Supplemental Data

Supplemental table 1. Rare nonsynonymous *TPP1* variants in BMF exome cohort

Family	Nucleotide substitution	Amino acid substitution	CADD score	gnomAD	Zygosity
1	c.280C>T	p.Val94Ile	12.74	1 report only 191/270022	Hom Het
2	c.284T>A	p.Leu95Gln	24.20	NR	Hom
3Ψ	c.131G>T*	p.Gly44Val	0.15	5/233084	Het
4∫	c.365A>G*	p.Gln122Arg	3.10	NR	Het
5ϕ	c.871A>G*	p.Thr291Ala	23.20	358/276656	Het

Hom, homozygous; Het, Heterozygous; significance; CADD, Combined Annotation Depletion Dependent PHRED score; gnomAD, allele frequency in the Genome Aggregation Database; NR= not reported. ∗ indicates variant of unknown significance. ϕ This case has been previously identified with homozygous *PARN* mutation.[1] Ψ This case has been identified with compound heterozygous mutations in the *TERT* gene. ∫ This case has been identified to have rare heterozygous variant of unknown significance in *TERT*, and *CTC1* genes.

Supplemental table 2. Features of individuals in families 1 and 2

Family	1	1	2	2
Family member	Index case	Elder sister	Index case	Elder brother
ACD variant	c.280C>T; p.V94I homozygous	c.280C>T; p.V94I heterozygous	c.284T>A; p.L95Q homozygous	?
Gender	Male	Female	Male	Male
Ethnic origin	Turkish	Turkish	Turkish	Turkish
Age[a]	38	60	12	8
Parents first cousins	Yes	Yes	Yes	Yes
Bone marrow failure	Yes[b]	No	Yes[c]	Yes[d]
Hemoglobin (g/l)	148	Normal[#]	116	60
Wbc ($x10^9$/l)	6.6	Normal[#]	3.2	0.6
Platelets ($x10^9$/l)	100	Normal[#]	65	5
Short stature	Yes[e]	Yes[f]	No	No
Pulmonary abnormalities	Yes, Asthma	Yes, Fibrosis	No	No
Limbal stem cell deficiency	Yes	Yes	No	No
Leucoplakia	No	No	Yes	No
Other features	Yes[g]	Yes[h]	Yes[i]	No
Telomere length	Short	Normal	Very short	?

[a]In years, at initial investigation; [b]normal hemoglobin and Wbc but low platelets; [c]hypocellular bone marrow (supplementary figure) associated with pancytopenia; [d]died of severe aplastic anemia (hypocellular bone marrow) aged 8 years; [e]height 1.55m (SDS -2.76); [f]height 1.49m (SDS -1.66); [g]extensive dental caries, cataracts, avascular necrosis of hips; [h]diabetes mellitus and cataracts; [i]immune deficiency, low B-lymphocytes and IgM levels; Wbc, white blood cell count; [#]blood count was reported as normal on local medical records but precise values were not recorded; ?, unknown (DNA not available).

Supplemental table 3
Rare homozygous variants in the index cases from families 1 and 2:

