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The N-terminal GQ motif of human telomerase reverse transcriptase (hTERT) is essential for telomerase function but not sufficient for cell immortalization

Renxiang Chen[1], Guangzhao Li[1], Aleksandra Dakic[2], Yun-Ling Zheng[3], Jenny Li[4], Xuefeng Liu[5*]

[1]Department of Pathology, Georgetown University Medical Center, Washington, DC; [2]Division of Neuroscience, National Institute of Aging, Bethesda, MD; [3]Department of Oncology, Georgetown University Medical Center, Washington, DC; [4]Department of Pathology, Wexner Medical Center, The Ohio State University, Columbus, OH; [5]Departments of Pathology, Urology and Radiation Oncology, Wexner Medical Center, Comprehensive Cancer Center, The Ohio State University, Columbus, OH. *Corresponding author: Xuefeng Liu, xuefeng.liu@osumc.edu

ABSTRACT

Telomerase is a reverse transcriptase that adds telomeric DNA repeats at the end of chromosomes and prevents senescence, apoptosis, and genome instability. The core components of TERT are a catalytic protein subunit, the telomerase reverse transcriptase (TERT), and an RNA subunit, the telomerase RNA (TR). The N-terminus of TERT has been implicated in processive DNA synthesis that is facilitated by telomere-binding domains in TERT called anchor sites. In this study, we characterized three evolutionarily conserved residues in human TERT, G145, Q169 and G172. We performed site-directed mutagenesis and created hTERT G145A, Q169A and G172A mutants. All three hTERT mutants were not able to immortalize human foreskin fibroblasts (HFFs) and maintain telomere length in cells. The G145A mutant did not abrogate hTERT enzymatic activity, while the Q169 and G172 mutants exhibited severely reduced levels and enzymatic activity. When compared to wild-type hTERT, the G145 mutant still could interact with telomeric DNA in cells. In contrast, substitutions of Q169 and G172 impaired the interaction between hTERT and telomeres in cells. Thus, the characterization of these three conserved residue mutants in the GQ motif suggested that the hTERT N-terminal DNA binding is necessary for telomerase function, but not sufficient for maintaining telomere length and immortalizing cells.

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Introduction

Telomeres are protective structures at the ends of eukaryotic chromosomes, consisting of repetitive DNA sequences (TTAGGG in humans) and associated proteins. These structures safeguard genomic integrity by preventing chromosome degradation, end-to-end fusions, and recognition of DNA damage. Telomeres shorten with each cell division due to the end-replication problem, eventually triggering cellular senescence or apoptosis. Telomerase is a ribonucleoprotein that is responsible for adding telomeric DNA repeat onto the 3'-ends of chromosomes in most organisms, which enables cells to maintain chromosome length and stability and prevents senescence, apoptosis, and genome instability [1, 2]. In most human somatic cells, telomerase expression is repressed, leading to progressive telomere shortening with each cell division. In contrast, the majority of human tumor cells express telomerase, which stabilizes telomere length, contributing to cellular immortality and unchecked proliferation [3]. Telomerase is a unique reverse transcriptase composed of two core subunits: the catalytic protein subunit, telomerase reverse transcriptase (TERT), and the RNA subunit, telomerase RNA (TERC) [4-6]. Extensive research has focused on the role of telomerase in aging, cancer, and various diseases [7-9]. Beyond its canonical role in telomere maintenance, numerous non-canonical functions of telomerase have been discovered. These include its involvement in regulating DNA replication and repair, gene expression, and various cell signaling pathways. Such functions may contribute to critical cellular processes like cell cycle progression, survival, proliferation, differentiation, apoptosis, metabolism, regeneration, and tumorigenesis [10-13]. Evidence supporting the independent non-canonical roles of TERT and TERC is rapidly accumulating [10, 13-22].

