A homozygous missense variant in *UBE2T* is associated with a mild Fanconi anemia phenotype

Laura Schultz-Rogers,[1] Francis P. Lach,[2*] Kimberly A. Rickman,[2] Alejandro Ferrer,[1] Abhishek A. Mangaonkar,[3] Tanya L. Schwab,[4] Christopher T. Schmitz,[4] Karl J. Clark,[4] Nikita R. Dsouza,[5] Michael T. Zimmermann,[5,6] Mark Litzow,[3] Nicole Jacobi,[7] Eric W. Klee,[1,8] Agata Smogorzewska[2#] and Mrinal M. Patnaik[3#]*

[1]Center for Individualized Medicine, Mayo Clinic, Rochester, MN; [2]Laboratory of Genome Maintenance, The Rockefeller University, New York, NY; [3]Department of Hematology, Mayo Clinic, Rochester, MN; [4]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN; [5]Bioinformatics Research and Development Laboratory, Genomics Sciences and Precision Medicine Center, Medical College of Wisconsin, Milwaukee, WI; [6]Clinical and Translational Sciences Institute, Medical College of Wisconsin, Milwaukee, WI; [7]Department of Hematology Oncology, Hennepin County Medical Center, Minneapolis, MN and [8]Department of Clinical Genomics, Mayo Clinic, Rochester, MN, USA

**LS-R and FPL contributed equally as co-first authors.*

#EWK, AS and MMP contributed equally as co-senior authors.

Correspondence: MRINAL PATNAIK - patnaik.mrinal@mayo.edu

AGATA SMOGORZEWSKA - asmogorzewska@rockefeller.edu

doi:10.3324/haematol.2020.259275

Supplemental Information

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* # These authors contributed equally
†Co-corresponding authors
[1]Center for Individualized Medicine, Mayo Clinic, Rochester, MN 55905, USA
[2]Laboratory of Genome Maintenance, The Rockefeller University, New York, NY 10065, USA
[3]Department of Hematology, Mayo Clinic, Rochester, MN 55905, USA
[4]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905, USA
[5]Bioinformatics Research and Development Laboratory, Genomics Sciences and Precision Medicine Center, Medical College of Wisconsin, Milwaukee, WI 53226, USA
[6]Clinical and Translational Sciences Institute, Medical College of Wisconsin, Milwaukee, WI 53226, USA
[7]Department of Hematology Oncology, Hennepin County Medical Center, Minneapolis, MN 55404, USA
[8]Department of Clinical Genomics, Mayo Clinic, Rochester, MN 55905, USA

Supplemental information includes:

1. **Methods**
2. **Two Supplemental Figures**
3. **Six Supplemental Tables**
4. **Supplemental References**

Methods:

Human subjects: The Institutional Review Boards of the Mayo Clinic and the Rockefeller University approved these studies. Written consent was obtained from the subject.

Sequencing: Sequencing for this patient was performed as previously described (1).

Molecular modeling: We used the experimental structure of the human UBE2T:FANCL complex (PDB 4ccg (2, 3)) to assess how the observed patient variant in UBE2T may affect stability or organization of the complex. We used molecular mechanics in Discovery Studio (4) to mutate proline-66 to threonine and obtain the change in the local conformation. Additional measurements of dihedral angles were performed in Chimera(5) and proteins were visualized using PyMOL.

Antibodies: HSPC150/UBE2T aa135-197 (Abcam ab154022), FANCD2 (Novus Biologicals NB100-182), alpha-Tubulin (T9026-.5ML), HA.11 Clone 16B12 (Biolegend 901514), Alexa Fluor 594 goat anti-mouse (Invitrogen A11005), Alexa Fluor 488 goat anti-rabbit (Invitrogen A-11008). Peroxidase AP goat anti-rabbit IgG (Jackson 111-035-003), Peroxidase AP goat anti-mouse IgG (Jackson 115-035-003).

