Supplemental Methods

Subjects

The study was approved by the institutional review board at the University of Texas Southwestern Medical Center. Written informed consent was obtained from all participants prior to their participation in the study.

Samples

Genomic DNA was isolated from circulating leukocytes using AutoPure (Qiagen) according to the manufacturer's instructions. Genomic DNA samples were sequenced from individuals with a rare mutation in *TERT* (n=137), *TERC* (n=10), *PARN* (n=32), or *RTEL1*(n=20), representing 44, 6, 8 and 7 different mutations, respectively. All exons of *TERT* and *TERC* were Sanger sequenced. *PARN* and *RTEL1* were sequenced by whole exome capture and next generation sequencing(1). All variants in these four genes were confirmed by Sanger sequencing. Genomic DNA samples from circulating leukocytes of the DHS2 cohort, a multiethnic population-based probability sample of Dallas County, were isolated using identical methods. Genomic DNA isolated from EBV-transformed lymphoblastoid cell lines from 100 Whites (AGPLONG3) and 32 African Americans (AGPLONG5), ranging in age from 98-108, were obtained from the Coriell Institute.

Sequencing and Allelic Discrimination (AD) Assay

Sanger sequencing of the *TERT* promoter was performed using the oligonucleotides listed in **Supplemental Table 5,** LA Taq DNA polymerase (Takara Clontech) and GC buffer I for 30 cycles. Negative controls (no DNA) were included in each experiment. Taqman allelic discrimination assays were used to test for both *TERT* promoter mutations in the DHS2 cohort. A 1:1 dilution of the PCR product generated for Sanger sequencing (see above) was the substrate for each AD assay. The oligonucleotides and probes for querying the -124 C/T mutation (also known as C228T, chr5:1,295,228 C>T) were purchased from Life Technologies (TERT_228; Cat# 4332072). The oligonucleotides and probes for querying the -146 C/T mutation (also known as C250T, chr5: 1,295,250 C>T) are listed in **Supplemental Table 5.** Sanger sequencing of the 132 DNA samples from centenarians was performed using the

Accuprime GC-rich DNA polymerase (ThermoFischer) with 1x BufferA, 5% DMSO, 1M Betaine and oligonucleotides listed in **Supplemental Table 5**.

Flow cytometry

Blood samples (1 ml) from subjects were stained with fluorochrome-conjugated antibodies and sorted by flow cytometry (FACS Aria SORP 5 instrument) into four populations: granulocytes (CD66b-APC positive, eBiosceince #17-0666), monocytes (CD14-PE positive, Tonbo Bio #35-9459), T-cells (CD3-VioletFluor450 positive, Tonbo Bio #75-0037), and B-Cells (CD19-PerCP-Cy5.5 positive, Tonbo Bio #65-0199). Genomic DNA was isolated from each cell fraction using QiaAmp columns (Qiagen).

Limiting dilution PCR

Phasing of the *TERT* promoter and coding DNA sequence (CDS) mutations were assessed by Sanger sequencing after two rounds of nested PCR. First-round, multiplex PCR was carried out in a single tube that contained primers that amplified both the promoter and CDS with comparable efficiency. PCR of diluted genomic DNA (approximately ~1 pg) was performed using Hotstar Taq polymerase (Qiagen) in 2-4% DMSO and 5x Q-solution for 35-40 cycles. One µL of the first round PCR product was used as a template to amplify the promoter and CDS regions in parallel separate PCR reactions using the appropriate nested primers (**Supplemental Table 5**). PCR products from second-round PCR were gel purified prior to sequencing if two different sized products were detected. At least 10 independent PCR reactions were performed and analyzed so that Sanger sequencing of the PCR products revealed single nucleotide peaks.

Telomere length assays

Telomere lengths of genomic DNA specimens were measured using a quantitative PCR assay as previously described(2). Flow-FISH telomere lengths were measured by Repeat Diagnostics Inc. (Vancouver, Canada).

Cell culture

Epstein-Barr virus (EBV)-transformed LCLs were established by incubating ~1 x 10^6 isolated lymphocytes with EBV shed from a marmoset cell line (B95-8, American Type Culture Collection) and the mitogen phytohemagglutinin. The cell lines were cultured in RPMI 1640 (HyClone) supplemented with 15% FBS, 2 mM L-glutamine, 1 mM pyruvate, penicillin (100 U/ml) and streptomycin (100 μg/ml), fed twice weekly and expanded to a suspension culture volume of at least 50 ml before storage and use.

Telomere Repeat Amplification Protocol

Cy5 fluorescent gel-based whole cell telomere repeat amplification protocol (TRAP) was performed as described(3). Prior to harvesting the cell lysates the cell cultures were first treated with 5 mM thymidine (Sigma) for 20 hours followed by 5 μg/ml aphidicolin (Sigma) for 16 hour to synchronize cells as described(4). A serial dilution (125-fold) of the protein extracts from each cell line was made and an appropriate volume of cell extract was selected that produced a linear response in all cell lines. Extension products were analyzed by visualizing the polyacrylamide gel on a Typhoon 9410 instrument. Data represent mean ± SD.

