Structural Analysis Reveals the Deleterious Effects of Telomerase Mutations in Bone Marrow Failure Syndromes*

Hunter Hoffman1, Cory Rice1, and Emmanuel Skordalakes1,2

From the 1Department of Gene Expression and Regulation, Wistar Institute, Philadelphia, Pennsylvania 19104 and the 2Department of Biochemistry and Molecular Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Edited by Joel Gottesfeld

Naturally occurring mutations in the ribonucleoprotein reverse transcriptase, telomerase, are associated with the bone marrow failure syndromes dyskeratosis congenita, aplastic anemia, and idiopathic pulmonary fibrosis. However, the mechanism by which these mutations impact telomerase function remains unknown. Here we present the structure of the human telomerase C-terminal extension (or thumb domain) determined by the method of single-wavelength anomalous diffraction to 2.31 Å resolution. We also used direct telomerase activity and nucleic acid binding assays to explain how naturally occurring mutations within this portion of telomerase contribute to human disease. The single mutations localize within three highly conserved regions of the telomerase thumb domain referred to as motifs E-I (thumb loop and helix), E-II, and E-III (the FVYL pocket, comprising the hydrophobic residues Phe-1012, Val-1025, Tyr-1089, and Leu-1092). Biochemical data show that the mutations associated with dyskeratosis congenita, aplastic anemia, and idiopathic pulmonary fibrosis disrupt the binding between the protein subunit reverse transcriptase of the telomerase and its nucleic acid substrates leading to loss of telomerase activity and processivity. Collectively our data show that although these mutations do not alter the overall stability or telomerase activity and processivity, these rare genetic disorders are associated with an impaired telomerase holoenzyme that is unable to correctly assemble with its nucleic acid substrates, leading to incomplete telomere extension and telomere attrition, which are hallmarks of these diseases.

Human telomerase is a ribonucleoprotein reverse transcriptase that replicates the ends of eukaryotic chromosomes (1). The protein subunit, TERT,2 consists of several domains (TEN, TRBD, RT (fingers and palm), and thumb) organized into a closed, ring configuration, generating a large cavity in the interior of the ring and where the RNA template and the DNA bind during telomere elongation (2–5). The closed configuration of the TERT ring is stabilized by extensive interactions between the thumb and TRBD domains as well as by protein-RNA interactions (3, 6).

Several invariable motifs in the interior cavity of the TERT ring coordinate the RNA template and telomeric overhang (RNADNA hybrid) and position the 3’ end of the DNA for catalysis (2, 3). These include motifs E-I and E-II of the thumb domain, which bind the RNADNA hybrid and stabilize the telomerase elongation complex (2, 7); the primer grip region that guides the DNA at the active site of the enzyme and motifs 2 and B’ of the fingers and palm domain, respectively, which position the RNA template above the active site of the enzyme for nucleotide binding and selectivity (2, 3).

Current evidence shows that the RNA binding domain of telomerase (TRBD) binds the template boundary element (TBE) and the activation domain (CR4/5) of telomerase RNA (8–12). The TBE is a stem loop located only a few nucleotides upstream of the RNA template and is coordinated by the T, CP, and CP2 motifs in Tetrahymena thermophila (13, 14) or T, CP, and TFLY in vertebrates (8). These TBE-binding motifs are located at the interface of the TRBD and fingers domains, and together they form a well-defined indentation on the surface of the protein providing the platform for a network of nonspecific interactions with the TBE (13). Binding of the TBE to TRBD positions the RNA template at the active site of the enzyme thus promoting nucleotide binding and selectivity (3). We previously proposed, and it was subsequently shown, that the TRBD-TBE interaction provides the steric block that prevents TERT from replicating beyond the 5’ end of the RNA template thus promoting telomerase repeat addition processivity (2, 4, 13).

The telomerase activation domain consists of the stem loops P6a/b, P6.1, and P5 (15) and is coordinated by the VSR motif of TRBD and motifs E-II and E-III (FVYL pocket) of the thumb domain (6, 9, 10). Although CR4/5 is primarily coordinated by the TRBD, its stem loop P6.1 extends across the TRBD-thumb interface and binds the FVYL pocket of the thumb domain (6). Extensive interactions between the CR4/5 and the terminal domains of the TERT ring most likely stabilize the telomerase ribonucleoprotein complex and lock the closed ring configuration of TERT during telomere elongation.

*This work was supported by National Institutes of Health Grant R01CA201312 and Wistar Cancer Center Support Grant P30 CA10815. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

† This article contains supplemental Table S1 and Figs. S1 and S2.

