Supplemental Data

Functional interaction between compound heterozygous *TERT* mutations causes severe Telomere Biology Disorder

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Supplementary table 1: previously-reported patients with biallelic deleterious *TERT* variants

Variants carried	Patient age	Clinical features	Reference
Gly110Ala + Arg446Profs*93 (in *trans*)	4	BMF, nail dystrophy, leukoplakia, microcephaly, cerebellar hypoplasia	Collopy et al., 2015[1]
Thr567Met (homozygous)	2	Pancytopenia, developmental delay, ataxia, cerebellar hypoplasia	Gramatges et al., 2013[2]
Arg669Trp (homozygous)	68	BMF, liver cirrhosis, nonspecific interstitial pneumonia	van der Vis et al., 2020[3]
Pro704Ser (homozygous)	31	BMF, short stature, elfin appearance, esophageal stricture, leukoplakia, abnormal pigmentation, ridged fingernails, avascular necrosis of both hips, tooth loss, chronic diarrhea, learning difficulties, pulmonary infiltrates	Du et al., 2008[4]
Arg811Cys (homozygous)	13	BMF, nail dystrophy, leukoplakia, abnormal skin pigmentation	Marrone et al., 2007[5]
Tyr846Cys + His876Gln (in *trans*)	8	Aplastic anemia	Du et al., 2008[4]
Arg901Trp (homozygous)	3	BMF, leukoplakia, microcephaly, cerebellar hypoplasia, developmental delay, low birthweight	Marrone et al., 2007[5]
Thr1129Pro (homozygous)	6, 8, 12 (siblings)	BMF	Stocklausner et al., 2015[6]

Supplementary table 2: List of DNA oligonucleotides used in this study

Purpose	Name	Sequence (5'-3')
TERT mutagenesis	L557P F	GCTGC**C**CAGGTCTTTCTTTTATGTCACGGAGACCACGTTTCA
	L557P R	GAAAGACCTG**G**GCAGCTCGACGACGTACACACTCATCAGCC
	K1050E F	GCC**G**AGAACGCAGGGATGTCGCTGGGGGCC
	K1050E R	CCCTGCGTTCT**C**GGCTTTCAGGATGGAGTAGCAGAGGGAGG
Telomerase substrate	Bio-L-18GGG	Biotin-TTAGGGTTAGGGTTAGGGTTAGGG
Loading control	LC30	CTAGACCTGTCATCACTAGACCTGTCATCA-Biotin
hTR northern probe	Telo4	CGGTGGAAGGCGGCAGGCCGAGGC
Telomere length qPCR	telc	TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
	telg	ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT
	albu	CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG
	albd	GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT

Supplementary methods

Telomere length analysis (Flow-FISH)

Telomere Flow-Fluorescence *In Situ* Hybridization (Flow-FISH) was performed in the clinically-accredited telomere length testing facility in the Haematology Department, Children's Hospital at Westmead, Australia, with a published protocol[7]. Briefly, mononuclear cells were isolated by Ficoll density centrifugation from duplicate samples of lithium-heparin peripheral blood. A known cell line with long telomeres (human tetraploid T-cell lymphoblastic line CCRF-CEM, GM03671C[8]) served as an internal reference standard for telomere length; telomere lengths of this cell line remain stable at ~ 27 kb over 4 months in culture, as measured by TRF. Samples were hybridized with a fluorescein isothiocyanate (FITC)-conjugated $(CCCTAA)_3$ peptide nucleic acid (PNA) probe (Panagene, Korea) prior to flow cytometry on a FACS CANTO II (BD Biosciences, USA) instrument and data displayed and analyzed with BD FACSDiva software (BD Biosciences). Calculation of Relative Telomere Length of the patient's mononuclear cells was performed by comparing the fluorescence of these cells with the tetraploid CCRF-CEM cell line and expressed as a percentage. Blood samples from 240 healthy individuals were collected and telomere lengths measured with the same Flow-FISH protocol in the Westmead facility, showing the normal percentiles for different age groups.

Telomere length analysis (qPCR)

A previously described monochrome multiplex qPCR telomere length assay[9,10] was used. Briefly, each reaction was performed on a BioRad CFX386 Touch PCR detection system (BioRad) in 384-well PCR plates. Twenty nanograms of DNA was added to a master mix containing 300 nM each of *telc* and *telg* telomere PCR primers (Supplementary Table 2), 350 nM each of *albu* and *albd* single-copy gene PCR primers and Rotor-Gene SYBR Green PCR Master Mix (Qiagen). Each patient sample was assayed in quadruplicate, and each batch of PCR reactions included four control DNA samples, also assayed in quadruplicate. Telomere content was measured using the ΔΔCT method: the difference between CT_{alb} and CT_{tel} was calculated for the reference sample and the control sample. Relative telomere length (TL) was then calculated using $2^{(CT\text{-}sample - CT\text{-}reference)}$. Values obtained from 240 healthy individuals show the normal percentiles for different age groups.

