Multiple Mechanisms Contribute to the Cell Growth Defects Impacted by Human Telomerase Insertion in Fingers Domain Mutations Associated with Premature Aging Diseases*

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Normal human stem cells rely on low levels of active telomerase to sustain their high replicative requirements. Deficiency in telomere maintenance mechanisms leads to the development of premature aging diseases, such as dyskeratosis congenita and aplastic anemia. Mutations in the unique “insertion in fingers domain” (IFD) in the human telomerase reverse transcriptase catalytic subunit (hTERT) have previously been identified and shown to be associated with dyskeratosis congenita and aplastic anemia. However, little is known about the molecular mechanisms impacted by these IFD mutations. We performed comparative functional analyses of disease-associated IFD variants at the molecular and cellular levels. We report that hTERT-P721R- and hTERT-R811C-expressing cells exhibited growth defects likely due to impaired TPP1-mediated recruitment of these variant enzymes to telomeres. We showed that activity and processivity of hTERT-T726M failed to be stimulated by TPP1-POT1 overexpression and that dGTP usage by this variant was less efficient compared with the wild-type enzyme. hTERT-P785L-expressing cells did not show growth defects, and this variant likely confers cell survival through increased DNA synthesis and robust activity stimulation by TPP1-POT1. Altogether, our data suggest that multiple mechanisms contribute to cell growth defects conferred by the IFD variants.

Eukaryotic chromosome ends resemble double-stranded breaks. To escape from the activation of the DNA damage response (DDR)4 pathway, nucleoprotein structures called telomeres shield the ends of the genetic material. In humans, telomeres are composed of tandem (TTAGGG)n repeats coated by the sequence-specific shelterin complex (POT1, TPP1, TRF1/2, RAP1, and TIN2). The shelterin complex is required for the regulation of telomere length homeostasis, suppressing the DDR machinery, and to maintain the protective T-loop capping structure. In the absence of telomere maintenance mechanisms, telomeres shorten following each round of cell division due to the end replication problem. Progressive telomere shortening serves as a molecular clock, dictating the onset of cellular senescence while acting as a barrier to tumorigenesis. In normal human stem cells, low levels of active telomerase, the enzyme responsible for counteracting telomere erosion, are essential to sustain their high proliferative requirement. Defects in the telomere maintenance machinery (e.g. telomerase complex) in the human stem cell compartments impair cell proliferation, leading to the development of telomeropathies. Telomeropathy is defined as the spectrum of diseases associated with aberrant telomere maintenance and universally characterized by short telomeres (1,2). Consistently, ~85% of human cancer cells express telomerase to maintain telomere length and to confer cellular immortalization (3).

Human telomerase is minimally composed of the human telomerase reverse transcriptase catalytic subunit (hTERT) and an integral RNA component (human telomerase RNA (hTR)), which serves as a template for de novo telomere synthesis. In humans, mutations associated with premature aging diseases have been identified in telomere- and telomerase-interacting proteins. Identification of DKC1 gene mutations coding for dyskerin, a core component of the telomerase holoenzyme complex responsible for maintaining hTR stability, established the first causal relationship between telomere maintenance defects and dyskeratosis congenita (4, 5). Premature aging syndrome patient cohort studies later identified mutations in other components of the telomerase ribonucleoprotein complex (hTERT, hTR, NOP10, NHP2, and TCAB1), the shelterin members (TRF1, TRF2, TPP1, and TIN2), and regulators of telomere length (RTEI1 and CTC1) (6–22).

Because of the abnormally fast rate of telomere erosion, patients suffering from premature aging diseases usually die of bone marrow failure and are prone to the development of malignancies due to increased genomic instability resulting from short telomeres (23–25). In contrast to hTERT mutations, the majority of hTERT mutations identified to date have only

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4 The abbreviations used are: DDR, DNA damage response; IFD, insertion in fingers domain; (h)TERT, (human) telomerase reverse transcriptase catalytic subunit; hTR, human telomerase RNA; AA, aplastic anemia; RRL, rabbit reticulocyte lysate; TRAP, telomeric repeat amplification protocol; RAP, repeat addition processivity; DPE, direct primer extension; IP, immunoprecipitation; Q-FISH, quantitative FISH; SFE, signal-free end; TIF, telomere dysfunction-induced foci; IF, immunofluorescence; a.u.f., arbitrary units of fluorescence; E, early; L, late; Alu sequences, short DNA sequences that result from digestion by Arthrobacter luteus restriction endonuclease.
been “associated” with premature aging diseases rather than being a direct disease cause (26).

In this study, we performed an in-depth molecular and cellular comparative analysis of four premature aging disease-associated hTERT variants located in the “insertion in fingers domain” (IFD) motif. P721R and T726M are heterozygous mutations identified in autosomal recessive dyskeratosis congenita (27, 28) and severe aplastic anemia (AA) patients, respectively (28, 29), whereas R811C is the first homozygous autosomal recessive dyskeratosis congenita hTERT mutation reported (30). P785L is a heterozygous hTERT mutation identified in a family of Pakistani ancestry in which one sibling presented with myelodysplastic syndrome followed by the development of acute myeloid leukemia, another affected sibling was diagnosed with AA, and two other siblings were asymptomatic (31).

Thus far, these IFD variants remain poorly characterized because data from rare patient samples, in vitro rabbit reticulocyte lysates (RRLs), and immunopurified telomerase from cell extracts showed limited defects in activity as assessed by telomeric repeat amplification protocol (TRAP) and some heterozygous carriers are asymptomatic (27, 29–31). Additionally, most hTERT mutations manifest phenotypically as haploinsufficient heterozygotes, and thus the observed activity is as an average of the hTERT-WT and hTERT variant activity (32). Furthermore, several studies used a PCR-based TRAP activity assay, which is semiquantitative and does not provide information on other aspects of telomerase catalytic functions, such as the critical parameter of repeat addition processivity (RAP). The characterization of these variants has been discontinued without further investigating their impact on other mechanisms that regulate telomerase function, such as telomere binding and recruitment to telomeres, holoenzyme assembly, and interaction with telomerase-associated proteins (e.g. TPP1).