1 To whom correspondence should be addressed. E-mail: skorda@wistar.org.
2 The abbreviations used are: TERT, telomerase reverse transcriptase; DKC, dyskeratosis congenital; AA, aplastic anemia; IPF, idiopathic pulmonary fibrosis; TRBD, telomerase RNA binding domain; TBE, template boundary element; CR4/5, telomerase RNA activation domain; FP, fluorescence polarization; SUMO, small ubiquitin-like modifier.
Several naturally occurring mutations within the thumb domain contribute to the rare genetic disorders dyskeratosis congenita (DKC), aplastic anemia (AA), and idiopathic pulmonary fibrosis (IPF). With the exception of AA, which is acquired, these disorders are inherited in an autosomal dominant pattern (16–19). DKC affects multiple parts of the body including nail shape and growth, skin discoloration, and oral leukoplakia (20–22). Patients affected by DKC may also develop aplastic anemia, a bone marrow failure disorder that interferes with the production of normal levels of red blood cells (23). DKC patients are also high risk for IPF, a disease associated with lung scaring and decrease in oxygen transport to the body (24).

In addition to AA and IPF, DKC patients are also high risk cases for cancers of the neck, head, genitals, and anus. In its most severe form, DKC patients develop Hoyeraal Hreidarsson syndrome usually associated with an unusually small cerebellum and impairment of motor skills (25).

Here we report the structure of the human telomerase thumb domain and explain the role of the naturally occurring mutations in human disease. The structure reveals that the majority of these mutations localize to the nucleic acid binding regions of the protein, which includes motifs E-I, E-II, and E-III. Biochemical assays show that these disease-associated mutations interfere with proper telomerase elongation complex formation leading to telomere attrition and loss of cell viability.

Results

Structure of Human Telomerase Thumb Domain—We identified a construct of the human telomerase thumb domain (hThumb) consisting of residues 961–1132 by limited proteolysis (Fig. 1A). A longer construct consisting of residues 941–1132, which contains the thumb loop, a portion of motif E-I (3), produced low protein yields sufficient for biochemical but not crystallographic studies. We subsequently solved the structure of hThumb 961–1132 to 2.31 Å resolution using the method of single-wavelength anomalous dispersion and a mercury derivative (Table 1). The structure revealed clear density for residues 968–986 of motif E-I, D, topology schematic of hThumb. The same color scheme as that of B is used. E, sequence alignment of human and Tribolium thumb domains. F, structural overlay of the human (salmon) and Tribolium (lime green) thumb domains.
TABLE 1
Data collection, phasing, and refinement statistics

|                          | Native       | Phasing: Hg derivative 1 |
|--------------------------|--------------|-------------------------|
| **Data collection**       |              |                         |
| Wavelength (Å)           | 1.5418       | 1.5418                  |
| Space group              | P32_1        | P32_1                   |
| Cell dimensions: a, b, c (Å) | 92.4, 50.2   | 92.9, 49.9              |
| Resolution (Å)           | 20–2.31      | 30–2.9                  |
| (I/σI)                   | (2.25–2.31)  | (3.05–2.9)              |
| Completeness (%)         | 98.9 (98.3)  | 99.6 (96.4)             |
| Redundancy               | 12 (12.7)    | 28 (29.4)               |
| **Phasing analysis**     |              |                         |
| Resolution (Å)           | 20–2.31      |                         |
| Number of sites          | 11191        |                         |
| SKEW (Phenix)            | 0.20         |                         |
| CORR RMS (Phenix)        | 0.06         |                         |
| Mean figure of merit     | 0.37         |                         |
| **Refinement**           |              |                         |
| Resolution (Å)           | 20–2.31      |                         |
| No. reflections          | 11191        |                         |
| B-factors                |              |                         |
| Protein                  | 43.6         |                         |
| Water                    | 59.6         |                         |
| Root mean square deviations |          |                         |
| Bond lengths (Å)         | 0.008        |                         |
| Bond angles (°)          | 1.186        |                         |
| Ramachandran plot (%)    | 97           |                         |
| Most favored             |              |                         |
| Allowed                  | 3            |                         |

* SKEW, distribution of electron density values in an experimentally phased map.
* CORR RMS, a measure of how contiguous the solvent and non-solvent regions are in the map.

Bone Marrow Failure Syndromes Impair TERT-NA Assembly

(1, E, and F). Within this domain there are three conserved motifs (E-I, E-II, and E-III; Fig. 1, B and C) involved in nucleic acid binding (2, 3). Of note is the displacement of part of motif E-I by the same helix of a crystallographic symmetry related molecule (supplemental Fig. S1, A and B). The flexibility of a small portion of E-I of the isolated thumb domain can be attributed to the absence of contacts with the TRBD and RT domains (2, 3). Occupation of the displaced helix by a crystal symmetry-related one further confirms the structural and functional conservation of this helix in telomerases across species.