Family 1- index case

HUGO	Gene name	Variation in canonical transcript	gnomAD frequency
CD36	CD36 molecule (thrombospondin receptor)	ENST00000435819:c.T1079G:p.L360X	1.99E-04
NENF	neudesin neurotrophic factor	ENST00000366988:c.501_502insA:p.D167fs	2.17E-05
POSTN	periostin, osteoblast specific factor	ENST00000379747:c.G1388A:p.R463H	1.47E-04
HCCS	holocytochrome c synthase	ENST00000321143:c.C803T:p.S268L	1.68E-05
DENND4A	DENN/MADD domain containing 4A	ENST00000443035:c.G1237A:p.V413I	3.23E-05
ACSBG1	acyl-CoA synthetase bubblegum family member 1	ENST00000258873:c.G1190A:p.R397Q	6.06E-04
LIPE	lipase, hormone-sensitive	ENST00000244289:c.T1466C:p.L489P	4.06E-06
TMEM27	transmembrane protein 27	ENST00000380342:c.A227G:p.N76S	2.40E-05
NUP62	nucleoporin 62kDa	ENST00000596217:c.A995G:p.Q332R	8.17E-06
TNR	tenascin R	ENST00000367674:c.A538C:p.N180H	4.10E-03
BIRC7	baculoviral IAP repeat containing 7	ENST00000217169:c.G764T:p.C255F	1.13E-03
ZNF225	zinc finger protein 225	ENST00000262894:c.A1698T:p.R566S	3.40E-03
FCHO1	FCH domain only 1	ENST00000594202:c.A494G:p.E165G	6.71E-04
PLG	plasminogen	ENST00000308192:c.G2087C:p.R696P	VNF
KLC3	kinesin light chain 3	ENST00000391946:c.C722T:p.S241L	7.36E-04
OFCC1	orofacial cleft 1 candidate 1	ENST00000460363:c.689+1G>A	2.89E-04
VSTM4	V-set and transmembrane domain containing 4	ENST00000332853:c.G559A:p.V187M	4.33E-03
PARP6	poly (ADP-ribose) polymerase family, member 6	ENST00000569795:c.G166A:p.V56I	1.06E-04
SIGLEC10	sialic acid binding Ig-like lectin 10	ENST00000356298:c.G1438C:p.E480Q	VNF
ITIH6	inter-alpha-trypsin inhibitor heavy chain family, member 6	ENST00000218436:c.A271G:p.K91E	8.50E-05
EMC10	ER membrane protein complex subunit 10	ENST00000334976:c.C152T:p.T51M	1.71E-05
CSPG4	chondroitin sulfate proteoglycan 4	ENST00000308508:c.C3872T:p.S1291L	2.67E-03
DGKG	diacylglycerol kinase, gamma 90kDa	ENST00000265022:c.G1214A:p.R405K	1.63E-05
WDFY1	WD repeat and FYVE domain containing 1	ENST00000233055:c.933+3G>T	VNF
DMRT3	doublesex and mab-3 related transcription factor 3	ENST00000190165:c.C649T:p.R217C	4.50E-03
ACD	adrenocortical dysplasia homolog (mouse)	ENST00000393919:c.G280A:p.V94I	7.07E-04
CFI	complement factor I	ENST00000394634:c.A209C:p.N70T	3.66E-05
PIGB	phosphatidylinositol glycan anchor biosynthesis, class B	ENST00000164305:c.418-5T>C	2.67E-04
CSPG4	chondroitin sulfate proteoglycan 4	ENST00000308508:c.C3224T:p.T1075M	4.34E-05
OPHN1	oligophrenin 1	ENST00000355520:c.G133A:p.A45T	9.34E-04
TIMM44	translocase of inner mitochondrial membrane 44 homolog (yeast)	ENST00000595876:c.C376T:p.L126F	8.80E-04
TLR5	toll-like receptor 5	ENST00000540964:c.G2318A:p.S773N	5.69E-05
PID1	phosphotyrosine interaction domain containing 1	ENST00000354069:c.G95C:p.R32P	3.26E-03
PNKP	polynucleotide kinase 3'-phosphatase	ENST00000322344:c.G1519T:p.V507L	4.06E-06
MUC16	mucin 16, cell surface associated	ENST00000397910:c.C25532A:p.T8511N	8.15E-06
MUC4	mucin 4, cell surface associated	ENST00000463781:c.C10316A:p.T3439N	9.76E-05
TACR3	tachykinin receptor 3	ENST00000304883:c.A745G:p.I249V	1.38E-04
DSE	dermatan sulfate epimerase	ENST00000331677:c.A2005G:p.I669V	1.48E-04