In the present study, using HEK 293 and HeLa cells overexpressing the telomerase variants, we found that hTERT-P721R and hTERT-P785L displayed altered levels of telomerase activity, and under limiting amounts of dGTP all IFD variants showed lower levels of RAP compared with the WT enzyme. Furthermore, fluorescence in situ hybridization (FISH) experiments showed that all variants, except hTERT-T726M, exhibited a lower ability to localize to the telomeres, and hTERT-P721R and hTERT-R811C were defective in binding to telomeres as assessed by chromatin immunoprecipitation (ChIP). Importantly, only the P785L variant was responsive to the overexpression of TPP1-PO71 (hTR FISH and direct primer extension (DPE) assay). This suggests that P721R, T726M, and R811C mutants are potentially defective in interacting with TPP1 or other regulators of telomerase found in the cell extracts. Long term limited life span cell studies showed that these three latter variants impart cell growth defects resulting from higher levels of apoptosis compared with hTERT-WT–expressing cells accompanied by telomere shortening and elevated levels of short telomeres that activated a DDR at the telomeres. Importantly, the lack of prominent defects in telomerase activity may mask the importance of certain residues in regulating telomerase functions. Indeed, a previous study showed that hTERT enzymes harboring mutations located within the dissociates activities of telomerase (DAT) domain displayed high to moderate levels of in vitro telomerase activity, but when expressed in cells they failed to maintain telomeres and to confer cellular immortalization (33). Our data highlight the significance of characterizing hTERT variants at the biochemical and molecular levels because a combination of the different mechanisms regulating telomerase function, such as telomerase recruitment and association with interacting proteins, can contribute to severity of disease phenotypes.

Experimental Procedures

Plasmid Construction—The pMSCV-puroymycin-N-term-3XFLAG-hTERT was constructed as described previously (34) and used as a template for site-directed mutagenesis for the generation of pMSCV-puroymycin-N-term-3XFLAG-hTERT-P721R, -T726M, -P785L, and -R811C. pcDNA6/myc-His C-hTERT and pVAN3XFLAG-hTERT were used as templates for the generation of pcDNA6/myc-His C-hTERT-P721R, -T726M, -P785L, and -R811C and pVAN3XFLAG-hTERT-P721R, -T726M, -P785L, and -R811C.

Cell Culture, Retroviral Infection, and Transient Transfection—Cell culture, retroviral infection of HA5 cells, and transient transfections of HEK 293 and HeLa cells were performed as described previously (35). For the TPP1-PO71 direct primer extension rescue experiment, a 15-cm dish of HEK293 cells was transiently transfected with 6 μg of pcDNA6/myc-His C-hTERT-WT or variants and 6 μg of pbLueScript II SK (+)-hTR DNA in the presence or absence of 6 μg of pVAN3XFLAG-hTPP1 and 6 μg of pVAN3XFLAG-hPO71. For the TPP1-PO71 hTR FISH rescue experiment, HeLa cells in a 6-well dish were transiently transfected using 2 μg of pcDNA6/myc-His C-hTERT-WT or variants and 2 μg of pbLueScript II SK (+)-hTR DNA in the presence (+TPP1-PO71) or absence of 0.5 μg of pVAN3XFLAG-hTPP1 and 0.5 μg of pVAN3XFLAG-hPO71.

Protein Analysis by Western Blotting—Western blotting was performed as described previously (34, 35). Molecular mass markers ranging from 10 to 170 kDa were used (Thermo Scientific, PageRuler Prestained Protein Ladder, catalog number 26616, lot number 00211717). FLAG epitope was detected using monoclonal M2 anti-FLAG (Sigma, F3165, lot number SLBL1237V). hTERT was detected using goat monoclonal clone c-20 anti-FLAG (Santa Cruz Biotechnology, catalog number sc-7215, lot number B1110). Actin was detected using mouse monoclonal clone C4 anti-actin (Millipore, catalog number MAB1501, lot number 2065987). Tubulin was detected using mouse monoclonal clone B-5-1-2 anti-tubulin (Sigma, catalog number T5168, lot number 072M4809). The secondary antibodies used were as follows: rabbit anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma, catalog number A9040, lot number 098K4836) and polyclonal rabbit anti-goat IgG HRP conjugate (DakoCytomation, catalog number P0449, lot number 00085834).

DPE Assay—WT and variant telomerases were reconstituted in HEK 293 cells as described previously (35, 36). Unless otherwise stated, the direct primer extension experiment was performed and quantified as described previously (34–36).

Immunoprecipitation (IP) and Quantitative RT-PCR—HEK 293 cells from a 10-cm plate were transiently transfected with 3XFLAG-hTERT-WT or hTERT variants and hTR. Cells were
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collected by scraping and lysed in soft lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 10% glycerol, 150 mM NaCl, 5 μM 2-mercaptoethanol, 0.1% IGEPAL) (Sigma-Aldrich) supplemented with RNaseOUT (Invitrogen) and protease inhibitors. IP was performed using 5 μl of mouse monoclonal clone M2 anti-FLAG antibody (Sigma, catalog number F1804, lot number SLBF6631) immobilized on protein G-Sepharose 4 Fast Flow beads (GE Healthcare) and rotated at 4 °C for 2 h. After five washes with the soft lysis buffer, 50% of the beads were subjected to Western blotting analysis to assess protein IP efficiency, and the remaining 50% of the beads and 10% input sample were resuspended in 500 μl of TRIZol. RNA extraction was performed following the supplier’s instructions, and RNA was precipitated in the presence of 10 μg of glycogen (Ambion). cDNAs were obtained by performing reverse transcription on the isolated RNA. cDNA was used in quantitative PCR assay to amplify hTR and hypoxanthine phosphoribosyltransferase. Enrichment was calculated as described previously (37).

FISH—hTR-telomere FISH was performed as described previously using HeLa cells co-expressing hTERT-WT, hTERT-P721R, hTERT-T726M, hTERT-P785L, or hTERT-R811C and hTR in the presence or absence of overexpressed TPP1 and POT1 (34, 39–42).