Another highly conserved region of the hThumb is the FVYL pocket formed by the loops that connect motifs E-I, E-II, and E-III and was previously suggested to bind the P6.1 stem loop of CR4/5 (6). The FVYL pocket is located at the interface of the TRBD-thumb domain and is ~12 Å away from the nearest TRBD residue. The human FVYL pocket is the result of a small gap, generated by the organization of the tips and connecting loops of helices α2, α3, and α4, α5 (Fig. 1, B and D). The organization of the tips of these helices generates a gap 14 Å away and 8 Å deep and is sufficient to accommodate 2–3 nucleotide bases. The solvent-exposed, hTERT loop that connects helices α2 and α3 makes extensive interactions with the TRBD and contributes to the closed ring TERT configuration. In the absence of contacts with TRBD, this loop is flexible with Val-1025 partially occupying the FVYL pocket (Fig. 1, B and D). It is worth noting that the high salt crystal growth conditions may have also facilitated binding of Val-1025 into this hydrophobic region of the protein.

hThumb Disease Mutations Disrupt Protein-Nucleic Acid Binding—To better understand the role of hThumb mutations in human disease, we tested the ability of these mutant proteins to bind an RNADNA hybrid (hRNADNA) consisting of the human telomerase RNA template (CUAACCCU) and telomeric DNA (AGGGTTAG). To perform these assays, we overexpressed and purified to homogeneity the hThumb WT and R972H, R979W, C1015R, L1019F V1025F, N1028H, K1050E, and V1090M mutant proteins. We tested these proteins for binding to the hRNADNA hybrid using fluorescence polarization (FP) assays. It is worth noting that the V1025F mutant protein appears to aggregate in solution when overexpressed as an independent domain, and therefore we were unable to obtain a reliable Kₐ for this protein. The WT, hThumb binds the hRNADNA hybrid with ~450 nM binding affinity (Fig. 2, A and B, and supplemental Table S1). The mutant proteins located in motif E-I and E-II and make direct contacts with the hRNADNA hybrid (R972H, R979W, and K1050E) showed significantly lower affinity (2.6-, 4-, and 2.4-fold) for the hRNADNA hybrid than the WT protein. Similarly, the C1015R mutant, which contributes to the structural organization of this region of the protein and therefore the binding of the hRNADNA hybrid, also showed significantly lower affinity for the hRNADNA hybrid (4.6-fold) than the WT protein. Surprisingly, the L1019F showed almost wild type affinity for the hRNADNA hybrid (1.3-fold) compared with the WT protein (Fig. 2, A and B, and supplemental Table S1). Contrary to the mutations that contribute to hybrid binding, N1028H and V1090M showed a slight (1.5-fold) decrease in hRNADNA binding affinity, which is within the margin error. It is worth noting that in the full-length TERT, the hRNADNA hybrid makes additional contacts with the TRBD and RT domains (2, 6). We therefore expect a higher affinity for the hRNADNA in the context of the full-length protein, consistent with what has been previously reported (26).

hThumb Disease Mutations Impair Telomerase Activity and Processivity—We then asked how the disease associated mutations affect the activity and processivity of telomerase. To address this question, we performed direct telomerase activity assays. As a source of telomerase, we used lysates of HEK293T cells transfected with the plasmids pcDNA6-hTERT and pBS-U1-hTERT overexpressing telomerase. (The plasmids were a gift form Joachim Lingner’s laboratory at the Swiss Institute for Experimental Cancer Research.) Prior to carrying out the telomerase activity assays, we checked the levels of WT and mutant hTERT in cell lysates. We did so by Western blotting analysis, using a hTERT, specific antibody (abx120550, Abbeexa (27)), as described previously in Bryan et al. (6). Western blotting analysis showed that all hThumb WT and mutant proteins (R972H, K1050E, V1025F, and N1028W) express at nearly WT levels with subtle differences between them (Fig. 3A). The final activity and processivity of WT and mutant telomerases were adjusted for the subtle differences observed in the protein levels expressed and were quantified as previously described by Latrck and Cech (28) (details of activity and processivity quantifi-
Bone Marrow Failure Syndromes Impair TERT-NA Assembly

Depending on the severity of the TERT mutations, some mutant proteins show modest loss of telomerase activity and processivity, whereas others render the enzyme almost inactive (Fig. 3B). Mutations that make direct contacts with the hRNADNA hybrid or are important for the organization of these motifs show the most severe defects in telomerase activity and processivity. These include R972H and R979W of motif E-I and K1050E of motif E-II (Fig. 4, A–C). R972H and K1050E show 2-fold loss of activity and processivity each, relative to the WT telomerase. The severe R979W mutation results in 4-fold loss of activity and 20% of processivity compared with the WT enzyme (Fig. 3, B–D).

The TERT mutants C1015R and L1019F, which comprise part of motif E-I, are located in proximity to each other and are buried in the core of the protein. These two TERT mutations have significantly different effects in telomerase activity and processivity. C1015R shows 4-fold loss of activity and 20% loss of processivity relative to the wild type enzyme. In contrast, L1019F is almost 50% active compared with the WT enzyme, whereas its processivity is WT-like (Fig. 3, B–D).

The TERT mutations V1025F and N1028H comprise part of the loop that connects motifs E-I and E-II, while V1090M is part of motif EIII and they all form part of the FVYL pocket. These mutant proteins show a variety of changes in activity and processivity when compared with the WT protein. V1025F is 20% active and 70% processive relative to the WT telomerase; N1028H is 40% active, whereas its processivity is almost WT (90%), and V1090M is 80% active and processive when compared with the WT enzyme (Fig. 3, B–D). Interestingly, A1062T shows WT activity and processivity within the margin of error.

