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Homozygous OB-fold variants in telomere protein TPP1 are associated with dyskeratosis congenita like phenotypes

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To the Editor

Dyskeratosis congenita (DC) and its severe form Hoyeraal-Hreidarsson syndrome (HHS) are rare and have life threatening failure of hematopoiesis. Typically, DC patients present with disease features such as nail dystrophy, oral leukoplakia and abnormal skin pigmentation along with peripheral pancytopenia and marrow hypoplasia with strong predisposition to cancer.[1] In DC, hematopoietic failure occurs due to critical shortening of telomeres[2,3] enhancing DNA damage response[4,5] leading to premature senescence of hematopoietic stem cells.[6]

Telomeres are lengthened by the enzyme telomerase, which constitutes RNA template (TERC) and the catalytic reverse transcriptase (TERT). Variants affecting telomerase complex (*TERT* and *TERC*), telomerase stability (*DKC1, NOP10, NHP2, PARN and NAF1*), telomerase trafficking (*WRAP53*), and telomere replication (*CTC1* and *RTEL1*) have been identified in majority of DC and HHS cases. The shelterin complex proteins (TRF1, TRF2, RAP1, TIN2, POT1 and TPP1) that serve to protect telomeres are critical for telomerase function.[7] The first DC variants to be described in a shelterin component were found in *TINF2*.[8] Variants in *ACD* encoding TPP1 were later described in two independent families,[9,10] The first was in a family with a history of aplastic anemia, in which the index case had pancytopenia at 8 years of age and was heterozygous for the *ACD* variant c.499-501del; p.K170 del (K170Δ) that segregated as an autosomal dominant trait.[9] The proband's mother presented with thrombocytopenia in her twenty's and was later diagnosed with myelodysplasia. Her grandmother had mild macrocytic anaemia associated with hypocellular bone marrow. The second case, reported by Kocak et al in 2014,[10] had several features of HHS, had the same K170Δ variant on one allele along with c.1471 C>T; p.P491T on the other, suggesting an autosomal recessive inheritance.[10] It is of interest that in this family, the proband's father, who carried the K170Δ variant, had short telomeres but lacked any disease features. Structural and functional studies of K170Δ have not demonstrated a dominant negative effect on TPP1 function, but instead it is suggested that dosage of TEL patch (patch of amino acids involved in telomerase binding on the surface of TPP1) is responsible for telomere shortening.[11,12]

The evidence in support of causality in the BMF patients is largely derived from functional analysis of the variants themselves, rather than being statistically derived or based on the strength of a significant allelic series.[9-11] It remains to be established as to how loss of function (LOF) variants in *ACD* (frameshift, splice donor/acceptor or stop gain) can have a significant heterozygous frequency in the Genome aggregation database (gnomAD), where 57 of these variants are described (representing ~6 in 10,000 individuals). Given this frequency of heterozygous LOF *ACD* variants in the control population, together with the variable pattern of inheritance and very different clinical presentations in the only two families described to date, the case for *ACD* as a proven disease-causing locus remains uncertain.

By whole exome sequencing in a series of genetically uncharacterised patients (n=228) presenting with DC or constitutional bone marrow failure (BMF) from our DC Registry, we identified nonsynonymous *ACD* variants in 5 unrelated cases (supplemental Table 1). In two of these (index cases of families 1 and 2, Figure 1A) the *ACD* variants were homozygous (supplemental Table 1). The index case of family 1 harbors the homozygous variant c.280C>T;

