committee of the Tokai University Hospital and Kyoto University and obtained family informed consent from all subjects involved[14]. Our report included two cases in which mutations in the genes previously associated with FA were excluded by whole exome sequencing (WES) (listed as number 60 and 61 in Supplemental Table 1 in Hira *et al*.[14]) (Figure S1). Serendipitously, *UBE2T* mutations were found in both of them (Figure 1A-C). The two persons are hereafter designated PNGS-252 (Family 1-II-1 in Figure 1D) and PNGS-255 (Family 2-II-1 in Figure 1D) (Table 1). They were from unrelated families (Figure 1D) living in different geographic locations in Japan. Both individuals displayed typical FA phenotypes, with malformations and hematological abnormalities that necessitated haematopoietic stem cell transplantation (Table 1) (see Supplemental Note). Chromosome fragility in lymphocytes (described in Supplemental Table 2 in Hira *et al*.[14]) was consistent with the diagnosis of FA.

WES and validation by Sanger sequencing in PNGS-252 revealed an apparent homozygous c.4C>G missense alteration（RefSeq accession: NM_014176.3）, resulting in the amino acid substitution p.Gln2Glu (Figure 1A). This mutation must be very rare, because this is not listed in the NHLBI Exome Sequencing Project or the Human Genetic Variation Browser databases. The glutamine residue (Gln2) is highly conserved in the homologs found from vertebrates to worms excluding plants (Figure 1E, and the mutation is rated as “damaging” by both SIFT and PolyPhen predictions. The Gln2 is located in the N-terminal helix of UBE2T, which constitutes part of the hydrophobic E3-E2 interaction surface, near the conserved E2 UBC fold[15] (Figure 1C and 1F). Copy number analysis using WES data suggested that there was a

heterozygous deletion across the *UBE2T* locus in the PNGS-252 sample (data not shown). Indeed, our targeted array comparative genome hybridization (array-CGH) revealed an area of reduced hybridization signal encompassing almost the entire *UBE2T* (Figure 1A). The deletion junction carried 3 bp of microhomology (Figure S2A-D), suggesting that the junction arose from microhomology-mediated repair[16]. This person's father carried the genomic deletion, while the mother had the heterozygous c.4C>G mutation (Figure S3). There was no family history of malformations, hematological abnormalities, or cancer predisposition.

In the individual PNGS-255, WES revealed the c.4C>G mutation as well as a splice donor site mutation (c.180+5G>A) (Figure 1B-C). Both alterations were heterozygous, and on different chromosomes (Figure S4). Thus this individual was compound heterozygous for the *UBE2T* mutations. In bone marrow fibroblasts, we found a small fraction of *UBE2T* transcripts with skipped exon 2, resulting in a frameshift and premature stop codon (p.Gln37Argfs*47) (Figure S5). Family members of this person were not available for further evaluation. However, the results of SNP array analysis using the HumanOMni5 v1.0 array (Illumina, Inc.) suggested that a haplotype containing the c.4C>G mutation was shared by PNGS-252, her mother, and PNGS-255 (not shown). Thus they may have a common ancestral origin.

We extended WES to AP65P FA fibroblasts provided by the JCRB Cell Bank, and found the same *UBE2T* c.4C>G mutation. Moreover, 99.9% of the SNPs listed in dbSNP131 and identified in AP65P were identical to those in PNGS-252 (2244 out of 2247), demonstrating that AP65P was derived from PNGS-252 (Table 1). The AP65P individual has been reported as carrying no mutations in *FANCA*, *FANCG*, and *FANCC*[17]. We transformed the cells with human *TERT* (*hTERT*), and termed them

AP65P-hTERT. Unfortunately, we were unable to immortalize bone marrow fibroblasts from PNGS-255.