Family 2- index case

HUGO	Gene name	Variation in canonical transcript	gnomAD frequency
KSR2	kinase suppressor of ras 2	ENST00000339824:c.G2468A:p.R823H	7.71E-05
NOS1	nitric oxide synthase 1 (neuronal)	ENST00000338101:c.C55T:p.R19C	1.62E-04
SBNO1	strawberry notch homolog 1 (Drosophila)	ENST00000420886:c.G2050A:p.D684N	8.47E-04
UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7	ENST00000305231:c.G1018C:p.D340H	2.58E-04
ACD	adrenocortical dysplasia homolog (mouse)	ENST00000393919:c.T284A:p.L95Q	VNF
SH3BP2	SH3-domain binding protein 2	ENST00000503393:c.G1312A:p.V438M	2.69E-04
TACC3	transforming, acidic coiled-coil containing protein 3	ENST00000313288:c.G2347A:p.A783T	8.96E-04
MAP4K2	mitogen-activated protein kinase kinase kinase kinase 2	ENST00000294066:c.G1015A:p.A339T	4.75E-05
LRP5	low density lipoprotein receptor-related protein 5	ENST00000294304:c.G2785A:p.A929T	2.53E-05
SPEF2	sperm flagellar 2	ENST00000356031:c.C1633T:p.P545S	1.75E-03
C4orf19	chromosome 4 open reading frame 19	ENST00000284437:c.G682T:p.G228C	VNF
SLC22A10	solute carrier family 22, member 10	ENST00000332793:c.C1463A:p.T488N	2.32E-03
ITPR3	inositol 1,4,5-trisphosphate receptor, type 3	ENST00000374316:c.G3086T:p.G1029V	4.00E-04
RAI2	retinoic acid induced 2	ENST00000545871:c.C656A:p.S219Y	VNF
SPTBN2	spectrin, beta, non-erythrocytic 2	ENST00000533211:c.G3116A:p.R1039Q	7.87E-04
ZNF732	zinc finger protein 732	ENST00000419098:c.C1474T:p.H492Y	VNF
ECHDC3	enoyl CoA hydratase domain containing 3	ENST00000379215:c.C863T:p.T288M	7.45E-04
TNIP2	TNFAIP3 interacting protein 2	ENST00000315423:c.G424A:p.V142I	5.76E-04
MDC1	mediator of DNA-damage checkpoint 1	ENST00000376406:c.C5234T:p.P1745L	VNF
SATL1	spermidine/spermine N1-acetyl transferase-like 1	ENST00000509231:c.G890A:p.S297N	2.24E-05
OTUD4	OTU domain containing 4	ENST00000454497:c.T3093G:p.F1031L	4.78E-04
UBAP2	ubiquitin associated protein 2	ENST00000379238:c.C1045T:p.P349S	9.10E-04

VNF indicates novel variant of unknown significance.

Supplemental methods

Ethics statement: All patient samples were obtained with written consent under the approval of our local research ethics committee (London – City and East).

Exome Capture and variant calling: Whole exome sequencing was performed on the Hi Seq 2000 platform (Illumina) using the Truseq Exome enrichment kit (Illumina). Exome data was processed and analysed using the Phenopolis (https://phenopolis.org)[2]. Non-synonymous variants in genes whose function relates to telomere maintenance or regulation (Qiagen Human Telomeres and Telomerase RT2 Profile PCR Array) were then filtered. All relevant variants identified were validated by Sanger sequencing on a 3130xl Genetic Analyzer with a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems).

Cell culture, plasmids and treatments: EBV-infected LCLs, HEK 293 cells and HeLa cells were established and grown in RPMI Media 1640 or DMEM media, supplemented with penicillin and streptomycin, 2 mM L-glutamine (Life Technologies), and 10/20% (vol/vol) fetal bovine serum (FBS, Invitrogen). Site directed mutagenesis was performed on *pcDNA3.1+FLAG-TPP1* (Addgene plasmid # 53548) to generate constructs encoding OB-fold variants, p.L95Q and p.K170Δ. Wildtype (WT) and mutant *pcDNA3.1+FLAG-TPP1* along with *pLPC-myc-hPOT1* (Addgene plasmid #12387) and *pcDNA3.1+ TERT* cDNAs were co-transfected in to HEK293 or HeLa cells where indicated using Lipofectamine 2000.