Discussion

To better understand the role of the naturally occurring telomerase mutations associated with the bone marrow failure syndromes DKC, AA, and IPF, we solved the structure of human telomerase thumb domain and performed a host of biochemical assays all geared toward understanding how these mutations affect telomerase function. The hThumb structure is structurally conserved to the Tribolium thumb domain (tcThumb) (Fig. 1, D and E), supporting structural conservation across species. The structural similarity between the two proteins allowed us to generated a telomerase model consisting of an overlay between the tcTERT-hRNADNA complex (PDB code 3KYL) and the hThumb domain (Fig. 4A). The overlay places the hThumb motifs E-I and part of motif E-II in the interior cavity of the TERT ring, where the hRNADNA hybrid binds (Fig. 4A). There are three residues within these two motifs implicated in DKC. These include R972H, R979W, and K1050E. The charged side chains of Arg-972 and Arg-979 of motif E-I and Lys-1050 of motif E-II extend toward the center of the TERT ring where the hRNADNA hybrid is located (Fig. 4B). Arg-979 is within coordinating distance of the hRNADNA hybrid. Arg-972 and Lys-1050 are ~5.5 Å from the nearest nucleic acid residue, which is sufficient for solvent mediated coordination. Alternatively, a slight structural rearrangement of the nucleic acid or of motif E-I and E-II could place the Arg-972 and Lys-1050 side chains within direct coordinating distance of the hRNADNA hybrid. Altering the long positively charged side chain of Arg-972 and Arg-979 into the shorter, bulkier histidine or tryptophan side chains would most likely disrupt the canonical contacts with the nucleic acid substrate thus interfering with telomerase function. In fact, the proximity of Arg-979 to the RNA-DNA hybrid suggests that the large tryptophan side chain may interfere with productive telomeric DNA binding and telomerase, elongation complex assembly. Consistent with this hypothesis, R972H and R979W show 2.6- and 4-fold loss of hRNADNA binding affinity, respectively, relative to the WT enzyme (Fig. 3, B–D). Partial disruption of TERT-nucleic acid for R972H and K1050E leads to 50% loss of WT telomerase activity and processivity (Fig. 4, A–C). The more severe R979W TERT mutant shows 80 and 20% loss of WT activity and processivity, respectively (Figs. 3 and 4).

![Figure 2. WT and mutant hThumb RNA-DNA binding assays.](image-url)
Interestingly, the mutant TERT A1062T does not affect telomerase function. A1062T shows nearly wild type telomerase activity and processivity (Fig. 3, B–D). The biochemical data are further supported by the structure, which shows that A1062T is located at a solvent-exposed loop region of hThumb (Fig. 4C) and is at least 15 Å away from the nearest known functional site of TERT. Consistent with this hypothesis, it has been proposed that this mutation is also present in healthy subjects with telomere length within the normal range (16). It has therefore been proposed that this mutation may be a rare polymorphism, which does not contribute to the development of bone marrow failure.

Two additional mutations associated with AA and IPF are C1015R and L1019F, respectively. Cys-1015 and Leu-1019 comprise part of helix α2 of motif E-I (Figs. 1, B and C, and 5A). These residues are involved in direct interactions with helix α1, which contains Arg-972 and Arg-979, the two hThumb residues involved in interactions with the hRNADNA. Unlike Arg-972 and Arg-979, Cys-1015 and Leu-1019 are buried within the core of hThumb and are not in contact with the nucleic acid substrate (Fig. 5B). Specifically, Cys-1015 is coordinated by the hydrophobic side chains of Met-970 and Leu-1019 (Fig. 5B), whereas Leu-1019 is located adjacent to Cys-1015 and coordinated by Met-970 and Phe-1032 (Fig. 5B). Both residues are
Bone Marrow Failure Syndromes Impair TERT-NA Assembly

critical for the organization and positioning of helix α1 and possibly of the FYVL pocket. Substitution of these two residues with larger side chains would have an effect on the overall organization of the tertiary structure of hThumb and in particular of helix α1, which in turn would affect the fold of the protein and TERT nucleic acid binding. Consistent with this hypothesis, C1015R shows severe loss (4.6-fold lower than the WT protein) of nucleic acid binding (Fig. 2, A–C), and telomerase activity and processivity (4-fold loss of activity and 20% loss of processivity compared with the WT enzyme) (Fig. 3, B–D). Our data are further supported by telomere length studies on peripheral blood mononuclear cells from patients carrying the C1015R TERT mutation. Southern blot analysis of peripheral blood mononuclear cells from these patients showed marked telomere shortening frequently associated with low levels of telomerase or a dysfunctional telomerase holoenzyme (18). In contrast to C1015R, substitution of the branched leucine (Leu-1019) side chain with the aromatic side chain of phenylalanine may be less disruptive to the fold of the protein. This is reflected in the WT binding affinity of this mutant TERT protein for the hRNADNA and its WT processivity compared with the WT enzyme (Fig. 3, B–D). Close inspection of the L1019F binding site shows that it can accommodate the larger phenylalanine side chain with some subtle (if any) rearrangement of the side chains of the surrounding residues (Fig. 5B). However, the L1019F mutant shows 50% loss of WT telomerase activity, a defect that most likely contributes to IPF associated with this mutant.