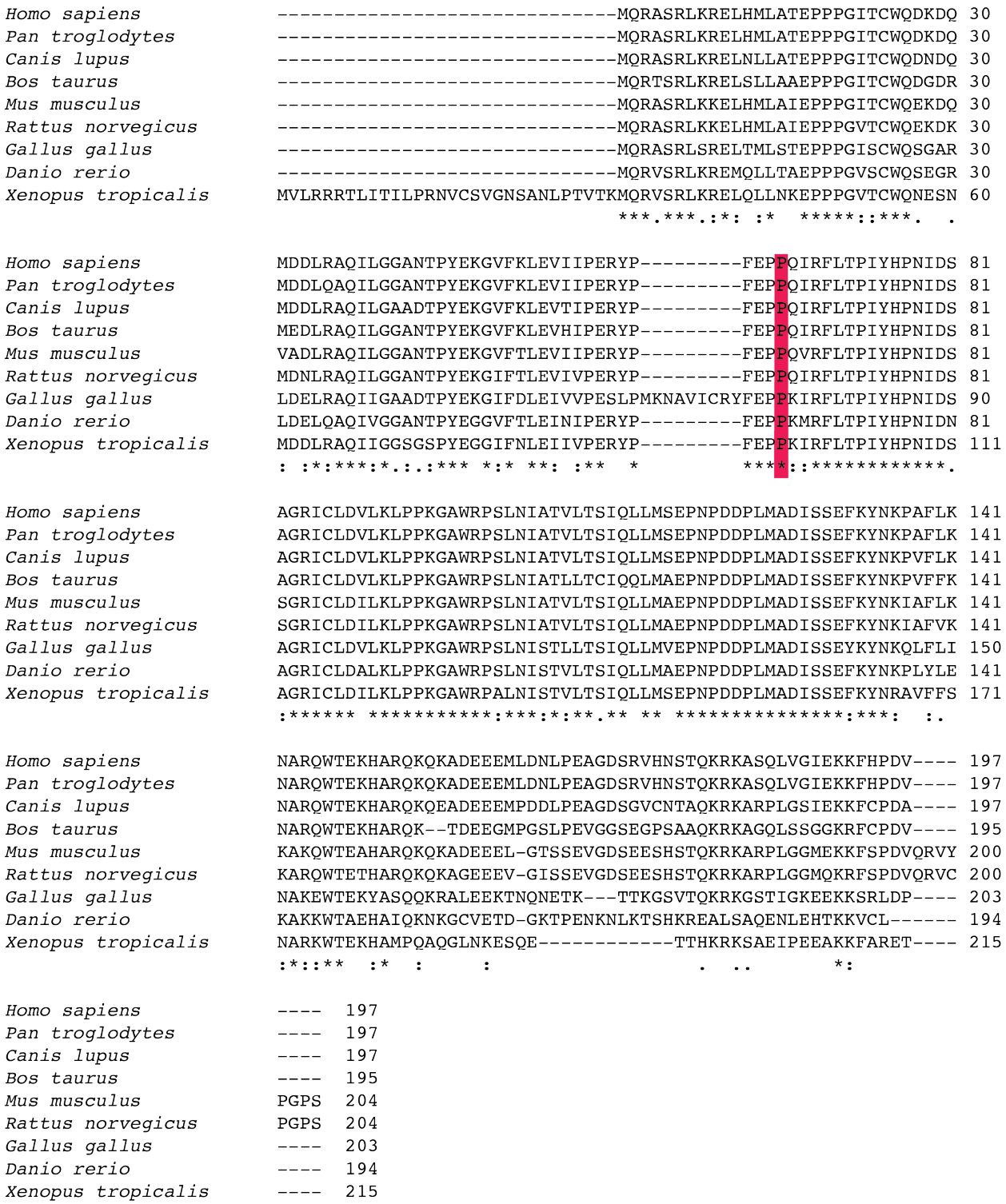
UBE2T cDNAs: UBE2T cDNA was obtained from the Human ORFeome V8.1 Library (GE Healthcare), cloned into pDONR223 and recombined with a pMSCV retroviral vector (MSCV C-HA-FLAG) using Gateway system (Invitrogen), resulting in a C-terminally HA-FLAG tagged UBE2T (MSCV C-HA-FLAG UBE2T) (6). The UBE2T P66T variant cDNA was obtained by subcloning the full-length cDNA from PM085 and recombining into pDONR223 and then into MSCV C-HA-FLAG. Primers used for cloning and sequencing are shown in Table S6.