Telomere length analysis (Southern blot)

Telomere length was analyzed by electrophoresis of telomeric restriction fragments on an agarose gel and hybridization with a radiolabeled telomeric probe as described[11]. Telomere terminal restriction fragments were prepared by *Hin*fI and *Rsa*I digestion of genomic DNA and 2 µg was loaded on a 1% (wt/vol) agarose gel in 0.5 × TBE. Pulsed-field gels were run at 6 V/cm for 14 h at 14°C, with an initial switch time of 1 s and a final switch time of 6 s. Gels were dried for 2 h at 60 °C, denatured and hybridized overnight to a 5'-^{32}P-labeled $(CCCTAA)_4$ oligonucleotide probe in Church and Gilbert hybridization buffer[12]. Gels were washed in 4 × SSC (0.06 M sodium citrate, 0.6 M NaCl, pH 7) and exposed to a phosphorimager screen overnight prior to visualization using a Typhoon TRIO Imager (GE Healthcare Life Sciences).

Over-expression and purification of human telomerase

The human *TERT* gene[13] driven by a cytomegalovirus (CMV) promoter was cloned into the *PmeI* restriction site of plasmid pcDNA3.1(+) (Invitrogen) with an N-terminal 6×FLAG tag followed by a PreScission Protease (Cytiva) cleavage site. The *TERC* gene under a U3 promoter and *DKC1* under a CMV promoter were cloned into a single plasmid in vector pcDNA3.1(+) using the *NruI* and *PmeI* restrictions sites, respectively. *TERT* variants 1670T>C (encoding L557P) and 3148A>G (encoding K1050E) were introduced into the FLAG-hTERT construct using the Takara In-Fusion HD Cloning Plus system following the manufacturer's recommendations, with the DNA changes incorporated into the *TERT* PCR primers (primer sequences listed in Supplementary Table 2). Template plasmid was removed by digestion with 100 U of *DpnI* (New England Biolabs) at 37 °C for 2 h, and recombinant plasmids were transformed into α-Select Gold competent cells (BioLine) and purified using a Hi-Speed Maxi-plasmid kit (Qiagen). All constructs were sequenced over the entire *TERT* gene to ensure only the variant of interest was introduced.

The telomerase complex was expressed in HEK293T cells and immunopurified as described previously[14], with minor modifications. Briefly, a 4:1 w/w mixture of plasmids encoding hTR/dyskerin and hTERT (either wild-type (WT) or the L557P or K1050E variants, or a 1:1 mixture of WT and each variant, or both variants) at a total concentration of 1 µg/mL were transfected into HEK293T cells using polyethylenimine (PEI) at a ratio of 3:1 PEI:DNA (w/w). Cells were harvested after 72 h and lysed with buffer A (20 mM HEPES-KOH (pH 8), 300 mM KCl, 2 mM $MgCl_2$, 10% v/v Glycerol, 0.1% v/v Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF)). hTERT was immunopurified from the clarified lysates with anti-FLAG M2

agarose beads (Sigma-Aldrich, #A2220) and eluted with buffer A containing 3×FLAG peptide (25 μM, Sigma-Aldrich) and 1 mM dithiothreitol (DTT) into LoBind tubes (Eppendorf).

Western blot analysis

To determine the amount of hTERT recovered during immunopurification (IP), lysates and IP eluates were electrophoresed on NuPAGE 4-12% Bis-Tris gels, electro-blotted onto a 0.45 μm nitrocellulose membrane (GE Healthcare) and probed with a mouse monoclonal primary antibody (anti-FLAG; Sigma-Aldrich #F3165) as described[15]. Membranes were visualized with Amersham ECL prime (GE Healthcare) on a Bio-Rad Chemidoc MP (Bio-Rad).