The telomerase FYVL pocket is also contained within this domain, comprises one of the two most conserved regions of hThumb (Consurf) (29), and most likely binds the P6.1 stem loop of the activation domain of telomerase (6). Three reported disease mutations (V1025F, N1028H, and V1090M) are located within this pocket of hThumb. We discussed some of these mutations previously in the context of the telomerase model published in PNAS (6, 30). With the hThumb structure at hand, we want to point out that these mutations indeed form part of the FYVL pocket of TERT (Fig. 5C). The precise mechanism of action of these mutations in telomerase function can only be obtained in the presence of a structure of hThumb bound to the P6.1 stem loop of CR4/5. However, we decided to test whether these mutations have an effect on hRNADNA binding. As we mentioned in the results section of the manuscript, V1025F most likely aggregates in solution, and we could not obtain an accurate Kd. However, FP assays of N1028H and V1090M show a subtle change in hRNADNA binding affinity (≈1.5-fold) compared with the WT enzyme (Fig. 2, A and B, and supplemental Table S1). This is not surprising because these two residues (N1028H and V1090M) comprise part of the loops that connect helices α2-α3 and α4-α5 respectively (Fig. 5C). They are located >20 Å away from the hRNADNA binding site and are not involved in the structural organization of this domain. The subtle changes in hRNADNA binding combined with the effect on P6.1 binding potentially explain the detrimental effects of these mutations in telomerase function and the development of the bone marrow failure-associated diseases.

Taken together, our data suggest that the disease-associated mutations discussed here do not alter the overall stability or expression of TERT, but instead, they are preventing proper nucleic acid interactions, resulting in significant decrease in telomerase activity and processivity. Consistent with the structural and biochemical assays, Southern blot analysis of DNA extracted from total peripheral blood white cells of patients carrying these disease mutations showed significantly shorter telomeres, a chromosomal defect usually associated with impaired telomerase activity (19, 31–33).
The role of TERT missense mutations associated with DC/AA/PF is very hard to establish because they are so rare. However, our structural and biochemical data provide significant insights into the role of these mutations in telomerase function and how they may contribute to human disease. Some of the main telomere-associated characteristics of these rare genetic disorders are significantly reduced telomerase activity and short telomeres. The severity of the disease is directly related to the degree of telomere shortening, which can be attributed in part to a dysfunctional telomerase.

**Experimental Procedures**

**Protein Expression and Purification**—The human thumb domain residues 961–1132 were cloned into a modified vector expressing a hexahistidine tag (His6 tag) followed by a tobacco etch virus-cleavable small ubiquitin-like modifier (SUMO) fusion protein at its N terminus. The protein was overexpressed in *Escherichia coli* ScarabXpress-1 (T7Lac) cells (Scarab Genomics) at 16 °C overnight using 1 mM isopropyl-β-D-thiogalactopyranoside (Gold Biotechnology). Cells were harvested by centrifugation and resuspended in buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 M KCl, 5% glycerol, 0.1 mM benzamidin, 0.1 M PMSF, and 15 mM imidazole prior to lysis via sonication. The hThumb protein was then purified over a nickel-nitrilotriacetic acid resin (MCLab) column. The His-SUMO tag was cleaved by tobacco etch virus endopeptidase overnight at 4 °C.

We further purified the protein over a porous HS resin (Applied Biosystems) column using a gradient of 25 mM Tris-HCl (pH 7.5), 0.5–1.5 M KCl, 5% glycerol, and 1 mM DTT. The protein was then concentrated and passed over a Superdex S200 size exclusion chromatography column (GE Healthcare) to remove any aggregation before moving on to crystallization.

**Protein Crystallization and Data Collection**—For crystallization studies, the purified protein was concentrated to 10 mg/ml and dialyzed in a buffer containing 5 mM NaHPO4 (pH 7.5), 100 mM KCl, and 1 mM TCEP. Crystals appeared within 3 days in sitting drop trays containing 0.8 M Li2SO4, and 50 mM Bis-Tris (pH 8.5) at 10 °C. Crystal diffraction was significantly improved using structural overlays, and the figures were prepared with PyMOL (38).