Cell culture and viral transfection/transduction: Primary fibroblasts from patients PM085, RA2627 (UBE2T/FANCT$^{-/-}$), RA3331 (SLX4/FANCP) (7), RA3226 (BRCA2/FANCD1) (8) from the International Fanconi Anemia Registry), HA239F (RAD50^{mut}) (9) and BJ normal fibroblasts (ATCC) were cultured in Dulbecco Modified Eagle medium (DMEM, Invitrogen) supplemented with 15% FBS (Atlanta Biologicals/BioTechne), 100 units of penicillin per milliliter, 0.1 mg of streptomycin per milliliter, non-essential amino acids, and glutamax (Invitrogen). cDNAs were delivered using retroviral transduction after packaging in HEK293T cells (Mirus). Fibroblasts were transduced in the presence of polybrene (4mg/ml) and selected in puromycin.

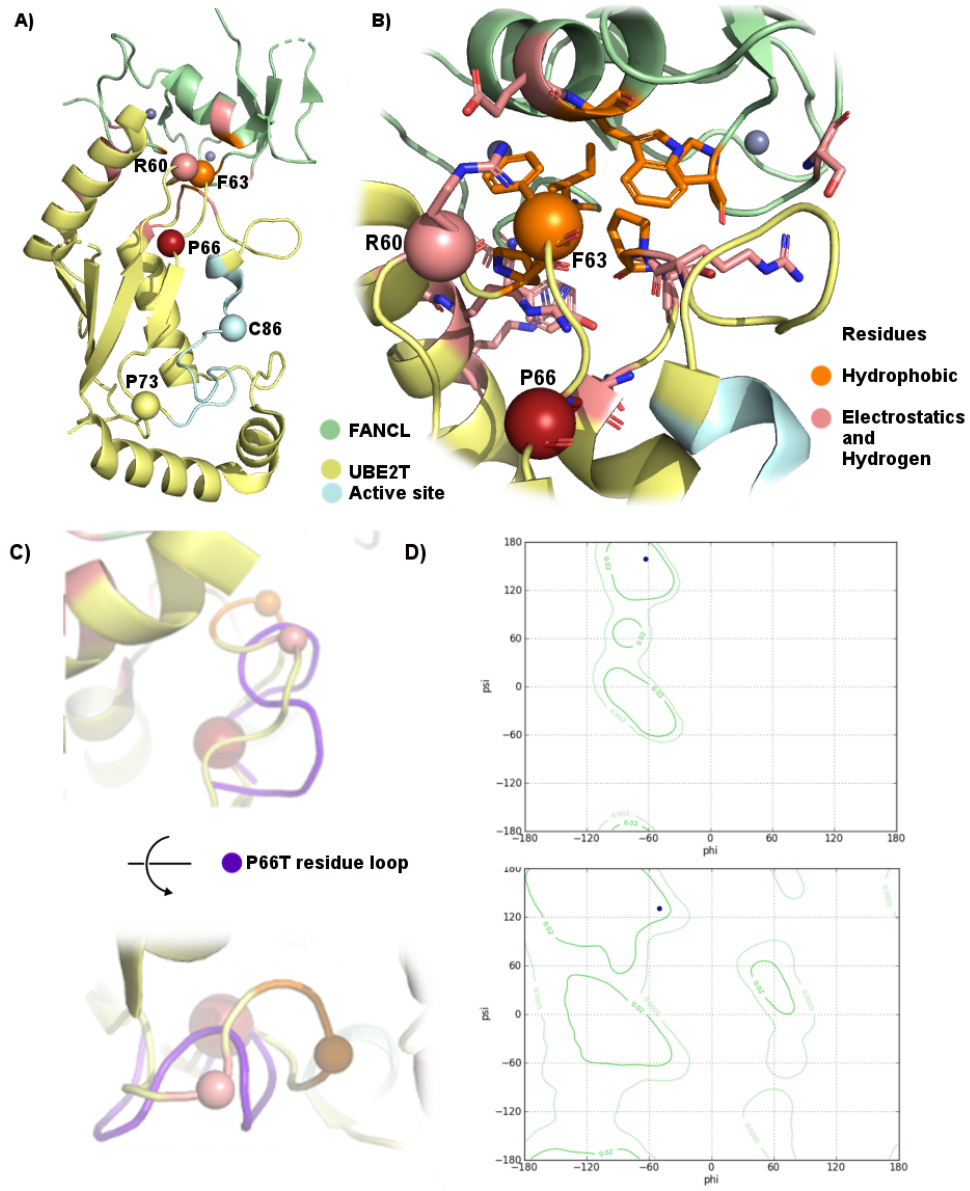
Sensitivity Assays: Transduced primary fibroblasts were seeded overnight and treated next day with mitomycin C (Sigma). Cells were grown for 3 to 4 days, passaged at appropriate ratios, and counted once nearly confluent with a Z2 particle counter (Beckman-Coulter). The percent survival relative to the untreated was then plotted per dose. Experiments were done in triplicate.