Direct telomerase activity assay

Relative telomerase activity was quantitated using a direct (i.e. non-PCR) assay, as described[14]. Briefly, equal amounts of immunopurified telomerase (based on hTERT quantitation on western blots) were incubated with 1 μM biotinylated DNA primer (Bio-L-18GGG; Supplementary Table 2) in 20 mM HEPES-KOH (pH 8), 150 mM KCl, 2 mM $MgCl_2$, 10 mM DTT, 5% glycerol, 0.05% Triton-X 100, 1 mM dATP, 1 mM dTTP, 5 μM dGTP and α-^{32}P-dGTP (20 μCi, Perkin-Elmer) at 37 °C for 1 h. Extension products were captured on Streptavidin M-280 Dynabeads (Thermo Fisher) together with a 5'-^{32}P-end-labeled oligonucleotide 5'-TTAGGGTTAGGG-biotin-3' as a recovery and loading control, washed to remove unincorporated dNTPs, suspended in 90% formamide/1×TBE supplemented with D-biotin (0.5 mM), and eluted by heating at 80°C for 10 min. Extension products were electrophoresed over a 10% polyacrylamide/8 M urea sequencing gel, which was exposed to a phosphorimager screen and imaged using a phosphorimager (Typhoon, GE Healthcare).

Northern dot-blot analysis of telomerase RNA

The ability of hTERT to bind to hTR was determined using Northern dot-blot analysis to quantitate hTR recovery after IP against hTERT, as described[14]. Briefly, equal amounts of IP samples (based on hTERT levels) were denatured and applied to a Hybond N^+ membrane (GE Healthcare) using a 96-well dot-blot apparatus (GE Healthcare). For quantitation of active enzyme, purified *in vitro*-transcribed hTR standards[16] (0.5 - 5 fmol) were spotted onto the same membrane. The membrane was hybridized at 55 °C overnight with a 5'-^{32}P-labeled DNA probe against hTR (Supplementary Table 2), in Church hybridization buffer. After washing, the membrane was exposed to a phosphorimager screen and imaged using a phosphorimager (Typhoon, GE Healthcare).

Telomerase-DNA binding assay

The equilibrium binding constant (K_D) between telomerase and its telomeric DNA substrate was determined using a pulldown assay with a biotinylated DNA substrate, as described[14]. Briefly, 50 pM of immunopurified telomerase (quantitated by northern dot-blot against hTR as described above) was incubated with a series of concentrations of Bio-L-18GGG (typically 0 nM - 400 nM, or 800 nM for some mutants) at 37 °C for 2 h, followed by recovery of the DNA-enzyme complex with blocked Neutravidin beads (ThermoFisher). The amount of telomerase remaining in the supernatant was quantitated using a northern dot-blot for hTR, as described above.