Interestingly, AP65P-hTERT cells displayed roughly similar protein levels of UBE2T as normal control cells (48BR), indicating that the p.Gln2Glu substitution does not significantly destabilize UBE2T protein (Figure 2A). We also detected the auto-monoubiquitinated form of UBE2T as previously described[7,8], suggesting that the mutant protein is able to receive activated ubiquitin from the E1 enzyme (Figure 2A). However, only faint amounts of long-form ID proteins were observed even after MMC stimulation (Figure 2A). As expected, AP65P-hTERT cells transduced with lentivirus encoding normal UBE2T, but not with the mutant, clearly restored the MMC-induced long form of FANCD2 (Figure 2A) as well as FANCD2 foci formation (Figure 2B). Furthermore, both the increased levels of MMC-induced chromosome breakage (Figure 2C) and the MMC sensitivity (Figure 3A) in AP65P-hTERT cells were suppressed by exogenous wild type UBE2T but not with UBE2T carrying p.Gln2Glu. Taken together, these results firmly established that the FA phenotype in these individuals is caused by the *UBE2T* mutations.

How, exactly, does the UBE2T alteration affect the activity of the protein in promoting monoubiquitination of the ID complex? We hypothesized that the p.Gln2Glu substitution might disrupt the FANCL-UBE2T interaction. Indeed, the p.Gln2Glu alteration drastically reduced the signal intensity in a mammalian two-hybrid assay (Figure 4A). This was confirmed by a GST pull-down experiment using purified recombinant human or chicken GST-FANCL and wild type or mutant UBE2T proteins (Figure 4B, 4C, and S6A). In an *in vitro* monoubiquitination assay[11], the mutated UBE2T protein displayed ~3-fold less efficiency in promoting FANCD2

monoubiquitination in the presence (Figure 4D) or absence (Figure S6B) of stimulator DNA, whereas auto-ubiquitination was normal compared to control proteins (Figure S6C). The p.Gln2Glu substitution abrogated FANCL monoubiquitination *in vitro* (Figure 4D, S6B and S6D); however, the FANCL-independent FANCI monoubiquitination was not affected (Figure S6D)[12]. These results are well explained by the specific disruption of the FANCL-UBE2T interaction by the p.Gln2Glu substitution.

In conclusion, we propose that *UBE2T(FANCT)* mutations define a FA subtype. This is also a rare example of a mutated E2 enzyme causing an inherited human disorder, like *UBE2A*[18]. The p.Gln2Glu substitution is probably hypomorphic, since a siUBE2T knockdown made AP65P-hTERT cells more sensitive to MMC and completely eliminated the trace FANCD2 monoubiquitination that could still be observed in the siLuc control knockdown cells (Figure 3B and 3C). Finally, it is interesting to note a recent report suggesting that *UBE2T* functions with an unknown E3 in nucleotide excision repair[19]. It is common for an E2 to function with a set of E3 ligases, since far fewer E2s (~38) are encoded in the genome than E3 ligases (600-1000)[20]. UBE2T may have a partner other than FANCL, such as BRCA1[21] or other E3s, as has been suggested by yeast two hybrid assays[22,23], raising the possibility that UBE2T might have a function outside the FA pathway. While a siUBE2T knockdown in AP65P-hTERT modestly sensitized cells to UV (Figure 3C), we detected only a marginal impact of *UBE2T* lentiviral transduction on UV survival (Figure 3D). These results suggest that the p.Gln4Glu substitution is a separation of function alteration that specifically reduces *UBE2T* function in the FA pathway but not in UV resistance. In line with this, neither of our FA-T individuals experienced any

photosensitivity. It thus remains unclear whether or how complete loss of *UBE2T* function would impact human phenotypes.

## Supplemental Data

Supplemental data include Case Reports, 6 Figures and 3 Tables.


## Acknowledgements

The use of *FANCT* as an alias for *UBE2T* was approved by the HUGO Gene Nomenclature Committee. We would like to thank the individuals PNGS-252 and 255 and their family members for making this work possible. We also thank Dr. Masao S. Sasaki (Professor Emeritus, Kyoto University) for his long-standing effort to collect Japanese FA samples, including AP65P fibroblasts; Dr. James Hejna (Graduate School of Biostudies, Kyoto University) for critical reading of the manuscript and English editing; Dr. Takayuki Yamashita (Gunma University) for GM6914 cells; Dr. Hiroyuki Miyoshi (RIKEN, currently at Keio University) and RIKEN Bio-resource Center (Tsukuba, Ibaragi, Japan) for a lentivirus construct (CSII-CMV-MCS-IRES-Bsd) and the packaging system; Dr. Settara C. Chandrasekharappa (NIH) for advice on the CGH array; Dr. Yoko Katsuki for advice on immunofluorescence; Ms. Tomoko Hirayama (JCRB) for a protocol for karyotyping in fibroblasts; Ms. Fumiko Tsuchida, Chinatsu Ohki, Akiko Watanabe, and Mao Hisano for expert technical help; Drs. Toshiyasu Taniguchi and Agata Smogorzewska for advice on anti-FANCD2/FANCI western blotting. The AP65P cell line (KURB1562) was kindly provided by JCRB Cell Bank, National Institute of Biomedical Innovation (Saito, Ibaraki, Osaka). This work was