Telomeric ChIP—ChIP was conducted and quantified as described previously (34, 43, 44) using HeLa cells co-expressing hTERT-WT, hTERT-P721R, hTERT-T726M, hTERT-P785L, or hTERT-R811C and hTR.

Quantitative FISH (Q-FISH) Analysis and Signal-free Ends (SFEs)—Metaphase spread analysis for telomere length measurement and detection of SFEs was performed as described previously (34, 35, 39).

Apoptosis Analysis by Fluorescence-activated Cell Sorting (FACS)—HA5 cells expressing WT or variant hTERTs were subjected to FACS analysis as described previously for quantification of apoptotic cells at early and late passages (34).

Immunofluorescence Combined with FISH for Detection of Telomere Dysfunction-induced Foci (TIF)—Combined immunofluorescence (IF)/FISH for the detection of TIF at 200 days postinfection was performed as described previously (34, 41). γ-H2AX foci were detected using mouse monoclonal anti-phosphohistone H2AX (Ser-139) clone JBW301 (Millipore, catalog number 05-636, lot number 2455602) and donkey FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, catalog number 705-095-003, lot number 72993).

Statistical Analysis—Statistical analysis was performed using the program GraphPad Prism. Parameters were analyzed using one-way analysis of variance. The Dunnett post-test was used to compare all samples with the control sample (WT). The Bonferroni post-test was used to compare all pairs of columns. The significance level was determined using the 95% confidence intervals. A p value ≥0.05 is not significant, 0.01–0.05 is significant (*), 0.001–0.01 is very significant (**), and 0.0001–0.001 is extremely significant (***)

Results

Disease-associated hTERT IFD Variants Display Altered Telomerase Activity and RAP That Can Be Affected in a dGTP-dependent Manner—Using site-directed mutagenesis, we generated four naturally occurring hTERT IFD mutations associated with premature aging diseases: P721R, T726M, P785L, and R811C (Fig. 1A). With the exception of P721R, the mutated hTERT IFD residues are poorly conserved as shown by the amino acid alignment generated using ClustalW, which compares the human telomerase IFD with the corresponding region from other organisms (Fig. 1A). Previously, data from patient samples, RRL, and immunopurified telomerase from cell extracts indicated that P721R, T726M, and R811C hTERT variants have slight defects in enzyme activity and processivity as assessed by TRAP or DPE experiments (26–30, 32). Although hTERT-P785L was shown to possess only 11% of the hTERT-WT telomerase activity by TRAP, many family members carrying the heterozygous mutation remain asymptomatic, suggesting that this mutation is a risk factor associated with AA rather than causing AA (31, 45). A recent study using telomerase reconstituted in RRL showed that P721R and T726M mutant enzymes exhibit, respectively, 65 and 79% WT telomerase activity by the DPE assay (32) and 20–100% WT activity by TRAP (26, 28). Another study by Dokal et al. (30) demonstrated that the R811C variant displayed less than 50% of WT TRAP activity but did not act in a dominant negative manner.

In the present study, we overexpressed the four hTERT IFD variants in HEK 293 cells. We confirmed comparable levels of protein expression by Western blotting analysis (Fig. 1B) and performed a DPE assay using two different concentrations of total dGTP (0.66 and 1.91 μM) in the reaction mixture to assess relative levels of DNA synthesis (activity) and RAP and their responsiveness to dGTP levels (Fig. 1, C–E). Under lower concentrations of dGTP, the P721R variant reconstituted a significantly lower level of total DNA synthesis (26.40 ± 9.36% (S.D.)), whereas hTERT-P785L displayed a higher level of telomerase activity (155.76 ± 24.13% (S.D.)) compared with hTERT-WT (activity set at 100%) (Fig. 1D). Total DNA synthesis exhibited by T726M and R811C mutant enzymes was not significantly different from that of WT telomerase (85.46 ± 28.45% (S.D.) and 78.89 ± 6.07% (S.D.), respectively) (Fig. 1D). Increasing the amount of dGTP to 1.91 μM had no significant effect on the telomerase activity of P721R, P785L, and R811C mutants (hTERT-P721R, 26.27 ± 8.99% (S.D.); hTERT-P785L, 176 ± 55.66% (S.D.); hTERT-R811C, 80.56 ± 1.06% (S.D.)) (Fig. 1D). Although not statistically significant, we did observe an increased level of DNA synthesis for hTERT-T726M at higher concentrations of dGTP (126.05 ± 36.88% (S.D.)) (Fig. 1D). At 0.66 μM dGTP present in the reaction, all mutants showed significantly lower levels of RAP in comparison with hTERT-WT (RAP set at 100%) (Fig. 1E). Interestingly, increasing the concentration of dGTP from 0.66 μM to 1.91 μM significantly enhanced RAP of hTERT-T726M (62.53 ± 23.85% (S.D.) versus 97.90 ± 0.33% (S.D.)) and modestly, but not significantly, increased the RAP of hTERT-P785L (64.81 ± 16.32% (S.D.) versus 84.46 ± 13.29% (S.D.)). The other hTERT mutants were not significantly responsive to the increase in dGTP levels (hTERT-P721R: 59.80 ± 14.67% (S.D.) versus 73.72 ± 5.61% (S.D.); hTERT-R811C: 58.90 ± 10.88% (S.D.) versus 77.73 ± 4.51% (S.D.)). Altogether, these data suggest that the catalytic defects of hTERT-T726M and hTERT-P785L are in part due to the lower ability to utilize dGTP in comparison with hTERT-WT, especially under conditions where dGTP is limiting. Our results
for hTERT-P721R and hTERT-P785L are significantly different from the previously reported data and could be due to different methods used to detect telomerase activity (TRAP versus DPE) or the use of non-purified telomerase extracts in our experiments (versus RRL or immunopurified telomerase). In RRL, regulators of the human telomerase are probably absent, whereas telomerase interaction with its regulators in human cells can be transient and cell cycle-regulated. Hence, the association with these proteins may or may not be occurring at the time of protein purification or can potentially be lost during the isolation process.