Quantitative PCR

RNA was isolated from pelleted LCLs using TRIzol (Ambion). After DNase I treatment, first-strand cDNA was generated using random hexamers and the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). qRT PCR reactions were performed using SYBR GreenER™ qPCR SuperMix (Invitrogen) with the following conditions: 50^{o}C of 2 min, 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec, 60^{o}C for 1 min, ending with a melt curve. Oligonucleotides used to amplify genes are listed in **Supplemental Table 5.** Data represent mean ± SD. Experiments were performed 3 times and a representative experiment is shown.

Lymphoblastoid cell line proliferation assay

After synchronization of cell cultures with thymidine/aphidicolin as described above, cells were stained with CellTrace^{TM} (Molecular Probes) as described(5). After four days, cells were sorted by flow cytometry (FACS Aria instrument) into three populations: low, medium and rapidly proliferating cells, each representing ~20% of all cells. Genomic DNA was isolated from each cell subpopulation using QiaAmp

columns (Qiagen) and PCR amplification of the *TERT* promoter mutation was performed across all samples in a single experiment as described above.

References

1. Stuart BD, Choi J, Zaidi S, Xing C, Holohan B, Chen R, Choi M, Dharwadkar P, Torres F, Girod CE, et al. Exome sequencing links mutations in PARN and RTEL1 with familial pulmonary fibrosis and telomere shortening. *Nat Genet.* 2015;47(5):512-7.
2. Stuart BD, Lee JS, Kozlitina J, Noth I, Devine MS, Glazer CS, Torres F, Kaza V, Girod CE, Jones KD, et al. Effect of telomere length on survival in patients with idiopathic pulmonary fibrosis: an observational cohort study with independent validation. *The lancet Respiratory medicine.* 2014;2(7):557-65.
3. Herbert B, Hochreiter AE, Wright WE, and Shay JS. Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. *Nature Protocols.* 2006;1(3):1583-9.
4. Zhu X, Kumar R, Mandal M, Sharma N, Sharma HW, Dhingra U, Sokoloski JA, Hsiao R, and Narayanan R. Cell cycle-dependent modulation of telomerase activity in tumor cells. *Proc Natl Acad Sci U S A.* 1996;93(12):6091-5.
5. Quah BJ, Warren HS, and Parish CR. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc.* 2007;2(9):2049-56.

Supplemental Table 1. Subjects with Telomere-related Gene Coding Sequence Mutations

Gene	DNA Change*	Impact on Protein*	No. of Subjects sequenced for *TERT* promoter mutations	No. of Subjects with -124C>T *TERT* promoter mutation	No. of Subjects with -146C>T *TERT* promoter mutation
TERT	c.97C>T	p.Pro33Ser	2		
TERT	c.293C>A	p.Ala98Asp	1		
TERT	c.377C>A	p.Thr126Lys	2		
TERT	c.416T>G	p.Leu139Arg	1		
TERT	c.430G>A	p.Val144Met	15	1	
TERT	c.569C>T	p.Ala190Val	1		
TERT	c.1002_1004delCTC	p.Ser335del	2		
TERT	c.1397G>C	p.Arg466Pro	1		
TERT	c.1417G>C	p.Val473Leu	1		
TERT	c.1456C>T	p.Arg486Cys	6		
TERT	c.1710G>T	p.Lys570Asn	1	1	
TERT	c.1892G>A	p.Arg631Gln	8	1	
TERT	c.1895C>T	p.Pro632Leu	2	1	
TERT	c.2006C>T	p.Arg669Gln	1		
TERT	c.2011C>T	p.Arg671Trp	1		
TERT	c.2033C>A	p.Ala678Asp	1		
TERT	c.2080G>A	p.Val694Met	1		
TERT	c.2105C>T	p.Pro702Leu	19		
TERT	c.2110C>T	p.Pro704Ser	3		
TERT	c.2240delT	p.Val747ValfsX32	6	1	
TERT	c.2266C>T	p.Arg756Cys	1		
TERT	c.[2431C>T;2433C>T]‡	p.Arg811Cys	2		
TERT	c.2469-2A>T	Splicing	3		1
TERT	c.2521C>T	p.Leu841Phe	1		
TERT	c.2539G>A	p.Gly847Ser	1		
TERT	c.2572C>T	p.Arg858Trp	1		
TERT	c.2581G>A	p.Gly861Arg	4		
TERT	c.2593C>T	p.Arg865Cys	2		
TERT	c.2594G>A	p.Arg865His	11		1
TERT	c.2599G>A	p.Val867Met	1		
TERT	c.2621C>G	p.Thr874Arg	3		
TERT	c.2647T>A	p.Phe883Ile	1	1	
TERT	c.2701C>T	p.Arg901Trp	2		
TERT	c.2775C>A	p.His925Gln	1		
TERT	c.2851C>T	p.Arg951Trp	4		
TERT	c.2869A>C	p.Ser957Arg	2		
TERT	c.2947C>T	p.His983Tyr	1		
TERT	c.2991delG	p.Val997ValfsX52	9	1	
TERT	c.3055C>T	p.Leu1019Phe	1		
TERT	c.3148A>G	p.Lys1050Glu	2		
TERT	c.3150G>C	p.Lys1050Asn	3		
TERT	c.3187G>A	p.Gly1063Ser	2		
TERT	c.3202G>A	p.Glu1068Lys	2		
TERT	c.3346_3522del177	p.Glu1116fsX11	2		
TERC	r.11g>t		1		
TERC	r.37a>g		4		
TERC	r.66c>a		1		
TERC	r.116c>t		1		
TERC	r.182g>c		2		
TERC	r.234c>g		1		
RTEL1	c.602delG	p.Gly201GlufsX15	3		
RTEL1	c.1451C>T	p.Pro484Leu	8		
RTEL1	c.1940C>T	p.Pro647Leu	1		
RTEL1	c.2005C>T	p.Gln669X	4		
RTEL1	c.2063C>G	p.Ser688Cys	1		
RTEL1	c.2205_2207delGAC	p.Asp736del	2		
RTEL1	c.3371A>C	p.His1124Pro	1		
PARN	c.246-2A>G	Splicing	8		
PARN	c.529C>T	p.Gln177X	11	1	
PARN	c.563_564insT	p.Ile188IlefsX7	2		
PARN	c.751delA	p.Arg251GlufsX14	1		
PARN	c.874delG	p.Asp292ThrfsX16	2		
PARN	c.1081+1G>A	Splicing	2		
PARN	c.1262A>G	p.Lys421Arg	5		
PARN	c.1749_1750delAG	p.Glu585AspfsX4	1		
Total			199	8	2