supported in part by grants from the Ministry of Health, Labor and Welfare of Japan. The authors declare no conflicts of interest.

**Web Resources**

The URLS for data presented herein are as follows:

European Genome-phenome Archive, https://www.ebi.ac.uk/ega/home

Human Genetic Variation Browser, http://www.genome.med.kyoto-u.ac.jp/SnpDB/

NHLBI Exome Sequencing Project, http://evs.gs.washington.edu/EVS/

Online Mendelian Inheritance in Man, http://www.omim.org

Polyphen-2, http://genetics.bwh.harvard.edu/pph2/

SIFT, http://sift.jcvi.org/

**Accession numbers**

The WES sequencing data have been deposited in the European Genome-phenome Archive (EGA) under the accession EGAS00001001103.

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**Table 1 Clinical features of the two FA cases.**

| Individual | PNGS-252 | PNGS-255 |
|---|---|---|
| Sex | Female | Male |
| Age at BMF (year)[a] | 7 | 3 |
| First allele | c.4C>G,<br>p.Gln2Glu | c.4C>G,<br>p.Gln2Glu |
| Second allele | ~23 kb deletion<br>(g.202288583_202309772del) | c.180+5G>A,<br>p.Gln37Argfs*47 |
| Physical abnormalities | Left hypoplastic thumb, Abnormalities of external genitalia, Short stature | Bilateral thumb polydactyly, Abnormal shape of left ear, Dysplasia of middle ear bone, Deafness, Facial nerve palsy |
| Hematological abnormalities | Severe aplastic anemia | MDS (Refractory anemia) evolving to AML |
| Age at HSCT (year)[b] | 13 | 8 |
| Outcome | Alive and well 12 years after HSCT | Died 5 months after HSCT |
| Solid tumors | None | None |
| Cells from JCRB Cell Bank | AP65P fibroblasts | Not available |
| *ALDH2* genotype | GA heterozygous | GA heterozygous |

[a]The onset of BMF was defined as described[24].

[b]Haematopoietic stem cell transplantation

**Figure titles and legends**

**Figure 1. Identification of *UBE2T* mutations.**

(A and B) Results of Sanger sequencing or array-CGH of the individual PNGS-252 (A) or PNGS-255 (B). In the array-CGH, one of the deletion junctions was outside of our probes installed, and therefore it is impossible to see the whole deletion. A red line indicates the approximate region of the genome deletion detected by genome PCR (Figure S2). A schematic summary is shown in (C). Genomic DNA was isolated from PHA-stimulated lymphocytes using a Puregene (Qiagen) kit. Genomic PCR was carried out using KOD-FX polymerase (TOYOBO) with primer pairs indicated in Table S1 and directly sequenced after ExoSAP-IT (Affymetrix) treatment or after purification from an agarose gel (Nucleospin, Takara). A custom CGH 4x180K array with a total of 179673 50mer probes in triplicate was designed using SureDesign (Agilent Technologies). The probes covered all of the known 16 genes associated with FA (including *FANCM* at the time of manufacturing) and related genes including *UBE2T*, *NBS1*, four *RAD51* paralogs, *FAAP20*, *FAAP24*, and *FAAP100*. Appropriate control probes were also included based on the recommendations from Agilent Technologies. After CGH slides were manufactured by Agilent Technologies, fluorochrome labeling of genomic DNA from the FA individuals and the reference subjects, hybridization with the CGH slides, scanning and preliminary analysis were done by Takara Bio company (Otsu, Shiga, Japan). Further analysis was carried out using Genomic workbench software (Agilent). Cys86 in UBE2T is the ubiquitin acceptor site, while Lys91 is an auto-ubiquitination site.