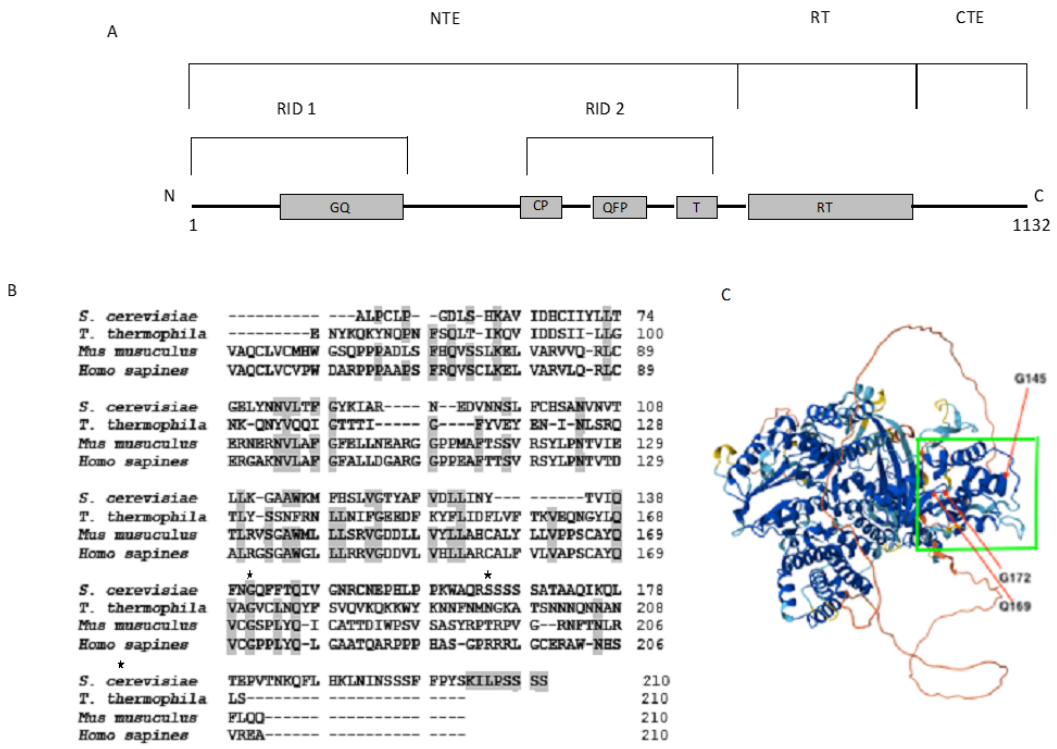

Figure 1. Linear structure and sequence of hTERT. **A.** Schematic representation of hTERT. The conserved GQ, CP, QFP, T motifs, and catalytic reverse transcriptase (RT) domains are depicted with gray boxes. The locations of RNA-interacting domains 1 and 2 (RID1 and RID2, respectively) are indicated. NTE: N-terminal extension; CTE: C-terminal extension. Schematic is drawn approximately to scale. **B.** Sequence alignment of N-termini of TERTs from various organisms. The conserved residues are shaded in gray. Residues studied in this paper are indicated by stars. From top to bottom, sequences are from *S. cerevisiae, T. thermophila, M. musculus, and H. sapiens*. **C.** Putative structure of hTERT. This structure was obtained with AlphaFold prediction [41, 42]. The green square is the RID1 domain with the three mutations we studied in the manuscript. The AlphaFold prediction is for theoretical modeling only and caution should be exercised in its use. It is provided 'as is' without any warranty of any kind, whether expressed or implied. For clarity, no warranty is given that the use of the information shall not infringe the rights of any third party. The information is not intended to be a substitute for professional medical advice, diagnosis, or treatment, and does not constitute medical or other professional advice. The AlphaFold Data have not been validated for, and are not approved for, any clinical use.