Western Blotting: Whole cell extracts were prepared by lysing cell pellets in Laemmli sample buffer (Bio-Rad) followed by sonication. Samples were heated to 100^{o}C for five minutes and run on 4%–12% Bis-tris or 3%–8% tris-acetate gradient gels (Invitrogen).

Immunofluorescence: Cells were fixed in 3.7% formaldehyde, permeabilized with 0.5% NP-40 in PBS, blocked in 0.5% (v/v) BSA, 0.2% cold water fish gelatin in PBS, and incubated with primary antibodies diluted 1:2000 in blocking buffer. Cells were washed and incubated with Alexa Flour secondary antibodies diluted 1:2500. Cells were washed and coverslips were embedded with DAPI Fluoromount-G (SouthernBiotech).



Supplemental Figure 1. Alignment of UBE2T from multiple species. The invariant Proline that corresponds to human Proline 66 is highlighted in red.



Supplemental Figure 2. 3D molecular structure of UBE2T WT (PDB 4ccg(2, 3) and modeled P66T variant. A. The structure of UBE2T:FANCL complex is shown. Represented in spheres are variants that affect FANCL binding or the FANCD2 ubiquitination or both. Highlighted in red is the case variant P66T. **B.** The interaction site of UBE2T and FANCL is displayed with residues interacting with either protein represented in sticks and color indicating the type of interaction. R60E has been previously shown to cause loss of FANCL binding and subsequently a loss of monoubiquitination of FANCD2 (2). F63A has been previously shown to decrease FANCD2 ubiquitination (10)**.** The displacement of the loop when Proline is mutated to Threonine. The loop is shown in the same conformation as previous panels and the loop for P66T shown in purple. The loop has shifted away from the FANCL interface. **D.** Ramachandran plots showing the backbone torsion angle range for (upper) proline compared to (lower) threonine. Threonine has a significantly larger range of motion compared to proline. The blue dot represents the phi/psi angles for P66 and T66, respectively, shown in panel (C).