Telomere lengths, FISH and ChIP: Whole blood telomere lengths were determined from genomic DNA using the monochrome multiplex quantitative PCR method as previously described.[3] Briefly, amplification of telomeric DNA (T) and a single copy gene (S) were quantified against standard curves obtained from dilution of a reference DNA sample. The T/S ratio, obtained in triplicate for each sample, is proportional to the telomere length. This ratio was normalized to the T/S ratio of a second reference sample that was run on every plate to give a relative T/S ratio. For fluorescence *in situ* hybridization (FISH) on meta phase spreads prepared by KaryoMAX™ Colcemid™ Solution (Thermo Fischer Cat n0:15212012) treatment to EBV transformed LCLs prepared were subjected to TelC-Cy3 FISH probe (PNA biosciences) hybridization to label telomeres and the images were captured at 63x magnification, for both control and patient LCLs. Flow-FISH telomere lengths were measured by Repeat Diagnostics Inc. (Vancouver, Canada; supplemental Figure 2). For telomere ChIP assays, *TPP1shRNA* treated HEK293 cells (supplemantal Figure 3A and B) were transfected with FLAG-TPP1 wildtype or OB-fold variants and the transfected cells were processed for ChIP. Briefly twenty-four hours post transfections with plasmids encoding TPP1-WT and OB-fold variants, cells were synchronized by adding 2mM thymidine (Sigma-Aldrich; cat no: T1895) for sixteen hours, released for eight hours in culture and treated with aphidicolin (Sigma Aldrich cat no: A0781) for further sixteen hours. Subsequently the S phase cells, verified by flow cytometry, were crosslinked using paraformaldehyde and the cell nuclei were lysed, sonicated and incubated with either mouse monoclonal anti-FLAG agarose beads (Sigma-Aldrich; cat no: 2426), or a POT1 antibody (Protein tech; 10581-1-AP) for immunoprecipitation of bound chromatin. A small aliquot (25%) of chromatin that was not subjected to immunoprecipitation was saved as input. DNA was extracted from all samples, dot blotted onto positively charged nylon membrane and probed for telomeric DNA following instructions in the Telo TAGGG kit (Sigma Aldrich).

Yeast strain generation: To make the yeast strain harbouring the Tpz1 L5Q mutation, site-directed mutagenesis was performed on wild-type plasmid pTpz1a-cHA3 (lab stock). In this

plasmid, tpz1 is linked to the kanamycin resistance cassette KanMX6, which confers resistance to G418. 1ug of plasmid was linearized with *PmeI* and transformed into a diploid strain in which one *tpz1* allele has been deleted and replaced with a nourseothricin resistance cassette (genotype h-/h+ ade6-M210/M216 tpz1:TKnatCM/+). Successful transformants (in which tpz1-L5Q recombined at the site of the natCM cassette) were selected for on a YES plate supplemented with 0.1mg/ml G418. Colonies were then replica plated onto YES supplemented with 0.1mg/ml nourseothricin, in order to confirm the loss of the natCM cassette. A successful transformant was selected and starved at 25°C on malt extract (ME) for 48 hours to induce sporulation. Cell walls were then digested using *Helix promatia* juice (Pall Life Sciences) at room temperature for 6hrs before plating spores on YE-G418. Haploid colonies (detectable by their pink shade on YE) were selected, gDNA extracted and the presence of the L5Q mutation was confirmed by Sanger sequencing. Expression of WT and mutant Tpz1 is verified by western blotting (supplemental Figure 3D).

Co-immunoprecipitation (CoIP) immunoblotting and: HEK293 cells were transfected with 2.5µg of either wild-type or mutant *pcDNA3.1+FLAG-TPP1*, 2.5µg *pLPC-myc-POT1*(Addgene plasmid #12387) and 2.5µg of *pcDNA3.1+TERT* and 2µg *pcDNA3.1+hTR* using lipofectamine 2000. *TPP1⊿OB-FLAG* was used for negative control. Twenty-four hours post transfection cells were lysed in whole cell lysis buffer (1M HEPES, 2M NaCl, 0.5M NaF, 1M Na3VO4, 0.5M EDTA, 0.1% NP40 and 1x cocktail of protease inhibitors) and centrifuged at 15000 g for 10 minutes. Nuclear lysates were prepared by re-suspending cells in hypotonic lysis buffer as previously described. Both whole cell and nuclear lysates were incubated with mouse monoclonal FLAG M2 agarose beads (Sigma Aldrich; cat no: 2426) and CoIP complexes were eluted with competing FLAG peptide (Sigma Aldrich; cat no: F3290) and immunoblotted on PVDF membrane using, rabbit anti-TERT (Abcam ab 32020) and rabbit anti-POT1 (Protein tech) for interaction analysis. The specificity of TERT antibody is verified by immuno blotting, where TERT expression is particularly driven in both control and patient cells upon EBV transformation when compared to whole blood (supplemental Figure 3E). This antibody used has been previously validated (Xi et al. 2014).[4] Quantification of TERT signal in cells expressing TPP1 OB-fold variants were calculated by obtaining the ratios of pull down (PD) towards the input (IN) and subsequently normalized to TPP-WT signal.