TERT uses a small RNA segment within the TERC subunit as a template to synthesize the G-rich strand of telomeres [1]. The TERT protein contains a central catalytic reverse transcriptase (RT) domain, a unique N-terminal extension, and a variable C-terminal extension [1]. The N-terminal extension consists of a non-conserved region, four conserved motifs-GQ, CP, QFP, and T—and a flexible linker located between the GQ and CP motifs [23]. The non-conserved region and the GQ motif together form RNA interaction domain 1 (RID1), while the CP, QFP, and T motifs constitute RNA interaction domain 2 (RID2) (Figure 1A).

The primary enzymatic activity of telomerase is reverse transcription. Telomerase recognizes, binds, and aligns the telomeric DNA primer within the active site to initiate reverse transcription. Unlike other reverse transcriptase, telomerase exhibits repeat addition processivity (type II processivity), a unique property that distinguishes it [1, 24]. An essential feature of type II processivity is that telomerase remains bound to the telomere throughout the entire repeat addition cycle. To achieve this, telomerase must contain an anchor site capable of interacting with the primer in the 5'-region of the RNA-DNA hybrid. This anchor site interaction is thought to enable telomerase to remain bound to the telomere, preventing dissociation when the DNA product unpairs from the RNA template before realignment [1, 24].

It has been reported that the telomerase anchor region is located in the N-terminus of TERT [23-30]. The structure of the N-terminal domain of Tetrahymena TERT, also known as the conserved TERT essential N-terminal (TEN) domain, was determined through X-ray crystallography [31]. The conserved residues located in a groove on the surface of this do-

main are essential for telomerase enzymatic activity. Several of these residues are specifically required for the sequence-specific binding of a single-stranded telomeric DNA primer [31]. Point mutation analysis of conserved residues within the GQ motif confirmed the functional importance of this motif [23]. Mutational analysis of the invariant glutamine (Q168 in tTERT) revealed significantly reduced enzymatic activity *in vitro* and impaired interaction between tTERT and telomeric single-stranded DNA (ssDNA) [28, 31]. Moreover, mutations of Q169 in hTERT resulted in reduced DNA synthesis levels *in vitro* but did not eliminate the ability of hTERT to interact with TERC or the telomeric DNA primer [32]. Additionally, alanine substitutions of two conserved glycine residues (G123 and G141 in yeast TERT) resulted in significantly reduced telomerase activity [23]. The G123A mutation displayed the most pronounced growth defects and telomere shortening, while G141A did not exhibit growth defects but resulted in telomere shortening of 150 to 200 base pairs [23].

In this study, we investigated the functional significance of three evolutionarily conserved residues within the N-terminal region of hTERT: G145, Q169, and G172. We generated alanine substitution mutants of these residues (hTERT G145A, Q169A, and G172A) and characterized their effects on cell immortalization, telomere length maintenance, telomerase activity, and telomere binding. All three hTERT mutants were unable to immortalize human foreskin fibroblasts (HFFs) or maintain telomere length in cells. The G145A substitution did not significantly impact hTERT enzymatic activity, while the Q169A and G172A mutants exhibited severely reduced enzymatic activity. Compared to wild-type hTERT, the G145 mutant retained the ability to interact with telomeric DNA in cells. In contrast, substitutions of Q169 and G172 impaired the interaction between hTERT and telomeres. Characterization of these conserved residue mutants in the GQ motif suggests that the GQ motif is necessary for telomerase function, but not sufficient for maintaining telomere length or immortalizing cells. Our findings shed light on the critical roles of these residues in hTERT function and provide insights into the mechanisms underlying telomerase-mediated cellular immortalization.

Material and Methods

Plasmids

The retrovirus vectors expressing hTERT constructs for stable cell lines were based on the pBABE-puro vector. Specific mutations in hTERT were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA), with different versions of tagged hTERT serving as templates. The mutations were confirmed by sequencing (Eurofins MWG Operon, Huntsville, AL).

Cell culture and Retroviruses

Retrovirus packaging cells, SD3443, were transfected with pBABE-FLAG-hTERT and pBABE-2XFLAG-hTERT constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The cell supernatant containing retroviruses was collected 24 hours after transfection.