(D) Pedigrees of the probands' families.

(E) Alignments of the N-terminal UBE2T amino acid sequences across species. The

following UBE2T amino acid sequences were aligned using Genetyx-Mac software with manual modifications. *H.sapiens*, NP_054895.3; *M. musculus*, NP_080300.1; *G. gallus*, XP_419230.2; *X. laevis*, NP_001080105.1; *D.rerio*, NP_001070763.1; *T.rubripes*, XP_003963365.2; *A.mellifera*, XP_003249077.1; *C.elegans*, NP_500272.2 ; *A.thaliana*, NP_566751.1; *O.sativa*, NP_001043518.1. The arrow indicates the Gln2 residue.

(F) Structure of the human FANCL Ring domain-UBE2T complex cited from Hodson *et al*.[15]. The Gln2 residue is highlighted by red lines.

**Figure 2. Functional characterization of the mutant UBE2T protein.**

**(**A) Western blotting of cell lysates from normal fibroblasts (48BR) and from PNGS-252 (AP65P-hTERT) with or without indicated lentiviral transduction. AP65P primary fibroblasts (designated KURB1562 in JCRB) were obtained from the JCRB Cell Bank, and transduced with an hTERT expressing retroviral vector pMSCV-hTERT-puro. For lentiviral production, HEK293T cells were transfected with CSII-CMV-MCS-IRES2-Bsd encoding human UBE2T-FLAG (either wild type or the p.Gln2Glu mutant) together with packaging plasmids pCAG-HIVgp and pCMV-VSV-G-RSV-Rev, using Lipofectamine 2000. After 48 h in culture, the 293T supernatants were filtered and added to the sparsely seeded AP65P-hTERT cells. Blastocidin S selection was started 2 days later (3 μg/ml). For western blotting, collected cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 5 mM EDTA, 20 mM β-glycerophosphate, 50 mM NaF, 1X protease inhibitor (Complete EDTA-free tablet Roche), 50 U/mL Benzonase, 1 mM PMSF). Samples were separated by

SDS-polyacrylamide gel electrophoresis, transferred to a membrane, and detected with indicated antibodies and ECL reagents (GE Healthcare) using a LAS4000 Mini apparatus (GE Healthcare). The antibodies used in this study are summarized in Table S3.

(B) FANCD2 foci formation. Cells were grown on coverslips and subjected to 100 ng/ml MMC for 24 h. Cells were then permeabilized with 0.5% Triton X-100/PBS for 10 min on ice, and fixed with 3% paraformaldehyde/2% sucrose at room temperature for 30 min followed by blocking with 2% BSA/0.05% Triton X-100/PBS for 30 min at room temperature and staining with primary antibodies diluted in PBS containing 2% BSA and 0.05% Triton X-100 overnight at 4°C. Primary antibodies were detected by anti-rabbit Alexa 488 (Molecular Probes). Nuclei were stained with Prolong Gold mounting agent (Life Tech). Immunofluorescence images were captured with Keyence Biorevo BZ-9000. The mean and SD from three independent experiments are shown.

(C) Number of chromosomal aberrations induced by MMC treatment. MMC-exposed cells (10 ng/ml for 24 h) were arrested at M phase with Colcemid (100 ng/ml) for 3 h, harvested and further treated with 2 ml hypotonic 0.9% Sodium Citrate for 22 min. Carnoy's solution (4 ml) was added, followed by centrifugation at 1200 rpm for 10 min. Cell pellets were washed once with 10 ml Carnoy's solution and resuspended in 1 ml Carnoy's solution. In each condition, 100 cells were scored.

**Figure 3. Cell survival curves after MMC or UV treatment.**

(A) MMC sensitivity assay. AP65P-hTERT cells were exposed to varying concentrations of MMC for 24 h, washed and seeded into 6 well plates. After 5-7 days surviving cells were counted using a LUNA digital automated cell counter (Logos

Biosystems) or stained with 0.006% Crystal violet/25% methanol solution. GM6914 (FA-A) and its complemented cells were included as control.