*The positions of the DNA, RNA and protein variants are described using TERT NM_198253.2 (isoform 1), TERC NR_001566.1, PARN NM_002582.3 (isoform 1) and RTEL1 NM_001283009.1 (isoform 3).

‡Both variants are found in two siblings, suggesting that they in *cis* and on the same allele.

Supplemental Table 2. Complete Blood Counts for Human Subjects with Somatic *TERT* promoter mutations

	UT822	UT2031	UT3224	UT387	UT248	UT2482	UT3410
Age	58	55	48	55	48	50	63
Gender	M	M	M	M	F	M	M
TERT CDS Mutation	V144M	R631Q	P632L	V747fs	R865H	F883I	V997fs
TERT Promoter Mutation	-124	-124	-124	-124	-146/-124	-124	-124
WBC (thousand/uL)	6.1	7.7	8.0	7.7	7.1	9.6	7.9
RBC (million/uL)	4.21 (L)	4.39	4.01 (L)	4.35	4.38	4.37	4.17
Hemoglobin (g/dL)	14.4	14.5	13.2	15.2	14.3	42.5	14.2
MCV (fL)	100	101.4 (H)	102.9 (H)	101.0 (H)	97.7	97	103 (H)
Platelet (thousand/uL)	158	166	204	159	227	146	108 (L)

Abbreviation: CDS, cDNA sequence; M, male; F, female; WBC, white blood cell count; RBC, red blood cell count; MCV, mean corpuscular volume

A (L) or (H) indicates values that are lower or higher, respectively, than the expected reference range for the laboratory.

Supplemental Table 3. Extended Lymphocyte Subset Quantification of Human Subjects

	***TERT* CDS + Promoter Mutation**			***TERT* CDS Mutation**		**Family Controls**	
	UT248	UT2031	UT3224	UT244	UT245	UT246	UT3442
Age	59	55	48	30	32	64	58
Gender	F	M	M	F	F	M	F
TERT CDS Mutation	R865H	R631Q	P632L	R865H	R865H	none	none
TERT Promoter Mutation	-146/-124	-124	-124	none	none	none	none
WBC (thousand/uL)	5.5	7.7	8.0	2.5 (L)	7.7	6.2	8.3
CD3+ (%)	66.0	50.6 (L)	68.5	73.5	81.7	76.3	80.9
CD3+ (cells/uL)	1252	475 (L)	921 (L)	652 (L)	1560	1509	1551
CD3+CD4+ (%)	48.6	38.8	42.2	55.6 (H)	47.6	55.1 (H)	55.6 (H)
CD3+CD4+ (cells/uL)	922	365 (L)	567	493 (L)	908	1090	1066
CD3+CD8+ (%)	15.5 (L)	10.5 (L)	24.4	17.2 (L)	30.5	20.9	20.5
CD3+CD8+ (cells/uL)	294 (L)	98 (L)	328 (L)	152 (L)	583	413	394
CD4/CD8 Ratio	3.1	3.7	1.7	3.2	1.6	2.6	2.7
CD3-CD19+ (%)	15.1	25 (H)	16.6	8.4	12.1	7.5	12.9
CD3-CD19+ (cells/uL)	286	235	224	75 (L)	231	148	246
CD2+ (%)	82.5	71.7	88.5	86.5	86.0	84.4	90.2
CD45RO+/CD45RA- (%)	49.9	43.0	47.2	36.4	36.4	39.4	54.5
CD45RA+/CD45RO- (%)	45.9	53.7	47.6	58.3	57.5	55.8	38.8
TCR-alpha/beta+ (%)	63.1	58.4	73.9	71.8	80.8	76.4	76.4
TCR-gamma/delta+ (%)	1.3	1.6	1.8	1.5	3.0	1.2	6.2