Primary human foreskin fibroblasts (HFFs) were cultured from neonatal foreskins as previously described [33, 34]. and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and penicillin/streptomycin.

Primary HFFs (passage 5) were infected with amphotropic pBABE-puro retroviruses expressing tagged-hTERT and its mutants. Infected HFFs were then selected in puromycin (500 ng/mL) for 5 days. After selection, resistant colonies were pooled and passaged when the cells reached 90% confluence (1:8 ratio). To assess the effect of hTERT mutants on cell immortalization, HFFs transfected with wild-type or mutant hTERT were serially passaged. The number of population doublings was calculated at each passage.

In vitro reconstitution of telomerase activity: BSSK-hTERT/hTR [35] and BSSK-mutant hTERT-hTR were used for *In vitro* transcription and translation with the coupled transcription/translation kit (Promega). These *in vitro* transcribed and translated products were used in the quantitative TRAP assays. These experiments were repeated three times with triplicate reactions each time.

Quantitative real-time TRAP (Q-TRAP)

HFFs were cultured in 100-mm dishes to 80-90% confluence, then harvested and lysed in 100 μL of TRAP buffer. Cell extracts were prepared from HFFs transfected with wild-type or mutant hTERT. The quantitative real-time TRAP assay was performed as previously described [36, 37]. These experiments were repeated three times with triplicate reactions each time.

Telomere length measurement

Genomic DNA was extracted from transduced HFF cells using the DNeasy Blood & Tissue Kit

(Qiagen). The average telomere length was then measured using a real-time PCR-based telomere assay, as previously described [38, 39]. Briefly, the ratio (T/S) of telomere repeat copy number (T) to the single-copy gene HBG1 (S) was determined using a Bio-Rad IQ5 thermocycler. Five nanograms of genomic DNA were amplified and detected using SYBR Green Supermix (Bio-Rad). The primers used for telomeres were as follows: 5'-CGGTTTGTTTGGGT TTGGGTTTGGGTTTGGGTTTGGGTT-3' and 5'-GGCTTGCCTTACCCTTACCCTTACCCT TACCCTTACCCT-3. For HBG1, the primers were 5′-TGTGCTGGCCCATCACTTTG-3′ and 5′-ACCAGCCACCACTTTCTGATAGG-3. The T/S ratio for each sample was calculated by normalizing the average HBG Ct value to the average telomere Ct value. These experiments were repeated three times with triplicate reactions each time.

Quantitative Telomere Chromatin Immunoprecipitation (ChIP) assay

To assess the interaction between hTERT and telomeres, ChIP assays were performed. HFFs were transfected with Flag-tagged wild-type or mutant hTERT expression vectors. Cells were cross-linked with formaldehyde, and chromatin was extracted. The chromatin was immunoprecipitated with an anti-Flag antibody or control IgG. The immunoprecipitated DNA was analyzed by quantitative PCR using primers specific for telomeric DNA. The quantitative telomere ChIP assay was previously described [40]. These experiments were repeated three times with triplicate reactions each time. Briefly, ChIP lysates from different cell lines were collected using the ChIP-IT Express Magnetic Chromatin Immunoprecipitation Kit (Active Motif, Carlsbad, CA), following the manufacturer's protocol. ChIP DNAs were then subjected to q-PCR using the following primers: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTT GGGTT-3' and 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' for telomere repeats.

Results

Mutations in the hTERT RID1 domain abolished cell immortalization and telomere elongation

We first tested whether the evolutionarily conserved residues in the RID1 domain are essential for cell immortalization. Based on sequence alignment, we selected three residues that are conserved across all available TERT sequences-G145, Q169, and G172-for further study (Figure 1 B and C). To assess the functional importance of these residues, we generated hTERT mutants with alanine substitutions at each position. These mutants were stably expressed in telomerase-negative human foreskin fibroblasts (HFFs). We found that none of the hTERT mutants were able to immortalize HFFs, in contrast to wild-type hTERT (Figure 2). Cells expressing the hTERT mutants stopped growing at approximately population doublings 30-50, while cells expressing wild-type hTERT continued to proliferate steadily. This result suggests that these conserved residues are crucial for cell immortalization.