**Fluorescence Polarization Assays**—We tested hThumb (WT and mutant)-hRNADNA binding using FP assays with the Envision Xcite multilabel plate reader (PerkinElmer Life Sciences). 20-μl binding reactions were carried out in a buffer containing 20 mM Hepes, pH 7.5, 100 mM KCl, 2 mM MgCl2, 1 mM EDTA, 2 mM DTT, 1 mg/ml BSA, 5% (v/v) glycerol. The hRNADNA probe consisting of the human telomerase RNA template and telomeric DNA linked together by a stable tetraloop (italics) (rCrUrArArCrUrGrCdTdTdCdGdG-GdCdAdGdGdGdTdTdAdG) was purchased with a 5'-6-FAM label from Integrated DNA Technologies. The final probe concentration was used at 2.5 μM, whereas the hThumb protein concentration ranged from 0 to 15 μM. We incubated the reactions at room temperature for 30 min and pipetted in triplicate into a black 384-well optiplate (PerkinElmer Life Sciences). The reactions were excited at 480 nm, and the emissions were measured at 535-nm wavelength. The millipolarization values were calculated by the Envision operating software (PerkinElmer Life Sciences). The data were fit and the binding constants were determined with a one-site fit all, nonlinear regression model using PRISM 5.0 (GraphPad Software).

**Cell Culture**—HEK293T cells were grown in DMEM (Gibco) and 10% fetal bovine serum (Gibco). WT and mutant pcDNA6-hTERT and WT pBS-U1-hTER were transfected in a 1:3 ratio into confluent cells following the standard Lipofectamine transfection reagent protocol (Thermo Fisher Scientific) as previously described by Cristofari et al. (39). The following day, the cells were transferred to a t25 flask and allowed to grow for an additional day. Two days post-transfection, the cells were trypsinized, pelleted, and stored at −80 °C until needed.

**Western Blotting Analysis**—For the Western blotting analysis, standard immunoblot protocols (40) were used with the following antibody dilutions: anti-human TERT antibody (Abbeza, product no. 120550 (27), 1:1000 dilution), anti-β-actin antibody conjugated to HRP (Abcam, product no. 49900, 1:1000 dilution), and secondary HRP-conjugated anti-sheep IgG antibody (Santa Cruz Biotechnology, product no. 2924, 1:1000 dilution) used to detect the human TERT antibody. Detection of antibody signal was done with chemiluminescence activated using 2 ml of Luminata Forte Western HRP Substrate (Millipore). The signal was detected and developed with a Fuji LAS-3000 scanner. Western blotting signals were quantified using ImageQuant TL (GE Healthcare) and normalized using the β-actin signal as a loading control.

**Direct Telomerase Assay**—Total cell lysates were prepared using 1X CHAPS lysis buffer (150 mM KCI, 50 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 32 units of RNasin, 10% glycerol, supplemented with protease inhibitor mixture and 5 mM Mg-mercaptoethanol). Endogenous, WT, and mutant lysates containing 2 μg of total protein were incubated in a direct assay reaction mix (50 mM Tris-HCl, pH 8, 50 mM KCl, 1 mM MgCl2, 1 mM spermidine, 5 mM Mg-mercaptoethanol, 0.04 mM dGTP, 0.5 mM dATP, 32 units of RNasin, 1 μM hTel Substrate TTAGGTTAGCGTTAGG) for 1 h at 30 °C. After incubation had finished, 10 nmol of a 32P-labeled loading control (single-stranded, yeast telomeric DNA, 10 bases long) was added to each sample. DNA was isolated by overnight precipitation in 3.6 M NH4OAc (pH 5), glycogen, and 100% ethanol at −80 °C. The following day, DNA was pelleted and resuspended in 20 μl of gel loading buffer (98% formamide, 10 mM EDTA, 0.05% xylene cyanol) and incubated at 95 °C for 5 min. Samples were then run on a 10% polyacrylamide (19:1), 8 μl urea gel for 2 h at 1800 V. The gel was then fixed with a 30% meth-
Bone Marrow Failure Syndromes Impair TERT-NA Assembly

anol, 10% acetic acid solution, and vacuum-dried at 80 °C for 1 h. The gel was exposed to a phosphor storage plate overnight and imaged the following day.

**Direct Assay Quantification**—The direct telomerase assays were quantified as described previously (28, 41). Briefly, the activity of each protein (WT or mutant) was determined as follows: we used ImageQuant TL (GE Healthcare) to determine the intensity of each band on the gel. The total intensity of each sample was adjusted based on the loading control. The intensity of each band was normalized for the number of radiolabeled nucleotides added per repeat. The total lane counts were then measured by taking the sum of the normalized band intensity over the entire lane.

The telomerase processivity for the WT and mutant hTERT proteins was quantified as follows: the intensity of each repeat was quantified using ImageQuant and normalized by dividing by the total number of hot Gs incorporated per repeat number. For each repeat (x), the number of telomeric products not extended (or fraction left behind) was calculated by dividing the total intensity of bands (1-x) by the total counts from that lane. We then plotted the natural log of 1 — the fraction left behind against each repeat number and the slope (m) from the linear regression (supplemental Fig. S2) of this graph was used to determine the processivity of each telomerase (WT or mutant), by dividing −ln(2)/m.

**Author Contributions**—E. S. and H. H. designed the experiments and wrote the manuscript. H. H. carried out the structural studies and telomerase activity assays. C. R. carried out the nucleic acid binding studies.