p.V94I, which has been reported once in the homozygous state and 191/270022 alleles in heterozygous state on gnomAD. This index case, aged 38 years had thrombocytopenia, short stature, pulmonary abnormalities and limbal stem cell deficiency (LSCD; supplemental Table 2 and supplemental Figure 1A-B). His older sister, who was heterozygous for this variant had short stature, pulmonary abnormalities and LSCD but no hematopoietic defect (supplemental Table 2). The index case from family 2 had a novel homozygous missense variant, c.284T>A, p.L95Q that has not been reported on gnomAD. The index case, aged 12 years presented with leukoplakia and subsequently developed BMF and immunodeficiency (supplemental Figure 1C-E and supplemental Table 2). His older brother had died of aplastic anemia (supplemental Table 2). No other obvious candidate genes that are previously known to cause BMF are identified in these two cases (supplemental Table 3). Both homozygous variants identified in families 1 and 2 are predicted to be damaging by CADD score (supplemental Table 1) and affect the highly conserved oligonucleotide binding (OB)-fold domain in TPP1 that is implicated in telomerase binding, recruitment and function at telomeres (Figure 1B and supplemental Figure 3C).[10,12]

Telomere length measurement by MMqPCR[13] revealed short telomeres in the index case of family 1, just below the tenth centile when compared to his heterozygous sister and controls (n=218; Figure 1C and supplemental Table 2). Index case from family 2 had very short telomeres, below the first centile as measured by MMqPCR (Figure 1C) and Flow-FISH method (supplemental Figure 2). His heterozygous parents also had short telomeres (Figure 1C, supplemental Figure 2 and supplemental Table 2) but were asymptomatic. Telomere labelling using a Tel-Cy3 probe (PNA bioscience) on metaphase spreads of EBV-transformed B-lymphoblasts (LCLs) derived from the index case of family 2 (L95Q) revealed reduced telomere signal when compared to age matched control (Figure 1D). Telomere chromatin immunoprecipitation (ChIP) assay in TPP1 shRNA treated HEK293 cells expressing TPP1-L95Q variant (supplemental; Figure 3A and B), revealed no significant change in its association with telomeres (Figure 1E). Furthermore, mimicking TPP1-L95Q variant in *S. pombe* (*Tpz1-L5Q*) also induced telomere shortening (Figure 1F and supplemental Figure 3C and D).[15] These data suggest that the TPP1-L95Q OB-fold variant interferes with telomere maintenance despite localising to telomeres.

The TEL patch in the OB-fold domain is essential for telomerase recruitment at telomeres (Figure 2A).[12,16] TPP1 OB-fold crystal structure reveals both V94 and L95 residues that are mutated in our cases are present in the N terminus of OB-fold (NOB) domain, forming a hydrophobic cleft that extends the TEL patch (Figure 2A).[12, 14] Mutating both these residues simultaneously reduces telomerase processivity, abrogates telomerase recruitment to telomeres, and shortens telomeres of human cells in culture.[14] In line with this, co-immunoprecipitation and quantitative TRAP analysis in HeLa nuclear lysates, demonstrated that both TPP1-V94I and TPP1-L95Q have impaired ability to bind TERT (Figure 2B and C) and reduced TPP1 associated telomerase activity in comparison to wild-type, when overexpressed (Figure 2D and E). These results indicate that both OB-fold residues V94 and L95 participate in TPP1–telomerase interaction and regulate telomerase activity. Telomerase association with TPP1 OB-fold, is important for its maturation, traffic and recruitment to telomeres via Cajal bodies.[17] In HeLa cells, when over expressed TPP1-L95Q showed diffused punctate in the nucleoplasm, which do not colocalise with coilin that resides in Cajal bodies (Figure 2F and G).

This is similar for TPPI-K170Δ and the other artificial TEL patch variants described previously.[9-12,14] Interestingly abnormal cytoplasmic staining of TPP1-L95Q (Figure 2H and I) and reduction in telomerase activity is observed in the patient cells (Figure 2J), indicating defects in telomerase maturation pathway.

In summary, we report the first instance of homozygous TPP1 OB-fold variants in patients from two unrelated families who have overlapping phenotypes with DC.[18] As these variants cluster in the TEL patch of TPP1, which is essential for telomerase recruitment to telomeres, they provide *in-vivo* evidence in humans for the biological importance of this TPP1 OB-fold domain.