To investigate the effect of mutations at G145, Q169, and G172 on telomere elongation, we performed a quantitative real-time PCR-based assay to measure the relative length of telomeres. As shown in Figure 3, unlike wild-type hTERT, which can elongate telomeres at late passage (passage 12), cells expressing hTERT mutants exhibited telomere shortening. Taken together, these data indicate that the evolutionarily conserved residues G145, Q169, and G172 in the RID1 domain are essential for cell immortalization and telomere maintenance and/or elongation hTERT G145A maintains telomerase enzymatic activity both in cells and *in vitro*, while Q169A and G172A do not have enzymatic activity

To determine whether mutations at G145, Q169, and G172 affect telomerase enzymatic activity, we assessed the enzymatic activity of these mutants both in cells and *in vitro* using a quantitative TRAP assay. Consistent with previous studies [29, 32], hTERT Q169A lost enzymatic activity in both cellular and *in vitro* assays. We also observed that hTERT G172A displayed a dramatic reduction in catalytic activity compared to wild-type hTERT. Surprisingly, we found that hTERT G145A retained telomerase enzymatic activity (Figure 4), suggesting that G145 is not essential for maintaining telomerase enzymatic activity.

hTERT G145A retains telomere binding activity in cells

The crystal structure of the Tetrahymena TERT TEN domain revealed an ssDNA-binding groove on its surface, which contains several phylogenetically conserved residues, including the Gln residue investigated here. The other two conserved residues, G144 and G171, are not exposed on the surface but contribute to the folding of the TEN domain and the local structure of the ssDNA-binding groove,