(B) Western blotting using the indicated antibodies (listed in Table S3). AP65P-hTERT cells were transfected with control (siLuc) or siRNAs targeting UBE2T (siUBE2T#1 and #2) (final 10 nM), seeded, and treated with MMC for 24 h or UV-irradiated. siRNAs (sequence listed in Table S2) were synthesized by Life Technologies and transfected using Lipofectamine RNAiMAX. (C) MMC or UV sensitivity in AP65P-hTERT cells depleted of mutated UBE2T protein.

(D) UV sensitivity in AP65P-hTERT cells with or without UBE2T lentiviral transduction. The sensitivity assays were repeated at least three times, and a representative data set with mean and SD of the triplicate cultures is shown.

**Figure 4. Functional interaction between FANCL and UBE2T**

(A) Mammalian two-hybrid assay. The assays were carried out as described[25]. Briefly, *UBE2T* and *FANCL* in the bait vector (pM) or prey vector (pVP16) were co-transfected into 293T cells with the reporter luciferase vector as well as an internal control (pRL Renilla Luciferase vector). Luminescence signals were quantified using a Dual-Glo Luciferase Reporter Assay System (Promega). The signal was first normalized to transfection efficiency using Renilla luciferase levels, and further divided by the value obtained by the empty bait and prey vector. The mean and SD of more than three independent experiments are shown. Statistical analysis was done using Student's t test, and p-values are indicated.

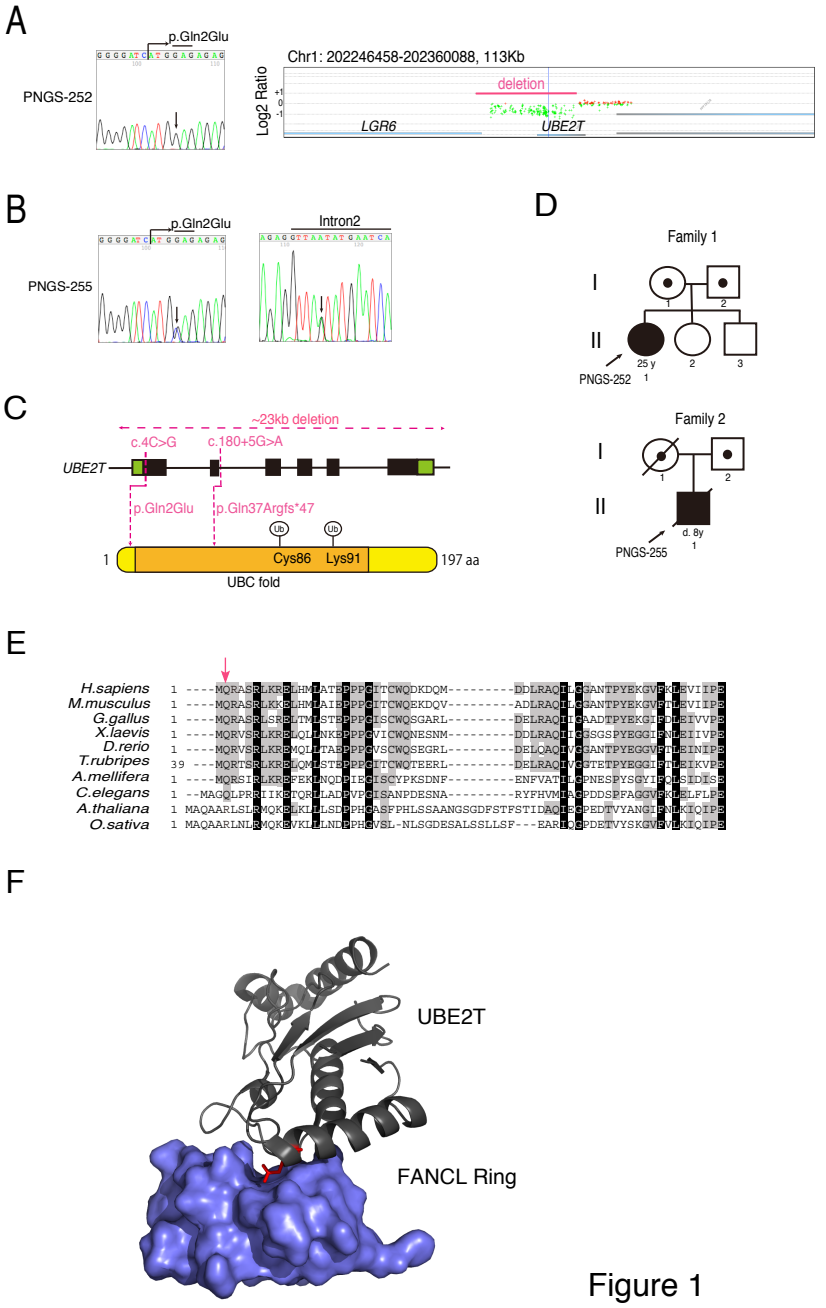
(B) Purified recombinant proteins detected by Coomassie brilliant blue (CBB) gel staining. Human UBE2T proteins with or without the p.Gln2Glu substitution were