FIGURE 1. Disease-associated hTERT IFD variants display altered telomerase activity and RAP that can be affected in a dGTP-dependent manner. A, schematic representation of TERT gene along with a sequence alignment of TERT IFDs from selected organisms using ClustalW2: Bos taurus (Bos), Canis lupus familiaris (Canis), Homo sapiens (Homo), Mus musculus (Mus), Tetrahymena thermophila (Tetrahymena), Saccharomyces cerevisiae (Saccharomyces), and Tribolium castaneum (Tribolium). Labels above the alignment indicate the prolines at amino acids 721 and 785, the threonine at position 726, and the arginine at residue 811 in hTERT and the corresponding residues at equivalent positions within the other TERTs. TERT is divided into three main domains labeled below the schematic: N-terminal extension (NTE), reverse transcriptase (RT), and C-terminal extension (CTE). Subdomains are indicated inside the TERT gene: TERT essential N-terminal domain (TEN), telomerase RNA binding domain (TRBD), fingers, IFD, palm, and thumb domains. B, representative immunoblot of hTERT using 50 μg of total protein from HEK 293 cells overexpressing WT or variant hTERTs and hTR used in C to confirm comparable hTERT protein expression. Blots were probed using antibodies against hTERT or the loading controls actin and tubulin. n = 3. C, representative gel of a direct primer extension experiment using mutant and WT telomerases expressed and reconstituted with co-expressed hTR in HEK 293 cells for assessment of telomerase total DNA synthesis (activity) and RAP levels using the telomeric substrate (T2AG3)3. Reactions were performed at 30 °C for 2 h under various dGTP concentrations identified above the lanes (0.66 or 1.91 μM). L.C., loading and recovery control (T2AG3)2. n = 3. D, quantitation of relative total DNA synthesis reconstituted by mutant and WT telomerases expressed in HEK 293 cells in C was performed by measuring the total incorporation of radiolabeled dGTP into the extension products. Values are normalized to the loading and recovery control and compared with their respective control sample (WT). Telomerase activity of WT enzyme for each dGTP condition is set to 100%. A p value of 0.01–0.05 is significant (*), and a p value of 0.001–0.01 is very significant (**). n = 3. E, quantitation of RAP reconstituted by mutant and WT telomerases expressed in HEK 293 cells in C was performed by measuring the extension products at +10 repeats over total product. RAP of WT telomerase for each dGTP concentration is set to 100%, and RAP of each variant is compared with their respective WT enzyme. A p value of 0.01–0.05 is significant (*), and a p value of 0.001–0.01 is very significant (**). n = 3. F, using an antibody against the FLAG tag, immunoprecipitation of WT or mutant telomerases reconstituted in HEK 293 was performed, and a representative immunoblot of hTERT protein post-IP for assessment of pulldown efficiency is shown. The blot was probed using an antibody against FLAG. n = 3. G, quantitation of percentage of WT or mutant hTERTs binding to hTR by RT-quantitative PCR. hTR binding efficiency of WT hTERT is set to 100%. Binding to hypoxanthine phosphoribosyltransferase (HPRT) is used as a negative control for nonspecific pulldown. All values are compared with WT hTERT binding to hTR for the calculation of statistical significance. A p value of 0.0001–0.001 is extremely significant (**). n = 3. Error bars indicate S.D.
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We overexpressed hTERT-WT or hTERT-variants with hTR in the presence or absence of TPPI-POT1 and assessed protein expression by Western blotting analysis (Fig. 2C). We then performed a direct primer extension experiment and measured telomerase activity and RAP. Results from three independent experiments showed that, with the exception of hTERT-WT and hTERT-P785L, there is no significant activity or RAP enhancement when TPPI-POT1 is co-expressed with the telomerase variants (hTERT-P721R, 66.44 ± 0.71% (S.D.) versus 69.35 ± 29.96% (S.D.); hTERT-T726M, 90.80 ± 10.39% (S.D.) versus 106.37 ± 7.81% (S.D.); hTERT-R811C, 75.27 ± 16.22% (S.D.) versus 88.92 ± 17.59% (S.D.)) (Fig. 2, A and B). hTERT-WT and hTERT-P785L displayed a robust stimulation of telomerase activity in the presence of TPPI-POT1 (hTERT WT, 100.00 ± 0.00% (S.D.) versus 151.82 ± 9.01% (S.D.); hTERT-P785L, 84.25 ± 4.89% (S.D.) versus 189.48 ± 27.77% (S.D.)) (Fig. 2B). When RAP was assessed, only hTERT-WT showed a significant increase in processivity in the presence of TPPI-POT1 (100.00 ± 0.00% (S.D.) versus 149.00 ± 16.82% (S.D.)), whereas all mutants remained unresponsive (hTERT-P721R, 84.00 ± 24.82% (S.D.) versus 87.00 ± 22.19% (S.D.); hTERT-T726M, 86.00 ± 0.76% (S.D.) versus 93.00 ± 9.10% (S.D.); hTERT-P785L, 81.00 ± 23.33% (S.D.) versus 90.32 ± 7.68% (S.D.); hTERT-R811C, 84.00 ± 4.56% (S.D.) versus 7.81% (S.D.)); hTERT-R811C, 75.27 ± 16.22% (S.D.) versus 88.92 ± 17.59% (S.D.)) (Fig. 2, A and B).

bly, we performed a co-IP experiment using 3XFLAG-tagged hTERT-WT and hTERT variants followed by the detection of bound hTR using quantitative RT-PCR (Fig. 1, F and G). In accordance with previously published data on hTERT-P721R obtained by Northern blotting analysis (26), we did not observe significant differences in binding to hTR between the hTERT mutants and hTERT-WT (Fig. 1G). We further confirmed the results by co-IF experiments using antibodies against colin (marker of Cajal bodies) and hTERT (data not shown). The hTRE-dependent localization of hTERT to Cajal bodies has been well studied (34, 36, 46, 47). Thus, defects observed in telomerase DNA synthesis and RAP are not due to inefficient assembly of hTERT with hTR, suggesting that other molecular defects underlie the observed phenotype.