Abbreviation: CDS, cDNA sequence; M, male; F, female; WBC, white blood cell count; RBC, red blood cell count; MCV, mean corpuscular volume

A (L) or (H) indicates values that are lower or higher, respectively, than the expected reference range. The reference range for the complete blood count was provided by Quest Diagnostics. The reference range for the extended lymphocyte subset panel was provided by the clinical laboratory and based on a published study that evaluated a large number of healthy donors (Sherer et al, J. Allergy Clin. Immunol., 2003, 112(5):973-980).

Supplemental Table 4. Haplotype analysis of diluted whole blood DNA from different subjects analyzed by limiting dilution PCR of the *TERT* promoter and coding sequence variants

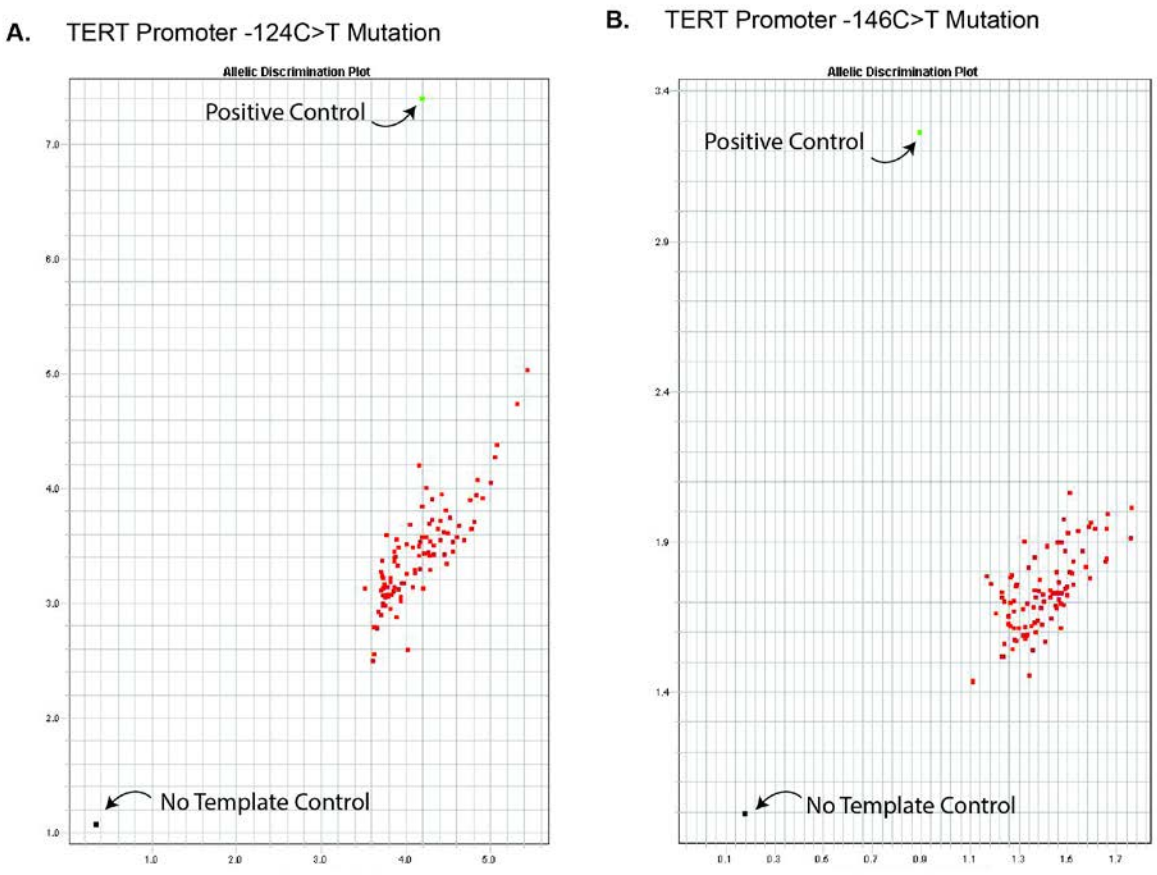
		-146 C>T Promoter Mutation in cis- with		-124 C>T Promoter Mutation in cis- with		Wildtype Promoter in cis-with	
Subject ID	No. samples	WT *TERT* CDS	Mutant *TERT* CDS	WT *TERT* CDS	Mutant *TERT* CDS	WT *TERT* CDS	Mutant *TERT* CDS
UT248	11	4 (36%)	0	1 (9%)	0	1 (10%)	5 (45%)
UT822	21	1 (5%)	0	5 (24%)	0	3 (14%)	12 (57%)
UT2923	15			6 (40%)	1 (7%)	1 (7%)	7 (47%)
UT387	10			4 (40%)	0	2 (20%)	4 (40%)
UT2210	13	5 (38%)	0			1 (8%)	7 (54%)
UT2482	12	4 (33%)	2 (17%)			4 (33%)	2 (17%)