produced in *E.coli*, and purified to homogeneity as previously described[11]. WT, wild type.

**(**C) *In vitro* pull-down assay between GST-human (h)FANCL and hUBE2T protein with or without the p.Gln2Glu substitution. Purified human UBE2T (6 μg) and GST-chicken FANCL or human FANCL (9 μg) were incubated at 37ºC for 1 h in 200 μl of reaction buffer containing 20 mM Tris-HCl (pH 7.5), 10% glycerol, 100 mM NaCl, 1 mM ZnOAc, 0.01% NP-40, and 5 mM 2-mercaptoethanol. Glutathione Sepharose 4B beads (3 μl; GE Healthcare) were added to the reaction mixtures, and reaction mixtures were gently mixed at 25ºC for 1 h. The beads were then washed twice with 1 ml of reaction buffer. The proteins bound to the beads were separated by 15% SDS-PAGE, and were visualized by Coomassie Brilliant Blue staining. *, read-through products.

(D) *In vitro* FANCD2 monoubiquitination assay in the presence of DNA. Execution of the *in vitro* FANCD2 ubiquitination reaction were as described[11]. As a control, FANCD2 protein carrying a Lys561Arg substitution blocking monoubiquitination was included. CBB, Coomassie brilliant blue. Western blotting with anti-HA (α-HA) was used to detect HA-tagged ubiquitin.

Figure 1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Log2 Ratio
deletion
LGR6
UBE2T
B
p.Gln2Glu
Intron2
PNGS-255
C
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c.180+5G>A
UBE2T
p.Gln2Glu
p.Gln37Argfs*47
Ub
Ub
1
Cys86
Lys91
197 aa
UBC fold
D
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I
II
1
2
25 y
1
2
3
PNGS-252
Family 2
I
II
1
2
d. 8y
1
PNGS-255
E
H.sapiens
M.musculus
G.gallus
X.laevis
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A.mellifera
C.elegans
A.thaliana
O.sativa
F
UBE2T
FANCL Ring