Table S1: Patient phenotypes associated with biallelic variants in *UBE2T*

Phenotype	Rickman *et al*	Hira *et al* Patient 1	Hira *et al* Patient 2	This report
UBE2T variant(s)	c.-64_468dup c.-64_468del	c.4C>G (p.Gln2Glu), g.202288583-202309772del	c.4C>G (p.Gln2Glu), c.180+5G>A (p.Gln37Argfs*47)	c.196C>A, (p.Pro66Thr)
Gender	Male	Female	Male	Female
Age at presentation	Birth	Birth	Birth	8 years old
Skeletal defects	Bilateral radial aplasia, absent thumbs	Left hypoplastic thumb	Bilateral thumb polydactyly	None
Dysmorphism	Micrognathia	Not reported	Abnormal left ear shape	None
Microcephaly	Yes	Not reported	Not reported	No
Skin findings	Café au lait spots	Not reported	Not reported	Intermittent urticarial rash
Cardiovascular	Ventricular septal defect and patent ductus arteriosus	Not reported	Not reported	None
Other	Absent left Kidney	Abnormalities of external genitalia	Left facial nerve palsy, dysplasia of the middle ear bone	Periodic fevers, menometrorrhagia
Endocrine	Hypothyroidism	Not reported	Not reported	Not reported
Short stature	Yes (5th percentile)	Yes (-2SD)	Not reported	142 cm, <10th centile
Intellectual Disability	No	Not reported	Not reported	No
Hearing loss	Yes, bilateral conductive	No	Yes, deafness	No
Bone age findings	Slightly greater than chronological age	Not reported	Not reported	Not reported
Family History	Thalassemia	Negative	Negative	Negative
Hematological Findings	Thrombocytopenia (resolved shortly after birth); somatic mosaicism, no bone marrow failure	Thrombocytopenia; severe aplastic anemia; bone marrow transplant age 13	Thrombocytopenia; MDS (refractory anemia) evolving to AML and requiring bone marrow transplant, death 5 months post-transplant at age 8	Originally presenting with mild leukopenia and thrombocytopenia; persistent macrocytosis and intermittent cytopenias
Clinical Chromosomal Breakage analysis	Peripheral blood: 5.8 breaks per cell in 85% of cell population, (DEB)*	0.48 breaks per cell, (DEB)	0.91 breaks per cell, (DEB)	Peripheral blood: 1.26 breaks per cell in 58% (MMC) and 0.52 breaks per cell in 36% (DEB) of cells **

Peripheral blood smear	Moderate neutropenia and microcytic red blood cells consistent with thalassemia trait	n/a	n/a	Round macrocytes and-or target cells are present
Bone marrow aspirate	Mildly hypocellular (35-45%) with trilineage hematopoiesis. No abnormal clones or leukemia	n/a	Cytogenetic analysis of bone marrow revealed complex karyotypes with a 3q abnormality	Moderately hypocellular (40-50%) with no evidence for dysplasia or a lymphoproliferative process. Normal cytogenetics

*Reference range for FA positive control for this report was 1.06-23.9 mean chromosome breaks per cell after DEB treatment.
**Reference range for FA positive control in this study was 0.56-12.52 aberrations per cell after MMC, and 0.42-13.24 aberrations per cell after DEB treatment.

Table S2: Most recent hematological findings for reported patient

Hb	12.8 gm/dL
WBC	2.9 x10(9)/L
ANC	1.4 x 10(9)/L
Platelets	175 x 10(9)/L
MCV	106.6

Table S3: Periodic fever gene panel

Gene
MEFV
MUK
LPIN2
TNFRSF1A
NLRP3
MPSTP1P1

Table S4 Custom targeted exome sequencing panel designed in collaboration with GeneDx for inherited bone marrow failure/unexplained cytopenias