Abbreviation: CDS, cDNA sequence

Supplemental Table 5. Oligonucleotides used for *TERT* Promoter Sanger Sequencing, Allelic Discrimination (AD) Assay, Limiting Dilution PCR and Quantitative (Q-)RT PCR Assays

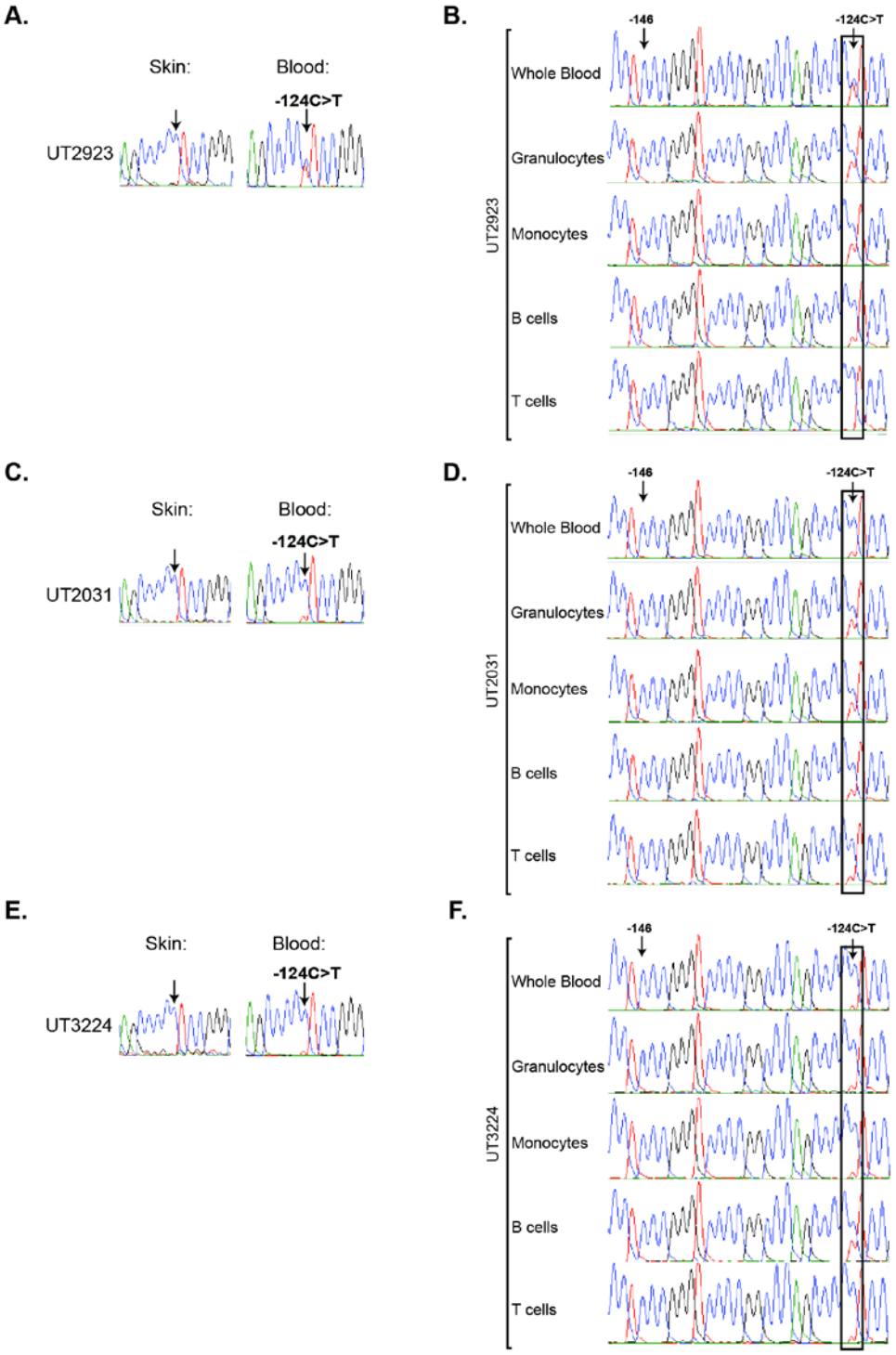
Assay, Primer	Oligonucleotide Name	Oligonucleotide Sequence, 5'-> 3'
Sanger sequencing and AD assay	TERT_Prom_A_F	CTGTGTCAAGGAGCCCAAG
Sanger sequencing and AD assay	TERT_Prom_A_R	AGCACCTCGCGGTAGTGG
-146 C>T AD assay internal oligo	TERT_C250T_F	CTTCCAGCTCCGCCTCCT
-146 C>T AD assay internal oligo	TERT_C250T_R	GGGCCGCGGAAAGGAA
-146 C>T AD assay VIC probe	VIC Probe	VIC-CGACCCCTCCCGG-MGBNFQ
-146 C>T AD assay FAM probe	FAM Probe	FAM-CGACCCCTTCCGG-MGBNFQ
Sequencing DNA from Centenarians	Out-TERT-F	ACGAACGTGGCCAGCGGCAG
Sequencing DNA from Centenarians	Out-TERT-R	CTGGCGTCCCTGCACCCTGG
Limiting dilution PCR, outside, CDS	Out-UT248/UT2210/UT2482-F	ACCCACTCCCTCCCGAAGGTGCC
Limiting dilution PCR, outside, CDS	Out-UT248/UT2210/UT2482-R	AGCTGGCACAGAATTGCACAAGCTGATGG
Limiting dilution PCR, inside, CDS	In-UT248/UT2210/UT2482-F	AGAGAGAGAGGACTTGGCAGAGAC
Limiting dilution PCR, inside, CDS	In-UT248/UT2210/UT2482-R	ACTGTAGAGAGCTCGTCTGTTGG
Limiting dilution PCR, outside, CDS	Out-UT387-F	CGTGAGGACAATGGTGCGGACCATGCC
Limiting dilution PCR, outside, CDS	Out-UT387-R	TTGGGGTCTGGAGTGGTGGGGGTCAG