Figure 2

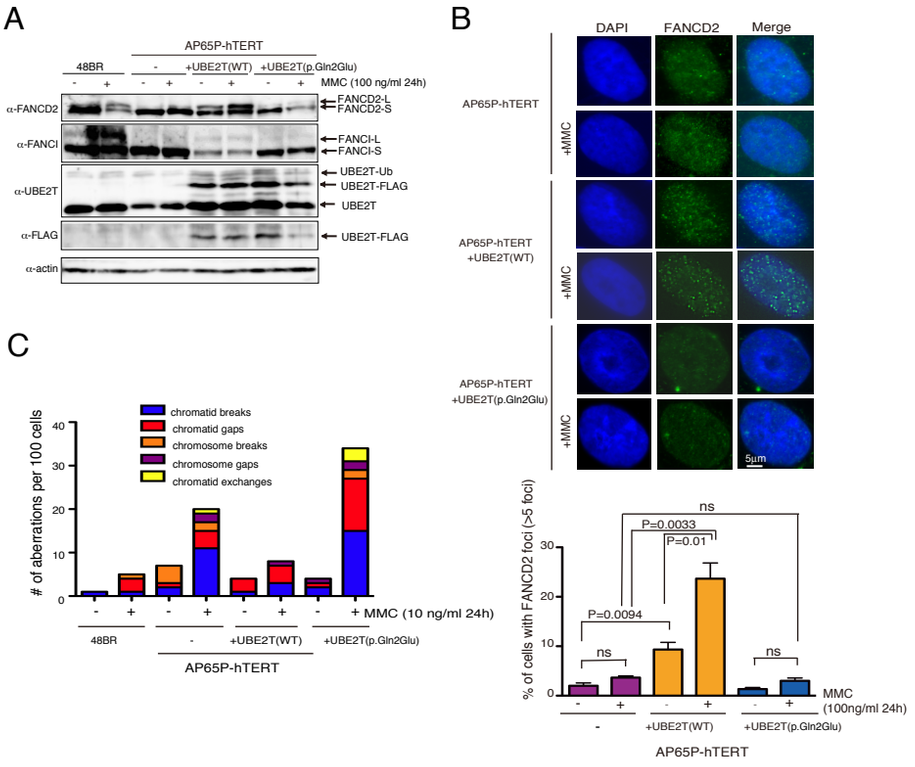


Figure 2

Figure 3

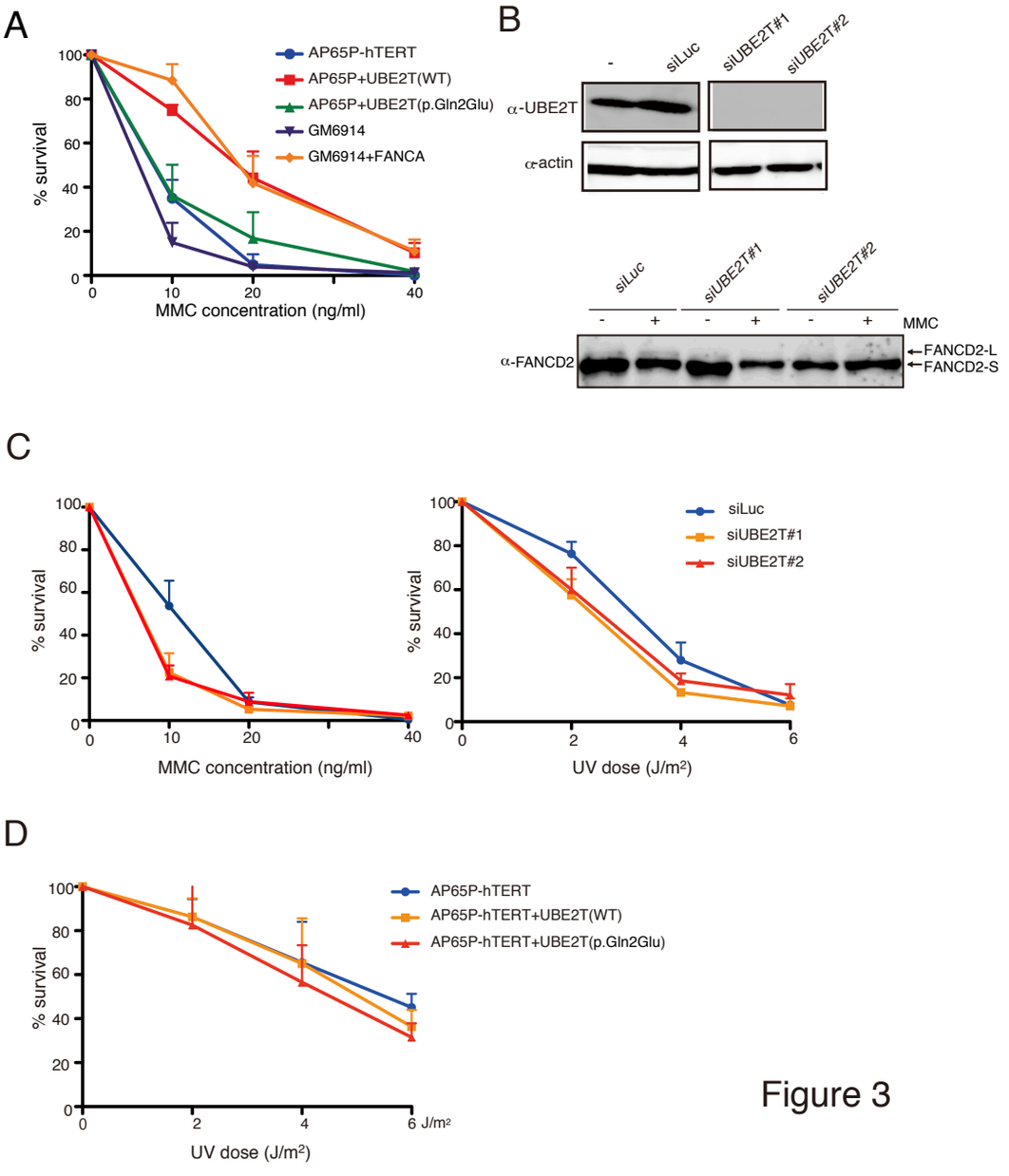