Immunocytochemistry: HeLa cells expressing FLAG tagged WT and OB-fold variant forms of TPP1 were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (TX100) in PBS, quenched in 50 mM NH4Cl, and blocked in 10% goat serum and 1% BSA in PBS containing 0.05% TX100 for 1 hr. Cells were incubated in the primary mouse monoclonal FLAG antibody (Sigma Aldirch; F1804) as well as rabbit polyclonal coilin antibody (Protein tech; 10967-1-AP) and the corresponding goat anti-mouse and anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 and 568 (Invitrogen) respectively in blocking solution for 1 hr separately. Cells were washed three times in PBS containing 0.05% TX100 between primary and secondary antibody incubations and mounted with vectashield containing DAPI (Vector Labs). For detection endogenous TPP1, immunocytochemistry on LCLs was performed by washing in warm PBS and spreading on polylysine coated slides and followed the steps as mentioned above. Images were collected with an LSM710 laser scanning confocal microscope

(Olympus) under relevant laser excitation, and the emitted signals were visualized with ZEN software (Zeiss)**.** TPP1 antibody specificity is verified by immunoblotting of protein lysates acquired from HEK293 cells, that are treated with *TPP1 shRNA that targets 3'UTR* (supplemental Figure 3A). The resistance of FLAG tagged TPP1 WT and variant expression against *TPP1 shRNA* is determined in HEK293 cell lysates by western blotting. TPP1 and TERT expression levels were also detected in both control and patient lymphoblastoid cell lysates using the same antibodies described above (supplemental Figure 3F).

Southern blotting: Southern blots were performed to measure telomere lengths in yeast strains. Briefly, 40ug of genomic DNA was digested overnight with *EcoRI* then separated on a 1% agarose gel, run at 40v for approximately 750 voltage hours. Samples were run alongside 3ul of Hyperladder IV (Bioline). DNA was blotted overnight onto a nitrocellulose membrane by capillary action, then crosslinked by UV light. The membrane was incubated for 12 hours with a ^{32}P-labelled telomeric probe in a hybridization oven at 55 °C. Unbound probe was washed away and membranes exposed overnight to a phosphorimager before developing.

TRAP assays: TPP1 associated telomerase activity we determined as described previously, briefly FLAG tagged TPP1-WT and OB-fold variant expressing cells (3×10^6) were lysed in lysis buffer supplied in the Telo TAGGG Telomerase PCR ELISA kit (sigma) for 30 min. The lysates were centrifuged at 14000 rpm for 20 min at 4 °C. The resulting supernatant was incubated with anti-FLAG M2-agarose beads (Sigma) and the immunoprecipitated proteins were eluted in 50 µL of elution buffer containing Flag peptides (200 µg/mL) as described previously.[5] The 2 µL of eluate was then diluted in 50 µL of PCR- TRAP reaction and subsequently quantified following kit instructions.the relative amounts of TPP1 in FLAG pulldown complexes were determined by western blotting. Telomerase activity in control and patient cells were determined by lysing 3 x 10^5 cells in 200 µL of lysis buffer and the subsequent supernatant was adjusted to no of cells in PCR-TRAP reaction. Gel based TRAP activity is determined by running the PCR products in non denaturing 20% TBE gels at 120 volts for 30 minutes and subsequently stained with SYBR™ Gold nucleic acid gel stain (Thermo Fischer).