Lower Levels of Telomerase Activity and Processivity of IFD Variants Cannot be Rescued by Overexpression of TPPI-POT1—Stimulation of telomerase catalytic functions by the shelterin duplex TPPI-POT1 is well established and has previously been used to assess the ability of telomerase to interact with TPPI-POT1 (34, 48–53). Based on earlier observations that the IFD variant hTERT-L805A with reduced activity and processivity can be partially stimulated by TPPI-POT1 overexpression (34), we investigated whether the hTERT IFD disease-associated mutants can also be stimulated by TPPI-POT1.

We overexpressed hTERT-WT or hTERT-variants with hTR in the presence or absence of TPPI-POT1 and assessed protein expression by Western blotting analysis (Fig. 2C). We then performed a direct primer extension experiment and measured telomerase activity and RAP. Results from three independent experiments showed that, with the exception of hTERT-WT and hTERT-P785L, there is no significant activity or RAP enhancement when TPPI-POT1 is co-expressed with the telomerase variants (hTERT-P721R, 66.44 ± 0.71% (S.D.) versus 69.35 ± 29.96% (S.D.); hTERT-T726M, 90.80 ± 10.39% (S.D.) versus 106.37 ± 7.81% (S.D.); hTERT-R811C, 75.27 ± 16.22% (S.D.) versus 88.92 ± 17.59% (S.D.)) (Fig. 2, A and B). hTERT-WT and hTERT-P785L displayed a robust stimulation of telomerase activity in the presence of TPPI-POT1 (hTERT WT, 100.00 ± 0.00% (S.D.) versus 151.82 ± 9.01% (S.D.); hTERT-P785L, 84.25 ± 4.89% (S.D.) versus 189.48 ± 27.77% (S.D.)) (Fig. 2B). When RAP was assessed, only hTERT-WT showed a significant increase in processivity in the presence of TPPI-POT1 (100.00 ± 0.00% (S.D.) versus 149.00 ± 16.82% (S.D.)), whereas all mutants remained unresponsive (hTERT-P721R, 84.00 ± 24.82% (S.D.) versus 87.00 ± 22.19% (S.D.); hTERT-T726M, 86.00 ± 0.76% (S.D.) versus 93.00 ± 9.10% (S.D.); hTERT-P785L, 81.00 ± 23.33% (S.D.) versus 90.32 ± 7.68% (S.D.); hTERT-R811C, 84.00 ± 4.56% (S.D.) versus 7.81% (S.D.)); hTERT-R811C, 75.27 ± 16.22% (S.D.) versus 88.92 ± 17.59% (S.D.)) (Fig. 2, A and B).

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Disease-associated hTERT variants display defects in telomere binding and localization to the telomeres that cannot be rescued by TPP1-POT1 overexpression with the exception of hTERT-P785L. A, representative ChIP experiment using M2 anti-FLAG antibody against 3XFLAG-tagged hTERT mutants or hTERT-WT co-expressed with hTR in HeLa cells. Immunoprecipitated chromatin was purified, slot blotted, and detected using radiolabeled oligonucleotide probes against telomeric DNA sequences ([T\(_\text{2}\)AG\(_\text{3}\)]

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\_) or Alu repeats ([Alu]), [Alu]). Quantitation of binding of mutant telomerases and WT telomerase to telomeric DNA as represented in A. Quantitation was performed by normalizing to input, and nonspecific binding to Alu sequences was taken into account for the calculations. Binding of hTERT-WT to telomeres was set at 100%. A p value of 0.05 is not significant (n.s.), a p value of 0.001–0.01 is significant (*), a p value of 0.001–0.005 is extremely significant (**), and a p value of 0.0001–0.001 is extremely significant (***) (n = 3). C, representative hTRISH experiment using mutant and WT telomerases expressed and reconstituted with co-expressed hTR in HeLa cells with or without co-expressed TPP1-POT1. Untransfected (Mock) sample was used as negative control to assess nonspecific signals. DAPI was used for detection of the nucleus. Oregon Green-conjugated telomeric probe ([T\(_\text{2}\)AG\(_\text{3}\)]

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\_) was used for the detection of telomeres. Three different Cy3-labeled hTR probes were used to detect telomerase localization. Yellow foci indicate colocalization of hTR (telomerase) at the telomeres. D, quantitation of average number of hTERT-telomere associations per cell as represented in C. A p value of 0.05 is not significant (n.s.), a p value of 0.01–0.05 is significant (*), a p value of 0.001–0.01 is very significant (**), and a p value of 0.0001–0.001 is extremely significant (***) (n = 2; n = 250 cells were counted per n. Error bars indicate S.D. 84.82 ± 12.46% (S.D.) (Fig. 2B). The differences in the levels of telomerase activity and RAP observed in this experiment compared with data obtained in Fig. 1, C–E, are due to variations in the experimental procedures, such as the incubation time (2 h in Fig. 1 versus 1 h in Fig. 2) and the transfection conditions (see "Experimental Procedures"). We propose that the interaction of hTERT-P712R, hTERT-T726M, and hTERT-R811C with TPP1 might be compromised. DNA synthesis of hTERT-P785L, but not RAP, can be stimulated by TPP1-POT1, potentially reflecting an increased recruitment of hTERT-P785L to the telomeric substrate. This might therefore allow the extension of more telomeric primer without the stimulation of RAP, thus leaving the levels of RAP unaltered. The ensuing implication is that the interaction of telomerase with TPP1 for the stimulation of telomerase RAP and telomerase recruitment to telomeres might be regulated independently.