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Author contribution

T.V. and I.D. are the principal investigators; H.T. and L.C performed most of the experiments; A.J.W., A.E., Z.S., V.P., J.F., K.T., and S.C contributed to the experiments. N.Y., T.A. and J.T. performed clinical analysis; and H.T., T.V., and I.D. wrote the manuscript.

Conflict-of-interest disclosure

None.

References

1. Collins J, Dokal I. Inherited BMF syndromes. *Hematology*. 2015; 20, 433-434.
2. Gramatges MM, Bertuch AA. Short telomeres: from dyskeratosis congenita to sporadic aplastic anemia and malignancy. *Transl Res*. 2013;162, 353–363.
3. Savage SA. Human telomeres and telomere biology disorders. *Prog Mol Biol Transl Sci*. 2014; 125, 41–66.
4. Kirwan M, Beswick R, Walne, AJ, et al. Dyskeratosis congenita and the DNA damage response. *Br J Haematol*. 2011; 153, 634–643.
5. Pereboeva L, Westin E, Patel T, et al. DNA damage responses and oxidative stress in dyskeratosis congenita. *PLoS One*. (2013); 8, e76473.
6. Townsley DM, Dumitriu B, Young NS. BMF and the telomeropathies. *Blood*. 2014; 124, 2775-2783.
7. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev*. 2005; 19, 2100-2110.
8. Savage SA, Giri N, Baerlocher GM, Orr N, Lansdorp PM and Alter BP. TINF2, a component of the shelterin telomere protection complex, is mutated in dyskeratosis congenita. *Am. J. Hum. Genet*. (2008); 82, 501-509.
9. Guo Y, Kartawinata M, Li J, et al. Inherited BMF associated with germline mutation of ACD,

the gene encoding telomere protein TPP1. *Blood*. 2014;124, 2767-2774.
10. Kocak H, Ballew BJ, Bisht K, et al. Hoyeraal-Hreidarsson syndrome caused by a germline mutation in the TEL patch of the telomere protein TPP1. *Genes Dev*. 2014; 28, 2090-2102.
11. Bisht K, Smith EM, Tesmer VM and Nandakumar J. Structural and functional consequences of a disease mutation in the telomere protein TPP1. *Proc. Natl. Acad. Sci. USA*. 2016; 113, 13021-13026.
12. Nandakumar J, Bell CF, Weidenfeld I, Zaug AJ, Leinwan L.A, Cech TR. The TEL patch of telomere protein TPP1 mediates telomerase recruitment and processivity. *Nature*. 2012; 492, 285-289.
13. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitation PCR method. *Nucleic Acids Res*. 2009; 37, e21.
14. Grill S, Tesmer VM, Nandakumar J. The N Terminus of the OB Domain of Telomere Protein TPP1 Is Critical for Telomerase Action. *Cell Rep*. 2018; 22 :1132-1140.
15. Armstrong CA, Pearson SR, Amelina H, Moiseeva V. and Tomita, K. Telomerase activation after recruitment in fission yeast. *Curr. Biol*. 2014; 24**,** 2006-2011.
16. Wang F, Podell ER, Zaug AJ, et al. The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature.* 2007; 445:506-510.
17. Zhong FL, Batista LF, Freund A, Pech MF, Venteicher A, Artandi SE. TPP1 OB-fold domain controls telomere maintenance by recruiting telomerase to chromosome ends. *Cell*. 2012; 150, 481-494.
18. Aslan D, Akata RF, Holme H, Vulliamy T, Dokal I. Limbal stem cell deficiency in patients with inherited stem cell disorder of dyskeratosis congenita. *Int Ophthalmol*. 2012; 32:615-622.