Gene	Coverage	Gene	Coverage	Gene	Coverage
ABCG5	100%	DNAJC21	100%	KRAS	100%
ABCG8	100%	DNMT3B	100%	LAMTOR2	100%
ACD	100%	DOCK8	100%	LIG4	100%
ACTN1	99.20%	ELANE	100%	LRBA	99.30%
ADA	100%	ERCC4	100%	MAGT1	100%
ADAMTS13	100%	ERCC6L2	100%	MECOM	100%
ALAS2	100%	ETV6	100%	MPL	100%
ANKRD26	99.60%	FADD	100%	MYH9	100%
AP3B1	100%	FANCA	100%	NAF1	96.10%
ATM	100%	FANCB	99%	NBEAL2	99.40%
BLM	100%	FANCC	100%	NBN	100%
BLOC1S6	100%	FANCD2	100%	NHEJ1	100%
BRCA1	100%	FANCE	100%	NHP2	100%
BRCA2	100%	FANCF	100%	NOP10	100%
BRIP1	100%	FANCG	100%	NPAT	100%
C3	100%	FANCI	100%	NRAS	100%
CARD11	100%	FANCL	100%	ORAI1	99.40%
CASP10	100%	FANCM	98.60%	PALB2	100%
CASP8	100%	FAS	100%	PARN	100%
CBL	99.80%	FASLG	100%	PAX5	100%
CD27	100%	FLI1	100%	PIK3CD	100%
CD3D	100%	FLNA	100%	PNP	100%
CD3E	100%	FOXN1	100%	POT1	100%
CD40LG	100%	FOXP3	100%	PRF1	100%
CD46	97%	FYB	97.30%	PRKACG	100%
CFB	100%	G6PC3	100%	PTPRC	97.70%
CFH	99.80%	GAR1	100%	RAB27A	100%
CFHR1	94.10%	GATA1	100%	RAC2	100%
CFHR3	100%	GATA2	100%	RAD50	99.60%
CFHR4	100%	GFI1	100%	RAD51C	100%
CFHR5	99.70%	GFI1B	100%	RAG1	100%
CFI	100%	GP1BA	100%	RAG2	100%
CHEK2	100%	GP1BB	100%	RBM8A	100%
CSF3R	100%	GP9	100%	RECQL4	100%
CTC1	100%	HAX1	100%	RPL11	100%
CTLA4	100%	HOXA11	100%	RPL15	82.10%
CXCR2	100%	IKZF1	10.30%	RPL26	100%
CXCR4	100%	IL2RG	100%	RPL35A	100%
CYCS	100%	IL7R	100%	RPL5	100%
DCLRE1B	100%	ITGA2B	100%	RPS10	100%
DCLRE1C	100%	ITGB3	100%	RPS19	100%
DDX41	100%	ITK	100%	RPS24	100%
DGKE	100%	JAGN1	100%	RPS26	100%
DKC1	100%	JAK3	99.30%	RPS7	99.30%

Gene	Coverage
RTEL1	100%
RUNX1	100%
SAMD9	100%
SAMD9L	100%
SBDS	100%
SH2D1A	100%
SIRT1	100%
SIRT4	100%
SRP54	100%
SRP72	100%
STAT3	100%
STAT5B	100%
STIM1	100%
STK4	100%
SIRT5	100%
SLC37A4	81.10%
SLC7A7	100%
SLFN14	100%
SLX4	100%
SRC	100%
STN1 [OBFC1]	100%
STX11	100%
STXBP2	100%
TAZ	100%
TBX1	91.10%
TCIRG1	100%
TERC	100%
TERF1	100%
TERF2IP	100%
TERT	100%
THBD	100%
TINF2	100%
TNFRSF13B	100%
TUBB1	100%
UBE2T	100%
UNC13D	100%
USB1	100%
VHL	100%
VPS13B	100%
VPS45	100%
VWF	98.10%
WAS	100%
WIPF1	100%
WRAP53	100%

Gene	Coverage
XIAP	100%
XRCC2	100%
ZAP70	100%

Table S5: *in silico* predictions for pathogenicity of p.Pro66Thr UBE2T variant

***in silico* Tool**	**Prediction of Variant Effect**
SIFT	Deleterious (score: 0)
MutationTaster	Disease causing (prob: 1)
PolyPhen2	Probably damaging (score: 1)
MCAP	Possibly pathogenic (score: 0.091)
PredictSNP2	Deleterious (87% expected accuracy)
CADD Score	31

Table S6. Primers used in the study

Name	Sequence
FPL474 cDNAF	GCGTTGCTGCGTTGTGAGG
FPL475 cDNAR	TTTCAGGTTTAAAAGATTTCAAAATACATA
FPL476 cDNAseq1F	GCATCCCAGGCAGCTCTTAGTGT
FPL756 UBE2Tex4F	CCCACCCTCCACCCTCAG
FPL757 UBE2Tex4R	TCAACCATTTACCCACAACTCACT
FPL758 UBE2Tex4F Seq	AAAAACTGGGGAGAACAACTGA
FPL759 UBE2T att B Fwd no stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCAGAGA GCTTCACGTCTGAAG
FPL760 UBE2T att B Rvs no stop	GGGGACCACTTTGTACAAGAAAGCTGGGTCAACATCAGG ATGAAATTTCTTTT

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