**Disease-associated hTERT Variants Display Defects in Telomere Binding and Localization to Telomeres That Cannot Be Rescued by TPP1-POT1 Overexpression with the Exception of hTERT-P785L**—We recently reported that specific residues within the IFD regulate telomerase localization and recruitment to telomeres in a TPP1-dependent manner (34). Because telomerase association and recruitment to telomeres are a prerequisite for telomerase-mediated telomere maintenance in cells, we sought to determine the ability of the IFD disease-associated variants to bind telomeres using ChIP (Fig. 3, A and B) and assess their in situ association with telomeres by hTR FISH (Fig. 3, C and D). We performed an IP against the FLAG epitope using HeLa cells co-expressing hTR and 3X-FLAG tagged hTERT (WT or variants) and analyzed the co-precipitated DNA by using radiolabeled probes against telomeric DNA ([T\(_\text{2}AG\(_\text{3}\)]

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\_) and Alu repeats as a control (Fig. 3B). hTERT-P721R and hTERT-R811C displayed significantly lower binding to telomeres (hTERT-P721R, 54.56 ± 12.86% (S.D.); hTERT-R811C, 54.03 ± 14.06% (S.D.)) compared with hTERT-WT (set as 100%) (Fig. 3B). hTERT-T726M (94.89 ± 8.4% (S.D.)) and hTERT-P785L (117.94 ± 15.56% (S.D.)) displayed similar telomerase binding ability as hTERT-WT. These data suggest that lower levels of telomerase activity or processivity observed for hTERT-P721R and hTERT-R811C can partly be caused by impaired ability to bind to telomeres and to interact with TPP1. The ability of IFD disease-associated variants to localize to the telomeres in cells was assessed by hTR FISH. The hTERT-dependent hTR localization to the telomeres has been characterized and extensively used as an indicator of telomerase localization to the telomeres (34, 39, 41, 42, 46, 54, 55). We have previously shown that specific IFD residues are implicated in the regulation of telomerase localization to the telomeres (34).
Disease-associated Telomerase IFD Variants

In the current work, we showed that all mutants (hTERT-P721R, 4.7 ± 0.0 (S.D.); hTERT-P785L, 4.2 ± 0.0 (S.D.); hTERT-R811C, 5.0 ± 0.2 (S.D.)), with the exception of hTERT-T726M (5.7 ± 0.1 (S.D.)), displayed a significantly lower number of hTR-telomere associations as compared with hTERT-WT (6.0 ± 0.3 (S.D.)) (Fig. 3, C and D). Curiously, despite hTERT-P785L not displaying defects in binding when assessed by ChIP, we did observe a considerably lower number of hTR-telomere associations by FISH compared with WT telomerase. Although the ChIP experiment allows the visualization of all bound telomeric DNA, the detection limit of hTR FISH might be lower, and the visibility of foci is dependent on cluster formation, which represents approximately an average of four to eight telomeres (56). Thus, we speculate that hTERT-P785L might be bound to several telomeres that did not necessarily form clusters and therefore are not detected by FISH.

The role of TPP1 in mediating telomerase recruitment to telomeres is well established (42, 57). Based on our DPE data showing that hTERT-P785L is the only mutant enzyme for which telomerase activity is stimulated by TPP1-POT1 overexpression, we investigated whether recruitment of the IFD variants to the telomeres can be enhanced by overexpressing TPP1-POT1 (Fig. 3C). In agreement with the DPE assay, only hTERT-P785L displayed a slight increase in telomerase-telomere association in the presence of co-expressed TPP1-POT1 (4.22 ± 0.00 (S.D.) versus 4.91 ± 0.20 (S.D.)), whereas the number of hTR-telomere colocalizations for hTERT-WT (5.99 ± 0.28 (S.D.) versus 6.19 ± 0.16 (S.D.)) and other hTERT variants remained unchanged in the presence of overexpressed TPP1-POT1 (hTERT-P721R, 4.76 ± 0.04 (S.D.) versus 4.82 ± 0.38 (S.D.); hTERT-T726M, 5.72 ± 0.13 (S.D.) versus 5.63 ± 0.44 S.D.; hTERT-R811C, 4.98 ± 0.21 (S.D.) versus 5.06 ± 0.33 (S.D.)) (Fig. 3D). The lack of a stimulation of the levels of hTR-telomere associations by TPP1 in hTERT-WT-transfected HeLa cells has been reported previously (34), and thus a lack of stimulation of hTERT-T726M, with numbers of hTR-telomere associations comparable with hTERT-WT, is not surprising. The absence of stimulation by TPP1-POT1 overexpression in HeLa cells overexpressing hTERT-P721R and hTERT-R811C further confirms that these IFD variants might be defective in telomere binding and recruitment as a result of impaired interaction with TPP1.

Limited Life Span HA5 Cells Expressing Disease-associated IFD Variants (P721R, T726M, and R811C) Display Growth Defects, Increased Levels of Apoptosis, Short Telomeres, and DNA Damage at Telomeres—We previously reported that several conserved IFD residues are important for telomerase function, telomere maintenance, and cell survival (34). HA5 cells, which are senescence-defective human embryonic kidney cells transformed with SV40, can undergo apoptosis due to defective telomere maintenance caused by the lack of hTERT expression (33, 34, 39, 58). Hence, we retrovirally infected HA5 cells with hTERT-P721R, hTERT-T726M, hTERT-P785L, hTERT-R811C, hTERT-WT, and empty vector control to determine whether the disease-associated IFD variants can regulate cell survival and telomere maintenance. After the infection, we followed the growth in culture of two clones from each cell line for ~300 days (Fig. 4, A–D). We assessed hTERT protein levels by Western blotting analysis to ensure comparable expression between hTERT-WT and hTERT variants at early (E; 20 days postinfection) and late (L; 200 days postinfection) passages (Fig. 4E). We plotted the number of population doublings as a function of number of days and compared the growth of hTERT-WT-, hTERT variant-, and vector control-infected cells (Fig. 4, A–D). In comparison with the hTERT-WT infected cells, we observed noticeable growth defects for hTERT-P721R-, hTERT-T726M-, and hTERT-R811C-infected cells, but hTERT-P785L-infected cells grew at the same rate as hTERT-WT-infected cells. This is consistent with the lack of apparent defects in telomerase activity or binding to telomeres by ChIP for hTERT-P785L.

Using FACS, we assessed whether the growth defects observed in the IFD variant-expressing cells resulted from an increase in the levels of apoptosis (Fig. 5). We did not further characterize hTERT-P785L-expressing HA5 cells because they did not display any growth defects. All disease-associated variant-infected cells we tested displayed higher levels of apoptosis (early and late apoptosis) at 200 days postinfection (hTERT-P721R-A, 44.7%; hTERT-T726M-A, 46.3%; hTERT-R811C-A, 62.0%) compared with hTERT-WT-A-expressing cells (17.9%), and elevated levels of cell death were already observable at 20 days postinfection for clone A of hTERT-R811C-infected cells (34.6%), consistent with a more severe growth defect phenotype observed in comparison with cells expressing the other mutant enzymes (Figs. 4D and 5). Because telomere maintenance is a prerequisite for cellular survival, insufficient telomerase catalytic functions lead to telomere shortening and eventual disruption of the T-loop structure. Uncapped telomeres, which are no longer protected, are recognized as double-stranded breaks, triggering the DNA damage response at the telomeres. It has been reported previously that in G1, the presence of five nonfusogenic dysfunctional telomeres is sufficient to trigger senescence in human cells (59). Because the senescence pathway in HA5 cells is inactive, an inability to resolve the DNA damage will trigger programmed cell death instead.