Supplementary references

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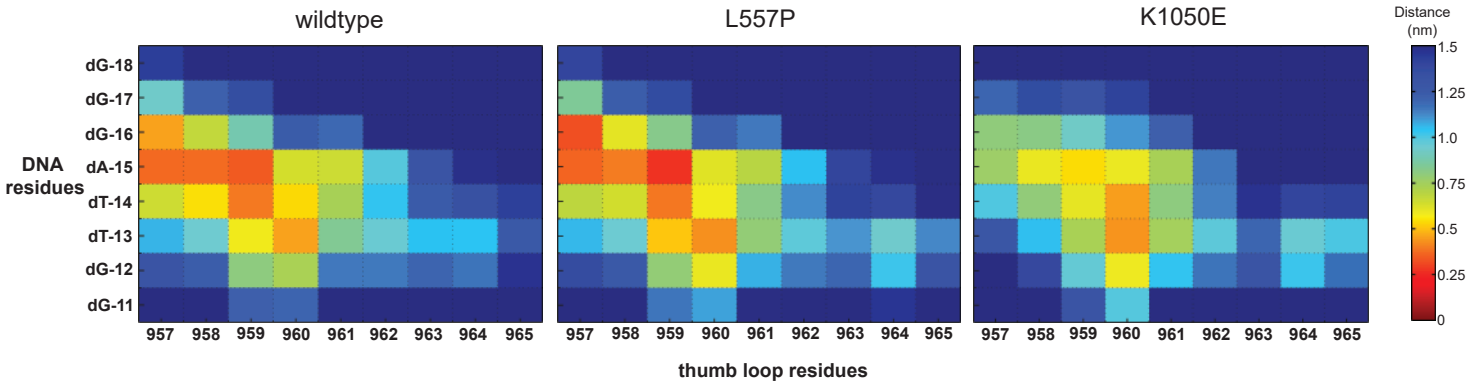
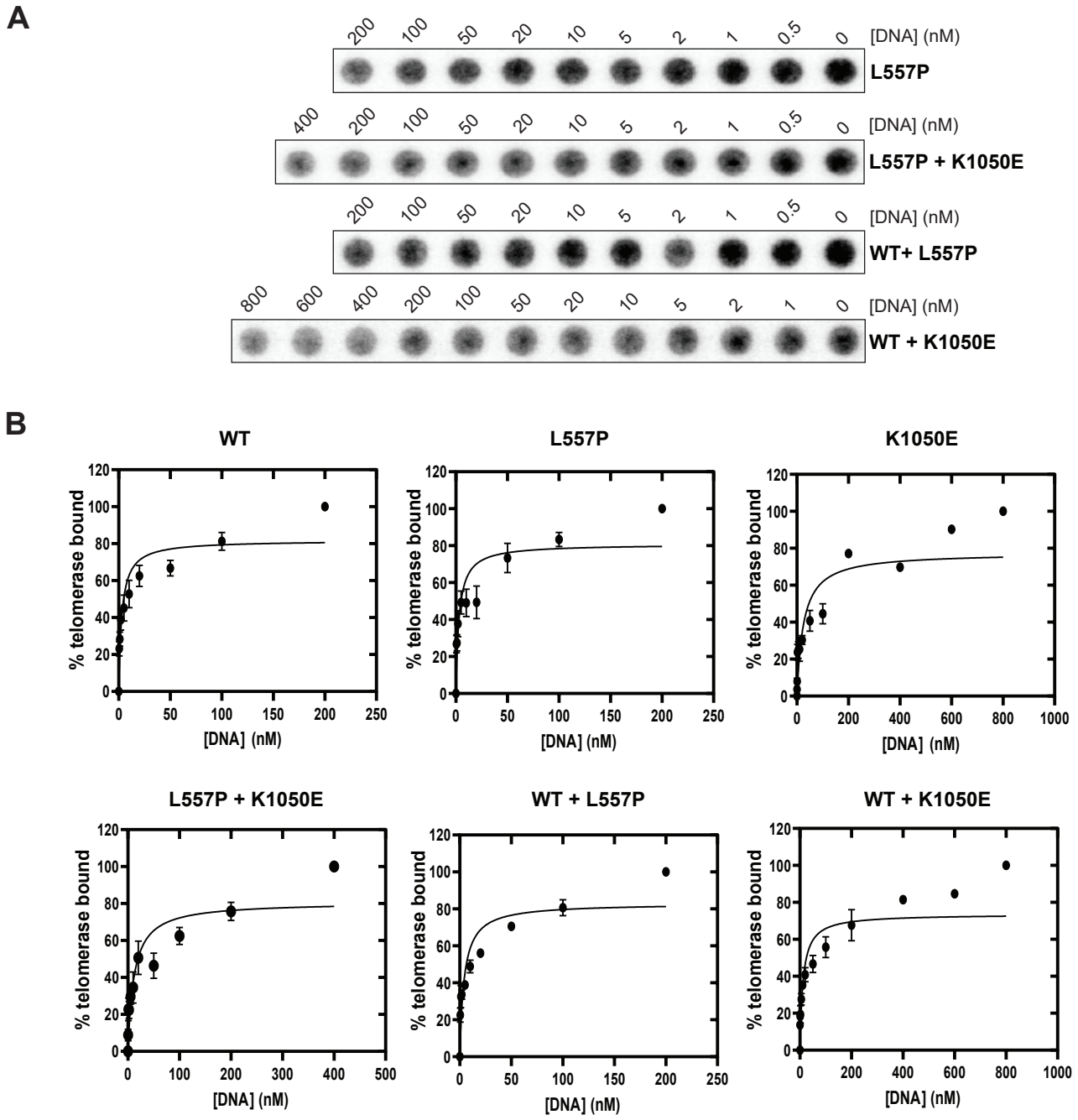
Niaz *et al*. Supplementary Figure 1



Supplementary Figure 1: Quantitation of distances between thumb loop residues of hTERT and DNA nucleotides. In the hTERT^{K1050E} simulation, the DNA substrate (residues 11-18) is shifted away from the thumb loop (hTERT residues 957-965) compared to wildtype. Heat map shows average distances; n=3.



Supplementary Figure 2: DNA binding assay to determine primer affinity (K_D) of telomerase variants.

(A) Northern dot-blot of the amount of telomerase remaining in solution after binding to a biotinylated DNA oligonucleotide primer (biotin-TTAGGG)$_3$) at the indicated primer concentrations, for all variants besides those shown in Figure 4C.

(B) Plots of the amount of telomerase removed from solution after binding to DNA at the indicated concentrations. The plots were fitted to the equation y = (B_{max}[S])/(K_D+[S]), where B_{max} is the maximal level of binding, [S] is the concentration of DNA, and K_D is the equilibrium binding constant (values shown in Figure 4D). Error bars represent the standard error of the mean (n = 3-7).