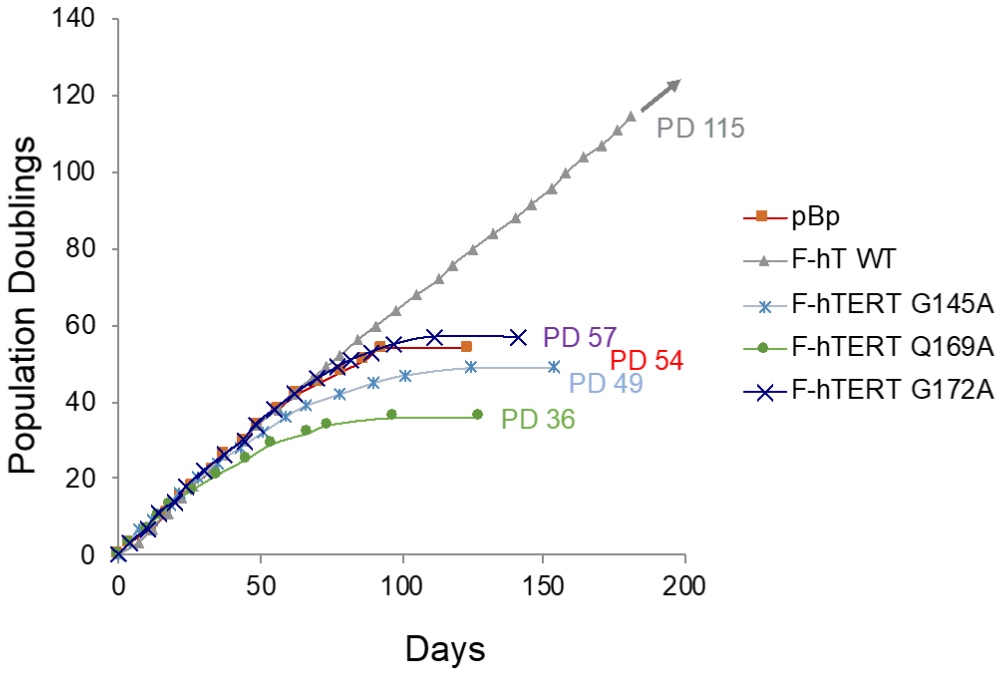

Figure 2. hTERT mutants cannot immortalize primary HFFs. Primary HFFs were transduced with retroviruses containing wild-type hTERT, hTERT mutants, and empty vectors and selected as described in Material and Methods. Cells were passed continuously, and the number of cell doublings was calculated and plotted versus the time in culture. Cultures that did not proliferate in 30 days were considered senescent and were terminated at the indicated times. This experiment was repeated twice with similar results. PD indicates population doubling, in this case, HFFs/wt HTERT reached 115 PD and continued proliferating. Other HFFs with mutant hTERT or control ended with 36- 57 PD. Conceptually, cells are considered "immortalized" once their PD is more than 50 more than the control.

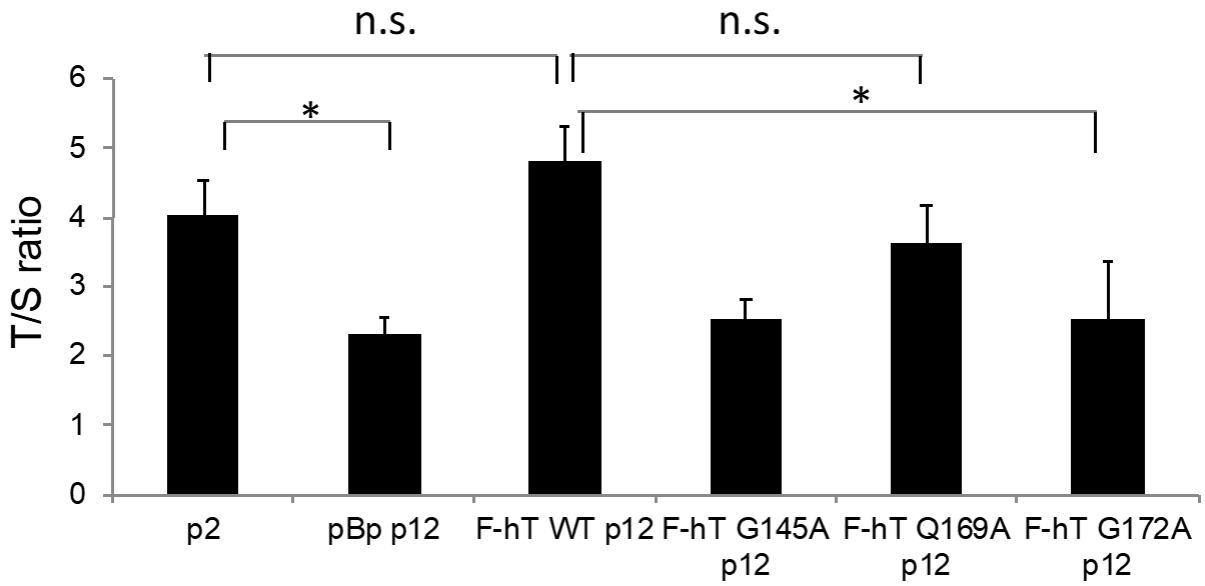

Figure 3. Relative telomere length in HFFs transduced with hTERT mutants. The relative telomere length represented as the T/S ratio was measured using quantitative PCR. At late passage, the telomere length of HFFs transduced with RID 1 mutant was shorter than that at early passage, while the telomere in cells expressing wild-type hTERT was elongated. These experiments were repeated three times with triplicate reactions each time. n.s.: $p > 0.05$; *: $p < 0.05$.