Based on the data collected from the DPE (Figs. 1, C–E, and 2), ChIP (Fig. 3, A and B), and hTR FISH experiments (Fig. 3, C and D), we speculated that the observed growth defects and higher levels of apoptosis might be a result of defective telomere maintenance and activation of the DDR pathway at the telomeres. Therefore, we measured the telomere lengths of HA5 cells expressing hTERT-WT or hTERT variants at 20 (E) and 200 (L) days postinfection by preparing metaphase spreads for Q-FISH analysis (Fig. 6A). At late passages, stable expression of hTERT variants failed to maintain telomeres and led to a decrease in the mean telomere length for all mutant-expressing cells (hTERT-P721R, 124.6–101.9 a.u.f.; hTERT-T726M, 144.4–114.5 a.u.f.; hTERT-R811C, 99.4–83.3 a.u.f.). Cells stably expressing hTERT-WT resulted in an increase in the mean telomere length 200 days postinfection (84.7–101.1 a.u.f.) (Fig. 6A) (34). Q-FISH analysis relies on Cy3 telomeric signal intensity measurement as a correlational readout of telomere length, disregarding chromosomal ends with very short telomeres that lack a detectable signal. As such, assessment of SFEs was necessary to complement the Q-FISH analysis (Fig. 6B). Our quantitation shows that hTERT variant-expressing cells have persistently higher levels of SFEs compared with hTERT-WT-expressing cells (E, 3.87%; L, 2.03%) both at early and late passage (Fig. 6B).
Although the levels of SFEs remain stable over time for hTERT-P721R-expressing cells (E, 8.13% versus L, 8.62%), hTERT-T726M- (E, 11.63% versus L, 18.94%) and hTERT-R811C-expressing cells (E, 18.77% versus L, 25.56%) have increased frequencies of SFEs at late passages (Fig. 6B). Levels of SFEs were highest in hTERT-R811C-expressing cells, consistent with the more pronounced growth defects and higher level of apoptosis observed in cells expressing this mutant enzyme.

The protective T-loop structure formation and maintenance are compromised when the telomere becomes critically short. Disruption of the protective cap results in telomere deprotection, recognition of the chromosomal ends as double-stranded breaks, and activation of the DDR at the telomeres. At late passages, hTERT variant-expressing cells all displayed telomere shortening and higher levels of SFEs as compared with the hTERT-WT-expressing cells. We predicted that late passage cells expressing the IFD variants also have high levels of TIF. We performed combined IF-FISH on HA5 cells at 200 days postinfection to evaluate DNA damage at the telomeres using an antibody against γH2AX (FITC) in combination with a Cy3-labeled peptide nucleic acid telomeric probe (Fig. 6C). The average number of colocalized foci (TIF) per cell and the percentage of TIF-positive cells (≥3 TIF/cell) were assessed. hTERT-P785L-expressing cells, which did not display growth defects compared with hTERT-WT-expressing cells, exhibited levels of TIF-positive cells or TIF/cell comparable with hTERT-WT-expressing cells (hTERT-P785L: clone 1, 0.15 TIF/cell, 0.97% TIF-positive cells and clone 2, 0.12 TIF/cell, 0.00% TIF-positive cells; hTERT-WT: clone 1, 0.15 TIF/cell, 0.00% TIF-positive cells and clone 2, 0.17 TIF/cell, 0.00% TIF-positive cells). For all other hTERT variant-expressing cells, both parameters are elevated (hTERT-P721R: clone A, 0.80 TIF/cell, 10.50% TIF-positive cells and clone D, 0.85 TIF/cell, 11.01% TIF-positive cells; hTERT-T726M: clone A, 0.92 TIF/cell, 10.33% TIF-positive cells and clone B, 0.86 TIF/cell, 10.65% TIF-positive cells; hTERT-R811C: clone A, 0.86 TIF/cell, 7.77% TIF-positive cells and clone B, 0.70 TIF/cell, 4.42 TIF-positive cells) in comparison with hTERT-WT-expressing cells (clone A, 0.12 TIF/cell, 0.00% TIF-positive cells; clone B, 0.19 TIF/cell, 0.45% TIF-positive cells). These data provide evidence that disease-associated hTERT IFD variants, with the exception of hTERT-P785L, can affect telomere maintenance and cellular survival.

**Discussion**

Establishing the correlation between gene mutation and patient phenotype is complicated by various aspects, including a limited amount of clinical data from patients and their relatives and limited biochemical and molecular characterization of specific mutant enzymes. Earlier work on the disease-associated IFD variants studied here suggests that hTERT-P721R, -T726M, and -P785L are mutations associated with premature aging diseases rather than having a direct causative link as they have been reported to only have slight defects with some heterozygous carriers presenting heterogeneous phenotypes or...
to be asymptomatic. hTERT-R811C, the first reported homozygous TERT mutation, was identified in a consanguineous family that presented features of classical autosomal recessive dyskeratosis congenita (30). For all hTERT mutants, the low availability of patient samples limits the amount of information that can be extracted, such as measurement of patient cell telomere length. Furthermore, methods for evaluation of telomerase catalytic function are often limited to activity measurements, therefore failing to take into consideration other parameters critical for telomerase and telomere length regulation.