Foot notes

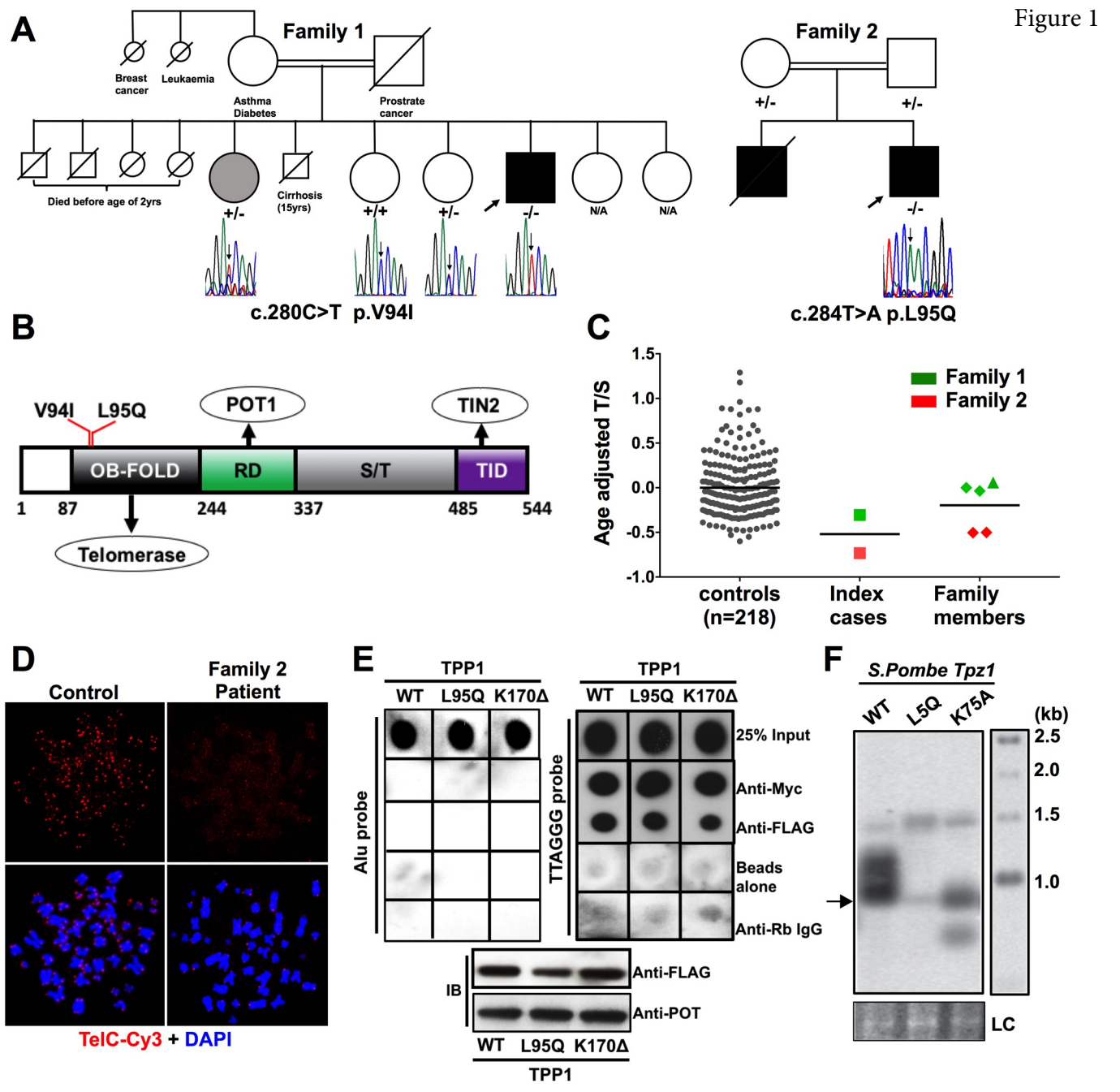
H.T. and L.C. contributed equally to this work.