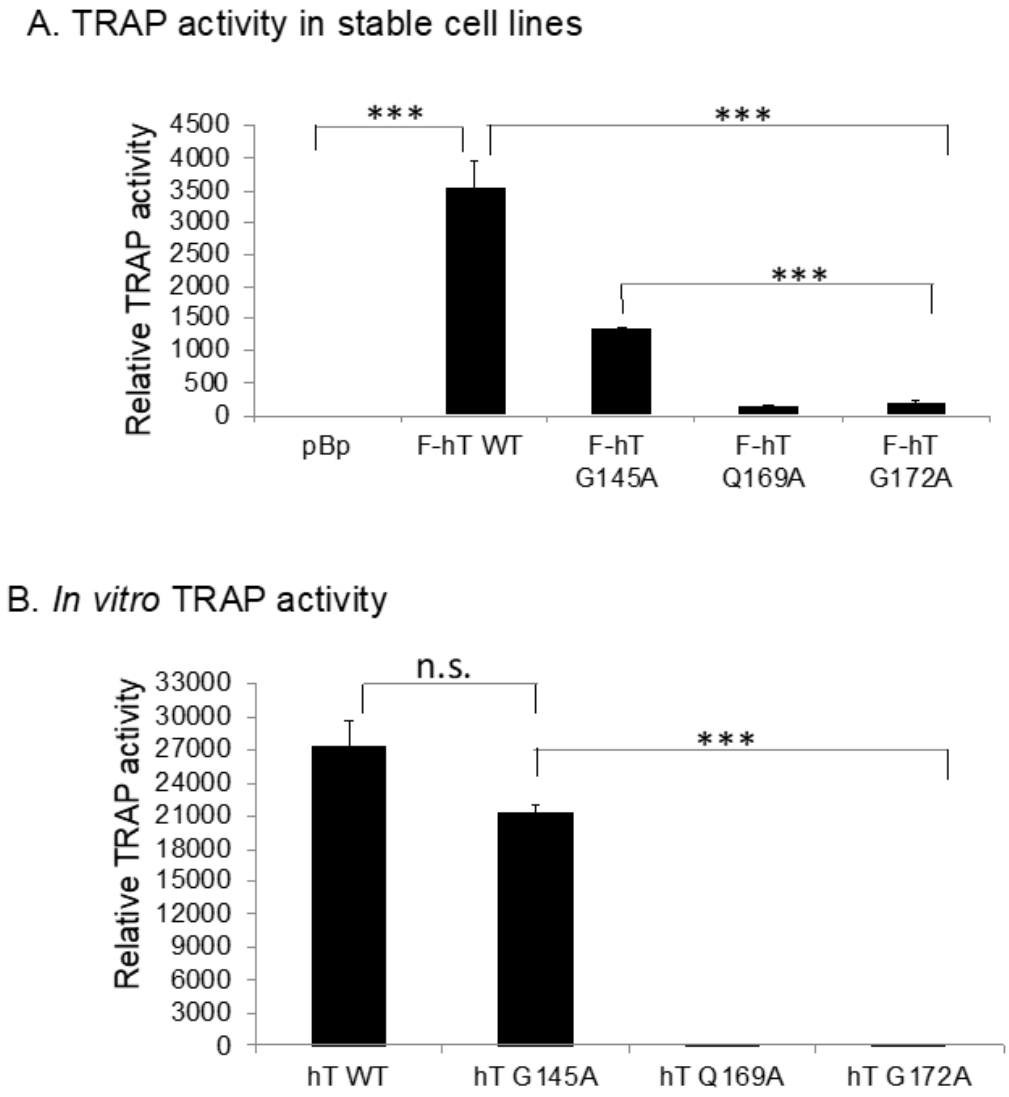

Figure 4. Telomerase activity of hTERT mutants. A. Telomerase activity of hTERT mutants in stable HFF cell lines. FLAG-tagged wild-type hTERT and hTERT mutants were transduced into HFFs, and the telomerase activity of each mutant was measured using quantitative real-time PCR. B. *In vitro* telomerase activity of hTERT mutants. Mutations were introduced into the BSSK hTERT-hTR construct, and the telomerase activity of each mutant was measured using quantitative real-time PCR. These experiments were repeated three times with triplicate reactions each time. ***: $p < 0.001$.