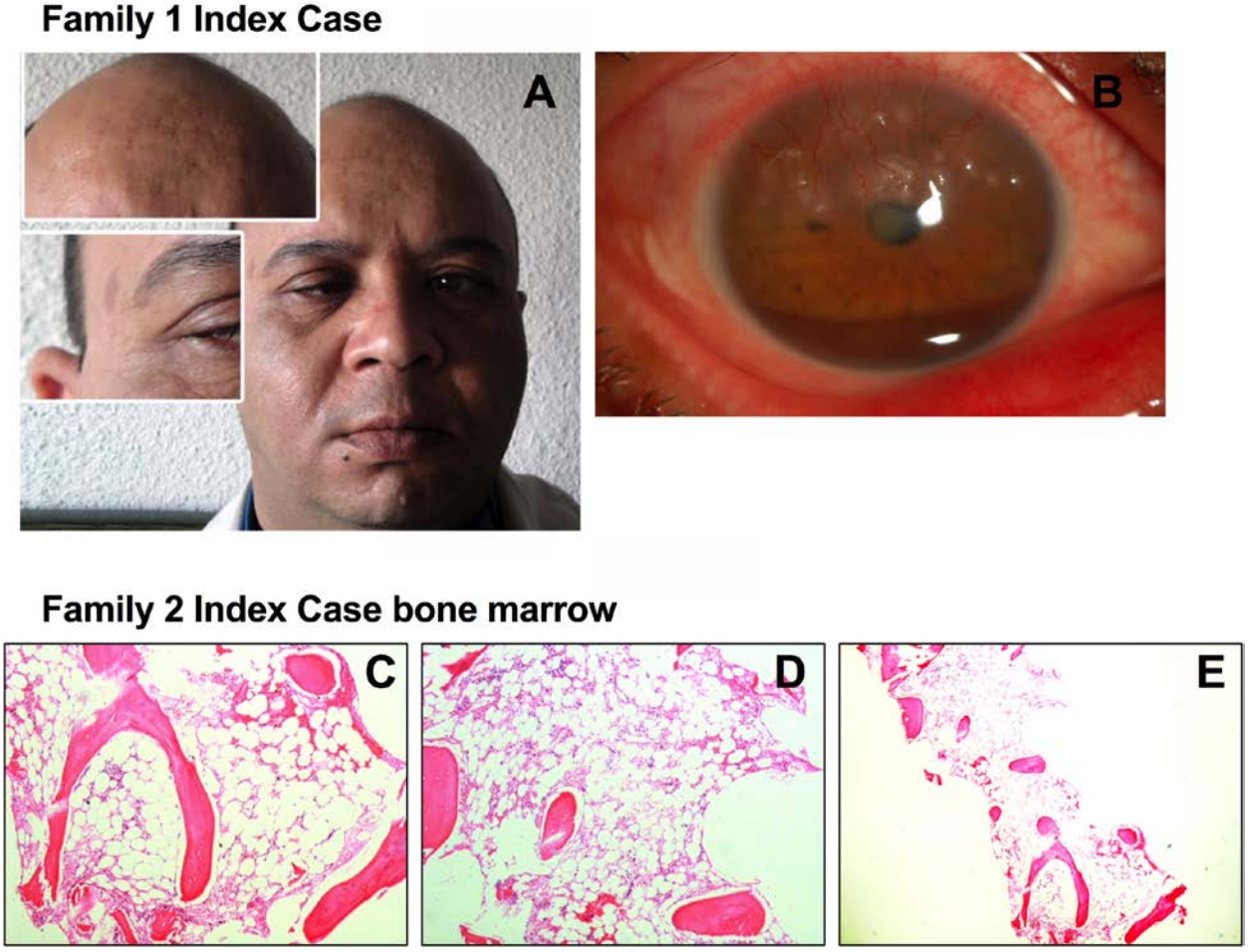
Structural analysis of the TPP1 OB-fold variants: TPP1 OB-fold crystal structure[6] (PDB id: 2I46) is obtained from Protein Data Bank (http://www.rcsb.org) and the location of OB-fold variants were visualised using Chimera visualised (https://www.cgl.ucsf.edu/chimera/).[7]

Supplemental references

1. Tummala H, Walne A, Collopy L, et al. Poly(A)-specific ribonuclease deficiency impacts telomere biology and causes dyskeratosis congenita. *J Clin Invest*. (2015); 125:2151-2160.
2. Pontikos N, Yu J, Moghul I,et al. Phenopolis: an open platform for harmonization and analysis of genetic and phenotypic data. *Bioinformatics*. (2017); 33:2421-2423.
3. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitation PCR method. *Nucleic Acids Res*, (2009); 37, e21.
4. Xi L, Cech TR. Inventory of telomerase components in human cells reveals multiple subpopulations of hTR and hTERT. Nucleic Acids Res. (2014); 42: 8565-8577.