Limiting dilution PCR, inside, CDS	In-UT387-F	CAGACACACACACATGCACACC
Limiting dilution PCR, inside, CDS	In-UT387-R	TGAGGGCAGAGGTGATGTCTG
Limiting dilution PCR, outside, CDS	Out-UT822-F	GGGCAACGGCAGACTTCGGCTGG
Limiting dilution PCR, outside, CDS	Out-UT822-R	AGAGCAGCGCAGGCGACTCAGG
Limiting dilution PCR, inside, CDS	In-UT822-F	CCCTGACGCTATGGTTCCAGG
Limiting dilution PCR, inside, CDS	In-UT822-R	TGTCCTGCCTGAAGGAGCTG
Limiting dilution PCR, outside, CDS	Out-UT2923-F	CTCCAGCCTGGGCAACAGAGTGAGACTCC
Limiting dilution PCR, outside, CDS	Out-UT2923-R	CCCTCGGTGATCTGGATGTGGCATGTCC
Limiting dilution PCR, inside, CDS	In- UT2923-F	GAAAGGCAAGGAGGCTAGTGG
Limiting dilution PCR, inside, CDS	In- UT2923-R	AAGTTCCTGCACTGGCTGATG
Limiting dilution PCR, outside, TERT	Out-TERT-F	ACGAACGTGGCCAGCGGCAG
Limiting dilution PCR, outside, TERT	Out-TERT-R	CTGGCGTCCCTGCACCCTGG
Limiting dilution PCR, inside, TERT	In-TERT-F	ACCTCGCGGTAGTGGCTGC
Limiting dilution PCR, inside, TERT	In-TERT-R	GTCCTGCCCCTTCACCTT
Q-RT PCR assay for hTERT	hTERT_F_qPCR	CGTACAGGTTTCACGCATGTG
Q-RT PCR assay for hTERT	hTERT_R_qPCR	ATGACGCGCAGGAAAAATGT
Q-RT PCR assay for hCyclophilin	hCyclophilin_F_qPCR	ATGTGGTGTTTGGCAAAGTTCTA
Q-RT PCR assay for hCyclophilin	hCyclophilin_R_qPCR	GGTTTATCCCGGCTGTCT
Q-RT PCR assay for hGAPDH	hGAPDH_F_qPCR	ACAGCAACAGGGTGGTGGAC
Q-RT PCR assay for hGAPDH	hGAPDH_R_qPCR	GACCATTGCTGGGGCTGGTG