Figure 3

Figure 4

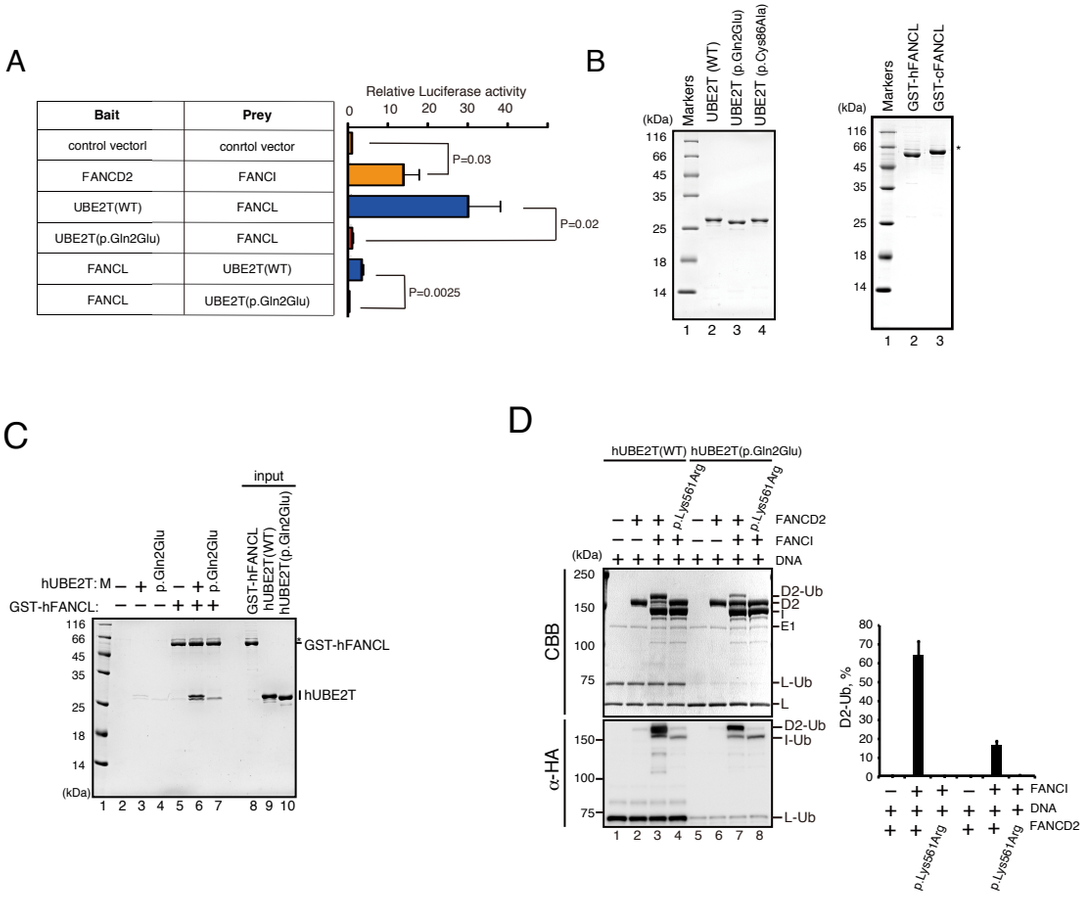


Figure 4

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