Figure legends

Figure 1. (A) Family trees showing the segregation of *ACD* variants. -/-, homozygous variant; +/-, heterozygous; +/+, wild type. Arrows indicate index cases that underwent whole exome sequencing. Grey colour indicates presence of some somatic features [short stature and limbal stem cell deficiency (LSCD)] but no hematopoietic abnormality. N/A; DNA not available (B). TPP1 topology showing three functional domains; telomerase interacting OB-fold, POT1 recruitment domain (RD) and TIN2 interacting domain (TID) separated by serine threonine (S/T) motif. (C) Whole blood telomere lengths of index cases and family members are indicated (family 1 in green and family 2 in red). Telomere lengths are reduced in index cases (squares) when compared with heterozygous (diamond) and wild type (triangle) family members and controls (n = 218). (D) Telomere FISH of EBV-transformed lymphoblasts derived from index case in family 2 and a healthy control. (E) Telomere ChIP of myc- POT1, FLAG- TPP1 wild type (WT) and OB-fold variants, expressed in HEK293 cells. Dot blot shows input chromatin used in each immunoprecipitation (top). Immunoprecipitation with an anti-POT1 antibody, anti-FLAG antibody, anti-rabbit (Rb) IgG control and protein G beads alone. Expression of TPP1 WT and

variants is demonstrated by immunoblotting in the bottom panel. TPP1 p.K170Δ variant was used as positive control as it has been previously shown to bind to telomeres.[9,10] (F) Telomere lengths of *S. pombe* strains harboring Tpz1 (orthologue of human *ACD*/TPP1) OB-fold variants. Tpz1 K75A yeast strains with previously confirmed short telomeres is used as a positive control.[15] Arrow indicates extremely faint telomeric band due to presence of very telomeric DNA present in L5Q strain. LC refers to loading control of genomic DNA stained by ethidium bromide. WT refers to wildtype.