**Acknowledgment**—We thank Dr. Joachim Lingner for providing the DNA primer.

**References**

1. Greider, C. W., and Blackburn, E. H. (1987) The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell* **51**, 887–898

2. Mitchell, M., Gillis, A., Futahashi, M., Fujiwara, H., and Skordalakes, E. (2010) Structural basis for telomerase catalytic subunit TERT binding to RNA template and telomeric DNA. *Nat. Struct. Mol. Biol.* **17**, 513–518

3. Gillis, A. J., Schuller, A. P., and Skordalakes, E. (2008) Structure of the *Tribolium castaneum* telomerase catalytic subunit TERT. *Nature* **455**, 633–637

4. Mason, M., Schuller, A., and Skordalakes, E. (2011) Telomerase structure function. *Curr. Opin. Struct. Biol.* **21**, 92–100

5. Sauerwald, A., Sandin, S., Cristofari, G., Scheres, S. H., Lingner, J., and Rhodes, D. (2013) Structure of active dimeric human telomerase. *Nat. Struct. Mol. Biol.* **20**, 454–460

6. Bryan, C., Rice, C., Hoffman, H., Harksheimer, M., Sweeney, M., and Skordalakes, E. (2015) Structural basis of telomerase inhibition by the highly specific BIBR1532. *Structure* **23**, 1934–1942

7. Huard, S., Moriarty, T. J., and Autexier, C. (2003) The C terminus of the human telomerase reverse transcriptase is a determinant of enzyme processivity. *Nucleic Acids Res.* **31**, 4059–4070

8. Harksheimer, M., Mason, M., Shuvavae, E., and Skordalakes, E. (2013) A motif in the vertebrate telomerase N-terminal linker of TERT contributes to RNA binding and telomerase activity and processivity. *Structure* **21**, 1870–1878

9. Huang, J., Brown, A. F., Wu, J., Xue, J., Bley, C. J., Rand, D. P., Wu, L., Zhang, R., Chen, J. I., and Lei, M. (2014) Structural basis for protein-RNA recognition in telomerase. *Nat. Struct. Mol. Biol.* **21**, 507–512

10. Bley, C. J., Qi, X., Rand, D. P., Borges, C. R., Nelson, R. W., and Chen, J. J. (2011) RNA-protein binding interface in the telomerase ribonucleoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 20333–20338

11. Jiang, J., Miracco, E. J., Hong, K., Eckert, B., Chan, H., Cash, D. D., Min, B., Zhou, Z. H., Collins, K., and Feigon, J. (2013) The architecture of Tetrahymena telomerase holoenzyme. *Nature* **496**, 187–192

12. Jiang, J., Chan, H., Cash, D. D., Miracco, E. J., Ogorkalek Loo, R. R., Upton, H. E., Cascio, D., O’Brien Johnson, R., Collins, K., Loo, J. A., Zhou, Z. H., and Feigon, J. (2015) Structure of Tetrahymena telomerase reveals previously unknown subunits, functions, and interactions. *Science* **350**, aab4070

13. Jansson, L. I., Akiyama, B. M., Ooms, A., Lu, C., Rubin, S. M., and Stone, M. D. (2015) Structural basis of template-boundary definition in Tetrahymena telomerase. *Nat. Struct. Mol. Biol.* **22**, 883–888

14. Rouida, S., and Skordalakes, E. (2007) Structure of the RNA-binding domain of telomerase: implications for RNA recognition and binding. *Structure* **15**, 1403–1412

15. Chen, J. L., Blasco, M. A., and Greider, C. W. (2000) Secondary structure of vertebrate telomerase RNA. *Cell* **100**, 503–514

16. Elder, J. K., Chen, J. J., Lancaster, L., Danoff, S., Su, S. C., Cogan, J. D., Vulto, I., Xie, M., Qi, X., Tuder, R. M., Phillips, J. A., 3rd, Lansdorp, P. M., Loyd, J. E., and Armanios, M. Y. (2008) Short telomerases are a risk factor for idiopathic pulmonary fibrosis. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 13051–13056

17. Calado, R. T., Regal, J. A., Hills, M., Yewdell, W. T., Dalmazo, L. F., Zago, M. A., Lansdorp, P. M., Hogge, D., Chanoik, S. J., Estey, E. H., Falco, R. P., and Young, N. S. (2009) Constitutional hypomorph telomerase mutations in patients with acute myeloid leukemia. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 1187–1192

18. Du, H. Y., Pumbo, E., Ivanovich, J., An, P., Maziarz, R. T., Reiss, U. M., Chimonas, D., Shimamura, A., Vlachos, A., Lipton, J. M., Goyal, R. K., Goldman, F., Wilson, D. B., Mason, P. J., and Bessler, M. (2009) TERC and TERT gene mutations in patients with bone marrow failure and the significance of telomere length measurements. *Blood* **113**, 309–316

19. Vulliami, T. J., Kirwan, M. J., Beswick, R., Hossain, U., Baqi, C., Ratcliffe, A., Marsh, J., Walne, A., and Dokal, I. (2011) Differences in disease severity but similar telomere lengths in genetic subgroups of patients with telomerase and shelterin mutations. *PloS One* **6**, e24383