Supplemental Figure 1



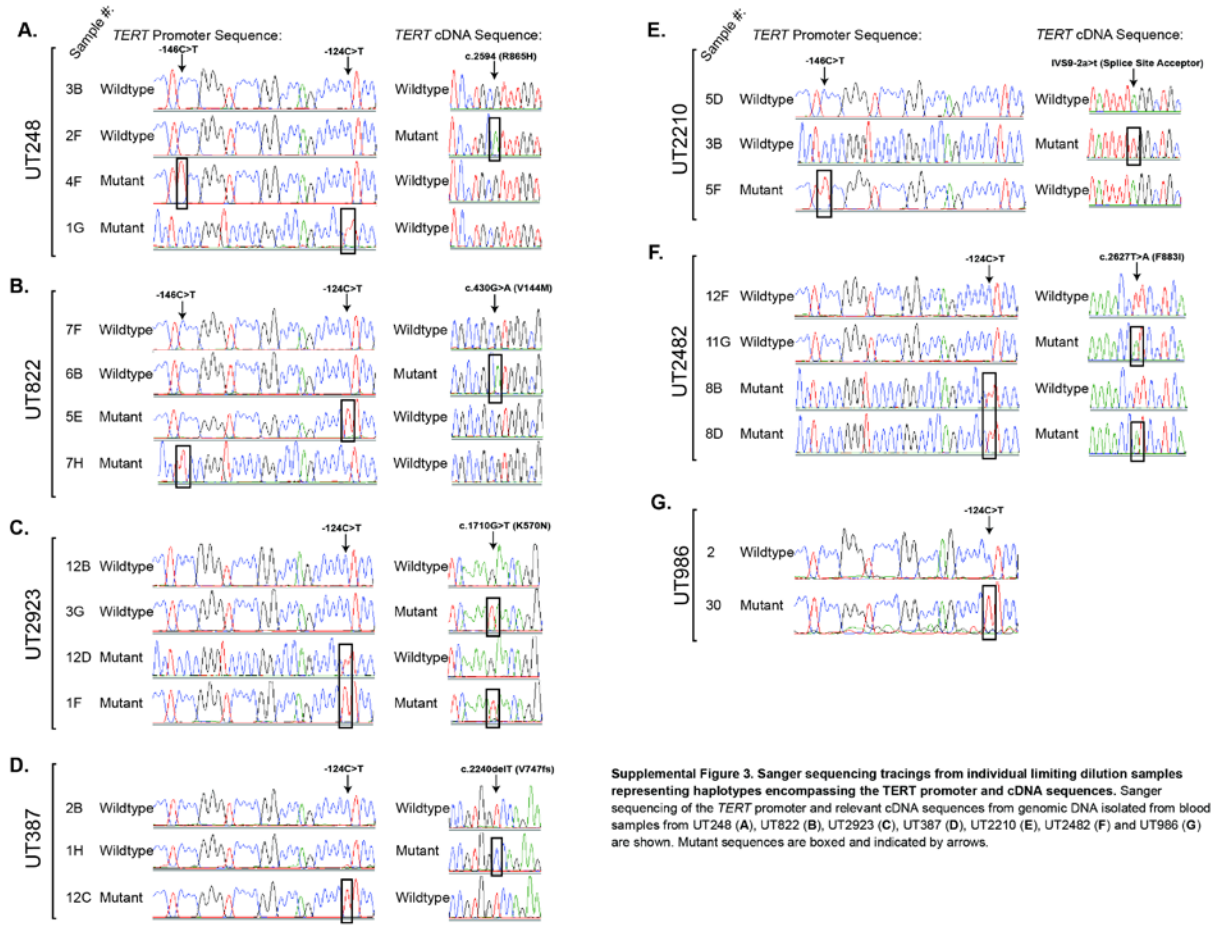
Supplemental Figure 1. Allelic discrimination assays for detection of the *TERT* -124 C>T (A) and the *TERT* -146 C>T (B) Promoter Mutations. One representative 96-well plate of samples is shown for each assay. The no template controls and the positive controls for each assay are indicated by arrows.