Our recent data demonstrated the novel implications of the IFD, located within the reverse transcriptase domain of hTERT, in the regulation of telomerase specific functions, such as mediating telomerase recruitment to the telomeres in a TPP1-dependent manner (34). A recent study exposing the Tetrahymena telomerase cryoelectron microscopy structure also suggests that the human TERT IFD may physically interact with the TPP1 oligonucleotide/oligosaccharide binding fold (60). Cech and co-workers (32) reported previously that several disease-associated hTERT variants that have normal levels of telomerase activity respond to TPP1-POT1 stimulation. However, they did not exclude the possibility that other mutant enzymes not analyzed in their study might be defective in TPP1 interaction. Hence, we sought to determine whether several disease-associated IFD variants might be impaired in other aspects of telomerase function, such as interacting with TPP1 and binding to telomeres.

Under our experimental conditions and in agreement with previously published data, the enzyme activity of T726M and R811C mutants is not significantly different compared with the WT enzyme. However, in contrast with earlier studies, we observed a significantly lower level of telomerase activity for hTERT-P721R, whereas hTERT-P785L instead had higher levels of DNA synthesis as compared with hTERT-WT (Fig. 1, C and D), which may be explained by the differences in experimental assays and conditions used. Based on the data obtained, we speculate that hTERT-P721R and hTERT-R811C are mutants defective in recruitment to telomeres due to impaired interaction with TPP1 as shown by the absence of activity and RAP stimulation by TPP1 as well as a lack of rescue of the number of hTR-telomere associations by TPP1 observed in the hTR FISH experiments. Reduced recruitment of mutant hTERTs likely contributed to telomere shortening in HA5 cells, leading to accumulation of very short telomeres, activation of DDR, and cell death. Furthermore, substitution of the proline

FIGURE 5. Disease-associated IFD variant-expressing cells with growth defects displayed higher levels of apoptosis in comparison with hTERT-WT-expressing cells. FACS analysis was performed at early (20 days postinfection) and late passages (200 days postinfection) of HA5 cells containing vector or expressing hTERT mutant enzymes or hTERT-WT (clones A and B for each, clone A and D for hTERT-P721R) to assess the percentage of apoptotic cells. hTERT-WT-expressing HA5 cells treated with staurosporine (STS) were used as a positive control for cell death. Representative annexin V/propidium iodide (PI) flow cytometry profiles of one clone (clone A) are shown. Percentages of early apoptotic (annexin V-positive, propidium iodide-negative; lower right quadrant) and late apoptotic (annexin V-positive, propidium iodide-positive; upper right quadrant) cells are given in each respective quadrant.
residue at residue 721 for an arginine is expected to have a structural impact because of the absence of the unique cyclic structure of proline that provides structural rigidity.

hTERT-T726M has no apparent defects in activity and telomere association and binding; thus we propose that this enzyme is less efficient in dGTP usage as compared with the WT enzyme and that the lack of catalytic stimulation by overexpressed TPP1-POT1 may contribute to the growth defects and phenotypes observed in HA5 cells. Finally, because hTERT-P785L-expressing cells showed no defects in telomere binding by ChIP and grew at the same rate as hTERT-WT-expressing cells, we speculate that this variant is associated with AA and is unlikely to contribute to disease phenotype. hTERT-P785L has high levels of total DNA synthesis, which can be further stimulated by overexpression of TPP1-POT1, thus possibly compensating for the lower levels of RAP observed. hTERT-P785L may confer the survival of the HA5 cells by maintaining telomeres in a distributive manner and responding robustly to TPP1 stimulation. It is also important to consider that overexpression of hTERT-P785L may have compensated for or suppressed cell growth defects that could be apparent at normal expression levels in patients.

Although hTERT-P721R and hTERT-T726M are reported to be heterozygous mutations and the WT allele can compensate for the less efficient allele, we found that when only the mutated version is expressed in HA5 cells significant growth defects accompanied by telomere shortening and higher levels of cell death were observed, suggesting that these variants have the ability to induce a premature aging phenotype. In patient cells, it is possible that the presence of hTERT heterozygous compound mutations or mutations in other genes that produce a more severe phenotype may mask the impact of the variants studied here. As such, although the mutation alone might not contribute to disease development, we propose that these associated mutations can potentially accelerate telomere attrition and contribute to disease progression and severity. Furthermore, hTERT-R811C-expressing HA5 cells mimicked the homozygous mutation in patient cells and showed that this variant can cause growth defects, telomere erosion, and uncapping, leading to high levels of apoptosis. These data suggest that the R811C variant has the potential to be directly disease-causing rather than only disease-associated.

In conclusion, our data suggest that the cell growth defects caused by the disease-associated IFD variants are the consequences of a combination of several faulty mechanisms in telomerase function and regulation, thus all contributing differently to the phenotypes observed in cell growth, cell survival, and telomere maintenance. Such impairments would include compromised interaction with TPP1, leading to ineffective recruitment to telomere maintenance. Furthermore, inability
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to interact efficiently with TPP1 prevents the stimulation of telomerase activity and RAP and, in combination with recruitment defects, leads to telomere shortening and activation of the DDR and ultimately triggers apoptosis. Additionally, although some IFD variants display only slight activity defects, it has been suggested previously that a small decrease of 10–20% is sufficient to cause telomere shortening over several years (32). The consequence of this slight activity defect will have a more important impact on patients who inherited shorter telomeres in comparison with those having longer telomeres (32). In the future, it would be interesting to study the interaction of these IFD variants with other telomerase-associated proteins, including the negative regulators hPif (61–63), the regulator of telomerase activity and recruitment Pinx1 (64, 65), TIN2 (66), and HOP1 (homeobox telomere-binding protein 1) (67). Furthermore, clinical data from two newly identified mutations in the IFD (M773T and V777L) strongly suggest that these variants might be disease-causing TERT variants (68). Understanding the biochemical and molecular defects of these and other mutant telomerase enzymes would be of primary importance for the design of therapeutic treatments.

Author Contributions—T. W. C. and C. A. designed the experiments, analyzed and interpreted the experimental data, and wrote the paper. T. W. C. performed all the experiments with the exception of the hTERT-P785L plasmid constructions, hTERT-P785L HA5 cell culture, a ChIP experiment for hTERT-P785L, and analysis of TIF for hTERT-P785L, which were performed by D. E. M. All authors reviewed the results, content, and interpretation of this work. All authors approved the final version of the manuscript.

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