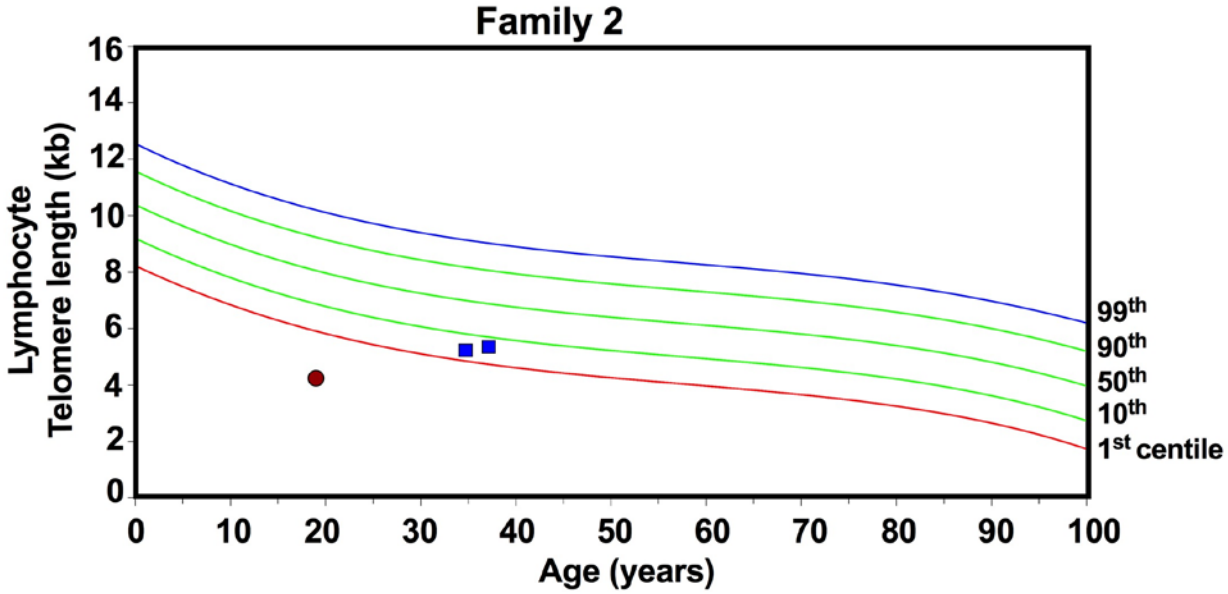
5. Zhang Y, Chen LY, Han X, et al. Phosphorylation of TPP1 regulates cell cycle-dependent telomerase recruitment. *Proc Natl Acad Sci* U S A. (2013), 110: 5457-5462.
6. Wang F, Podell ER, Zaug AJ, et al. The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature.* (2007); 445: 506-510.
7. Pettersen, EF, Goddard TD, et al. UCSF Chimera--a visualization system for exploratory research and analysis*. J. Comput. Chem*, (2004), 25; 1605-1612.