Supplemental Figure 2



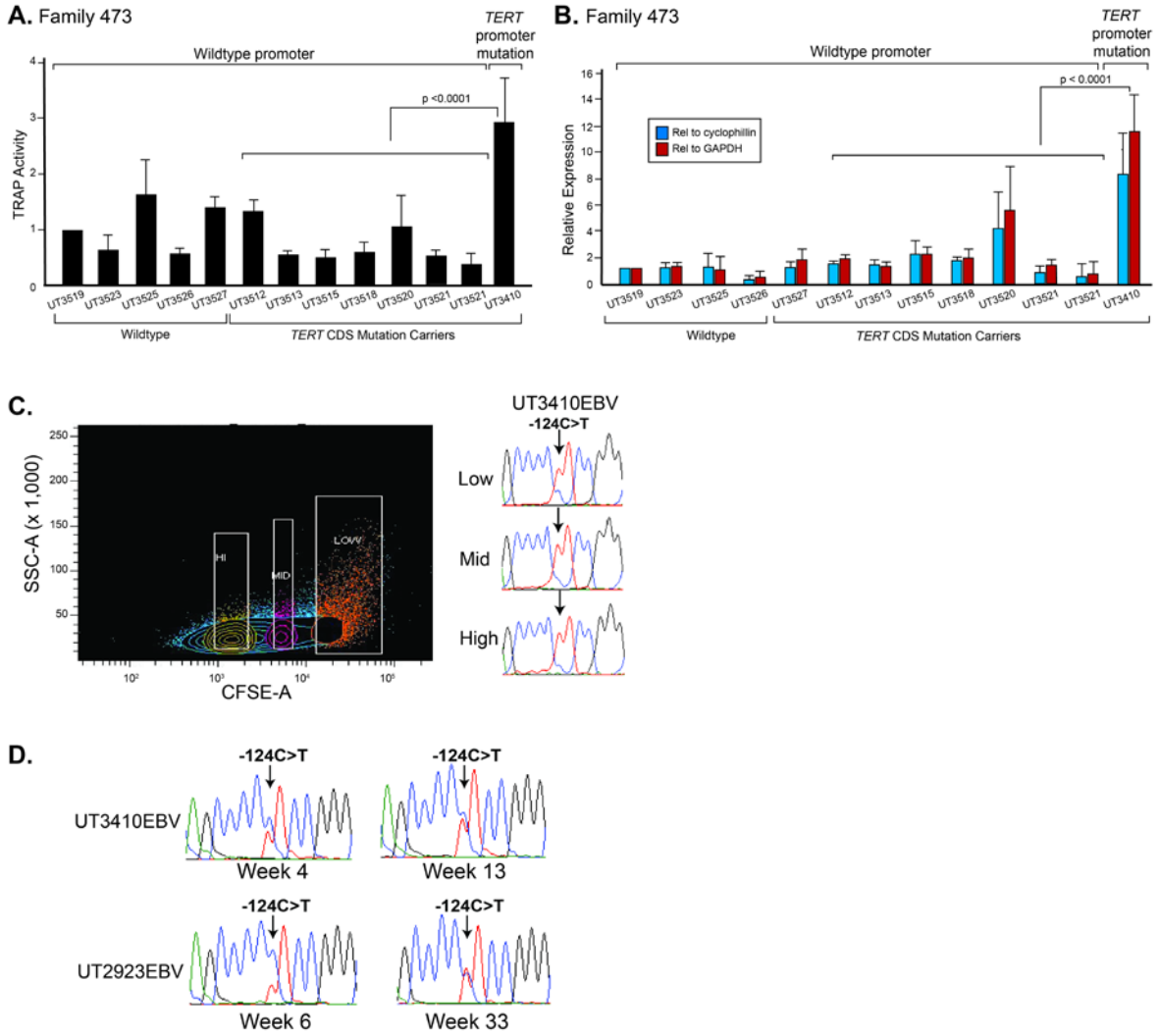
Supplemental Figure 2. Sanger sequences of the *TERT* promoter from skin, whole blood, granulocytes, monocytes, B cells and T cells of subjects with the *TERT* -124 C>T promoter mutation. Genomic DNA isolated from whole blood and skin was purified and subjected to Sanger sequencing of the *TERT* promoter (**A, C, E**). Blood samples were subjected to flow cytometry (see Methods). Genomic DNA from each fraction was purified and subjected to Sanger sequencing of the *TERT* promoter (**B, D, F**). The location of the -124 C>T mutation is indicated by the box.

Supplemental Figure 3



Supplemental Figure 3. Sanger sequencing tracings from individual limiting dilution samples representing haplotypes encompassing the TERT promoter and cDNA sequences. Sanger sequencing of the *TERT* promoter and relevant cDNA sequences from genomic DNA isolated from blood samples from UT248 (**A**), UT822 (**B**), UT2923 (**C**), UT387 (**D**), UT2210 (**E**), UT2482 (**F**) and UT986 (**G**) are shown. Mutant sequences are boxed and indicated by arrows.

Supplemental Figure 4



Supplemental Figure 4. *TERT* promoter mutation is associated with increased telomerase activity and higher proliferative capacity. A. Telomerase (TRAP) activity of equivalent numbers of EBV-transformed lymphoblastoid cells derived from different related individuals from family 473. The data are expressed relative to the activity of a wildtype control and represent the mean ± SD of 3 experiments. **B.** Expression of h*TERT* by real-time quantitative PCR relative to hCyclophilin and hGAPDH. The data are expressed relative to a wildtype control and represent the mean ± SD of triplicates of one representative experiment. Data was analyzed by a two-tailed Student's t-test in A and B. **C.** Flow sorting to separate EBV-transformed cells by proliferative capacity, followed by Sanger sequencing of the *TERT* promoter. **D.** Sanger sequencing of the *TERT* promoter from genomic DNA purified from the UT3410 and UT2923 EBV lymphoblastoid cell lines over time.