20. Bessler, M., Wilson, D. B., and Mason, P. J. (2010) Dyskeratosis congenita. *FEBS Lett.* **584**, 3831–3838

21. Dokal, I. (1996) Dyskeratosis congenita: an inherited bone marrow failure syndrome. *Br. J. Haematol.* **92**, 775–779

22. Kirwan, M., and Dokal, I. (2008) Dyskeratosis congenita: a genetic disorder of many faces. *Clin. Genet.* **73**, 103–112

23. Dokal, I., and Vulliami, T. (2003) Dyskeratosis congenita: its link to telomerase and aplastic anaemia. *Blood Rev.* **17**, 217–225

24. Tsakiri, K. D., Cronkhite, J. T., Kuan, P. J., Xing, C., Raghu, G., Weissler, J. C., Rosenblatt, R. L., Shay, J. W., and Garcia, C. K. (2007) Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 7552–7555

25. Knight, S. W., Heiss, N. S., Vulliami, T. J., Aafks, C. M., McMahon, C. Richard, J., Jones, A., Hennekam, R. C., Poustka, A., Mason, P. J., and Dokal, I. (1999) Unexplained aplastic anaemia, immunodeficiency, and cerebellar hypoplasia (Hoyeraal-Hreidarsson syndrome) due to mutations in the dyskeratosis congenita gene, DKC1. *Br. J. Haematol.* **107**, 335–339

26. Tomlinson, C. G., Holien, J. K., Mathias, J. A., Parker, M. W., and Bryan, T. M. (2016) The C-terminal extension of human telomerase reverse transcriptase is necessary for high affinity binding to telomeric DNA. *Biochimie* **128**, 114–121

27. Cohen, S. B., and Reddel, R. R. (2008) A sensitive direct human telomerase activity assay. *Nat. Methods* **5**, 355–360

28. Latrck, C. M., and Cech, T. R. (2010) POT1-TPP1 enhances telomerase processivity by slowing primer dissociation and aiding translocation. *EMBO J.* **29**, 924–933

29. Armon, A., Graur, D., and Ben-Tal, N. (2001) ConSurf: an algorithmic tool for the identification of functional regions in proteins by surface mapping of phylogenetic information. *J. Mol. Biol.* **307**, 447–465
30. Steczkiewicz, K., Zimmermann, M. T., Kurcinski, M., Lewis, B. A., Dobbs, D., Kloczkowski, A., Jernigan, R. L., Kolinski, A., and Ginalski, K. (2011) Human telomerase model shows the role of the TEN domain in advancing the double helix for the next polymerization step. Proc. Natl. Acad. Sci. U.S.A. 108, 9443–9448

31. Cronkhite, J. T., Xing, C., Raghu, G., Chin, K. M., Torres, F., Rosenblatt, R. L., and Garcia, C. K. (2008) Telomere shortening in familial and sporadic pulmonary fibrosis. Am. J. Respir. Crit. Care Med. 178, 729–737

32. Vulliamy, T. J., Walne, A., Baskaradas, A., Mason, P. J., Marrone, A., and Dokal, I. (2005) Mutations in the reverse transcriptase component of telomerase (TERT) in patients with bone marrow failure. Blood Cells Mol. Dis. 34, 257–263

33. Yamaguchi, H., Calado, R. T., Ly, H., Kajigaya, S., Baerlocher, G. M., Channock, S. J., Lansdorp, P. M., and Young, N. S. (2005) Mutations in TERT, the gene for telomerase reverse transcriptase, in aplastic anemia. N. Engl. J. Med. 352, 1413–1424

34. Kabsch, W. (2010) XDS. Acta Crystallogr. D Biol. Crystallogr. 66, 125–132

35. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221

36. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132

37. Vagin, A. A., Steiner, R. A., Lebedev, A. A., Potterson, L., McNicholas, S., Long, F., and Murshudov, G. N. (2004) REFMAC5 dictionary: organization of prior chemical knowledge and guidelines for its use. Acta Crystallogr. D Biol. Crystallogr. 60, 2184–2195

38. Delano, W. L. (2012) The PyMOL Molecular Graphics System, version 1.5.0.1. Schrodinger, LLC, New York

39. Cristofari, G., Reichenbach, P., Regamey, P. O., Banfi, D., Chambon, M., Turcatti, G., and Lingner, J. (2007) Low- to high-throughput analysis of telomerase modulators with Telospot. Nat. Methods 4, 851–853

40. Palmer, H. M. (2000) Using antibodies: a laboratory manual. J. Antimicrob. Chemother. 45, 413–413

41. Wang, F., and Lei, M. (2011) Human telomere POT1-TPP1 complex and its role in telomerase activity regulation. Methods Mol. Biol. 735, 173–187

Bone Marrow Failure Syndromes Impair TERT-NA Assembly

MARCH 17, 2017 • VOLUME 292 • NUMBER 11

JOURNAL OF BIOLOGICAL CHEMISTRY 4601