supplemental Figure 1



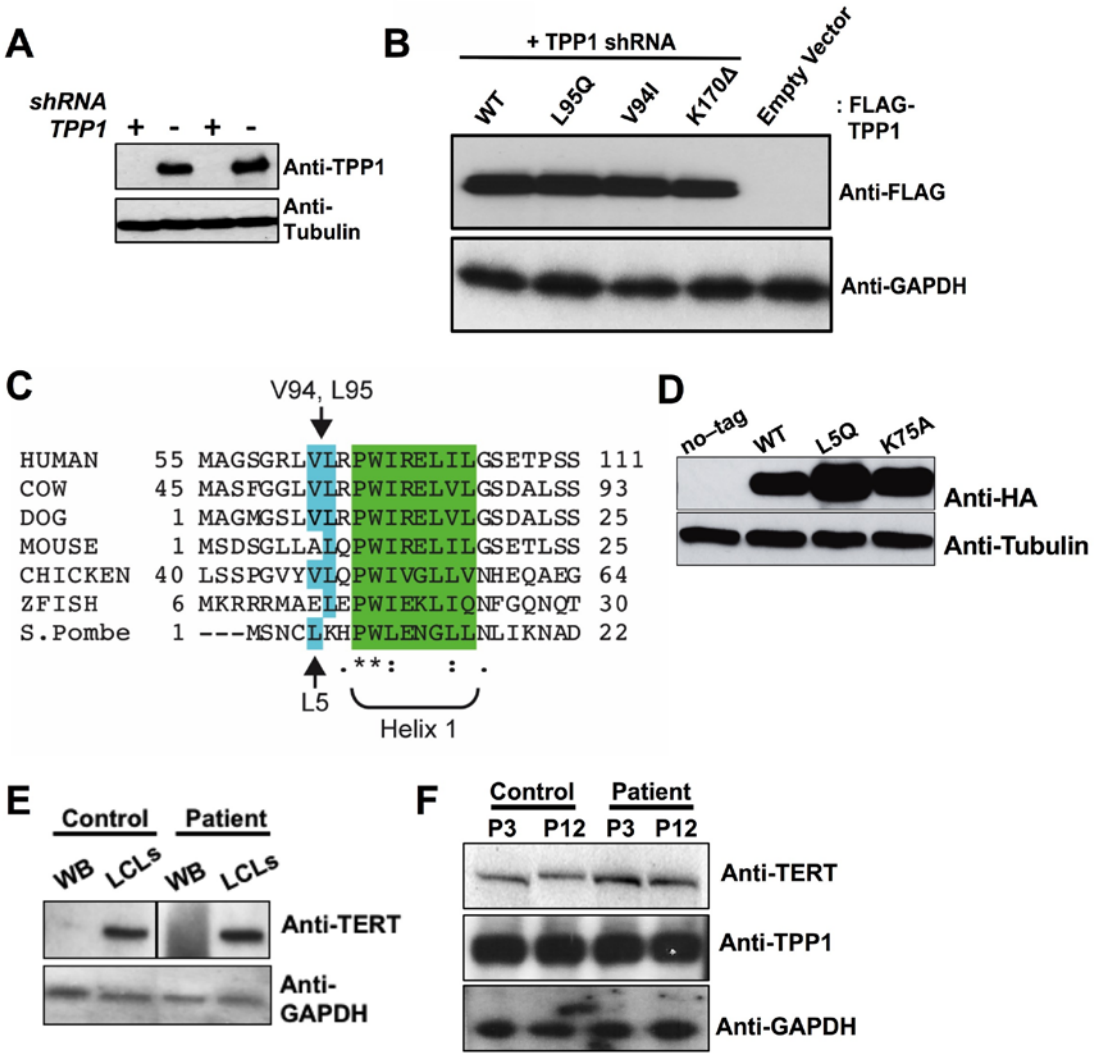
Clinical photographs of index case from family 1 showing (A) hyperpigmented skin lesions (B) corneal vascularization, a feature of limbal stem cell deficiency. (C-E) H and E staining reveals hypoplastic bone marrow in index case of family 2.

supplemental Figure 2



Telomere length analysis by Flow FISH revealed short telomeres below 1st centile in the index case (red circle) when compared to parents (blue squares) below 10th centile.

supplemental Figure 3



(A) Immunoblotting of HEK293 cell lysates transfected with *TPP1* shRNA. Anti-Tubulin is used to determine loading controls (B) FLAG tagged TPP1 WT and OB-fold varants were expressed in TPP1 shRNA expressing HEK293 cells. (C) Evolutionary conservation of the valine 94 and leucine 95 residue in vertebrates TPP1 and *S.* pombe Tpz1. Secondary structures were analysed using PHYRE2 Protein Fold Recognition Server. The patient variant L95Q has been highlighted with the corresponding amino acid in Tpz1. (D) Immunoblotting confirms the expression of *S.pombe TPZ1*. Anti-tubulin is used to determine the loading control (E) Immunoblotting of control and patient lymphoblastoid cell lysates (LCLs) show endogenous

expression levels of TERT in whole blood (WB) and EBV transformed control and patient cells. (F) Immunoblotting of control and patient lymphoblastoid cell lysates show endogenous expression levels of TERT, and TPP1 at different passages (P3 and P12). Anti- GAPDH is used to determine loading controls