Figure 2. (A) *In silico* model of the TPP1 OB-fold crystal structure (PDB id: 2I46)[16] using UCSF CHIMERA, an extensible molecular modelling software. Previously described TPP1-TEL patch residues are denoted in black [12] and the patient variants in BMF patients identified to date are denoted in red. OB-fold ribbon structure is depicted in blue and surface hydrophobicity preset is shown with amino acid hydrophobicity in the Kyte-Doolittle scale with colors ranging from dodger blue for the most hydrophilic to white at 0.0 to orange for the most hydrophobic. 'NOB' refers to N terminus of OB-fold domain that is recently described in telomerase binding.[14] (B and C) Immunoblotting of FLAG pull down (PD) complexes from nuclear extracts of HEK293 cells that are treated with TPP1 3'UTR shRNA and expressing shRNA resistant FLAG-TPP1 variants along with TERT and POT1. 'IN' refers to 15% Input. Note the reduction in TERT signal pulled by the TPP1 OB-fold variants compared to input and WT. (D and E) HeLa cells expressing Flag-tagged wild-type TPP1 were analyzed for TPP1-associated endogenous telomerase activity by immunoprecipitation and subsequent TRAP analysis. Results from TRAP assay were normalized based on the amount of eluted TPP1 protein. (F and G) Double immunofluorescence was used to detect the FLAG-TPP1 proteins (green) and coilin (a Cajal body marker, red) in HeLa cells. Yellow (merged green + red) spots indicated by arrows show co-localization of TPP1 with coilin are quantified by counting cells from different fields of view (n=2). (H and I) Endogenous TPP1 co-localisation with coilin in control and patient (TPP1 p.L95Q) B lymphoblastoid cells; WT refers to wildtype. (J) Relative levels of telomerase activity in the patient and age matched control B lymphoblastoid cells at passage 5 were determined by TRAP assay.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Family 1
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Leukaemia
Asthma
Diabetes
Prostrate cancer
Died before age of 2yrs
Cirrhosis (15yrs)
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c.280C>T p.V94I
Family 2
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controls (n=218)
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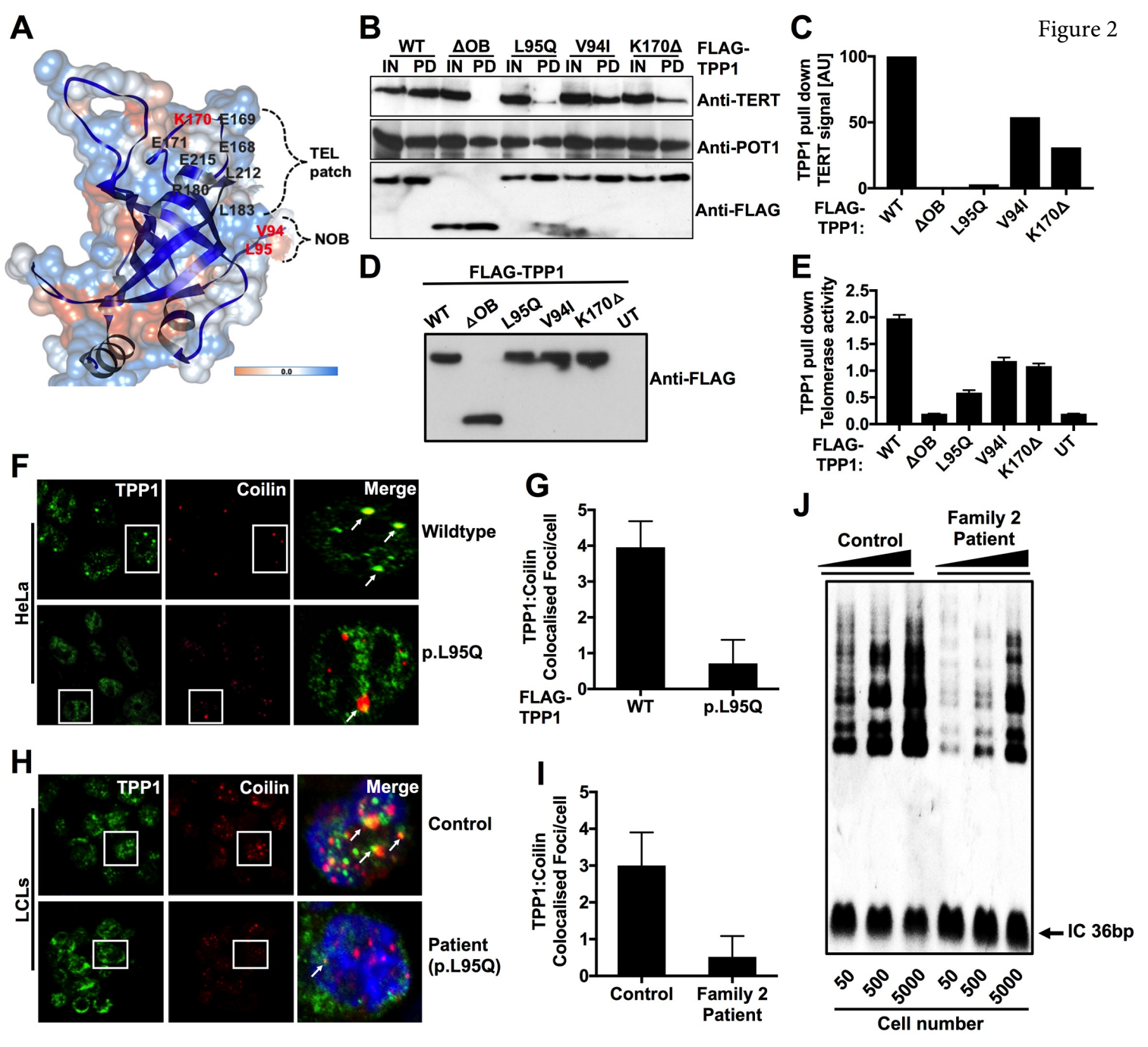
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Wildtype
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TPP1:Coilin Colocalised Foci/cell
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Cell number

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