respectively [31]. To further study the effect of hTERT mutations on the functions of telomerase, we used the quantitative telomere ChIP assay we developed [40], to characterize physical interactions between hTERT WT, G145A, Q169A and G172A and telomeres. HFF cells transduced with 2XFLAG-tagged hTERT constructs were lysed and immunoprecipitated using IgG and anti-FLAG antibodies. ChIP DNAs were then subjected to q-PCR, with signals generated by IgG set to 1. As shown in Figure 5, both 2XFLAG-hTERT WT and 2XFLAG-hTERT G145A were able to bind telomeric DNA sequences, whereas the Q169A and G172A mutants lost the ability to interact with telomeres, suggesting that G145 is not required for DNA binding. Therefore, residues in the RID1 domain that are essential for telomerase activity are also necessary for DNA binding. Taken together, these data suggest that the interaction of hTERT with telomeric DNA is required for telomerase activity, but it is not sufficient for maintaining telomere length and extending cell lifespan.

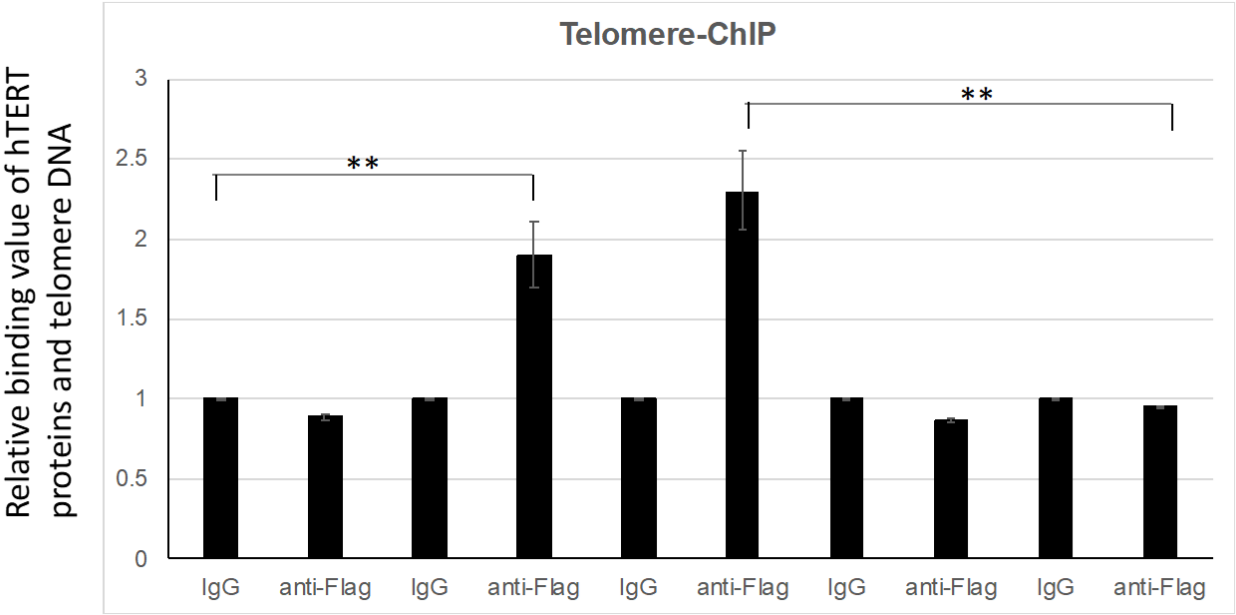

Figure 5. Interaction of hTERT mutants with telomere in cells using quantitative ChIP assay. Primary HFFs transduced with empty vector, wild-type hTERT, and mutants were used in the ChIP assay. The normal IgG was used for negative control and the signal from IgG was set to 1. These experiments were repeated three times with triplicate reactions each time. **: $p < 0.01$.

Discussion

The ability of telomerase to interact with DNA at sites outside its catalytic center is crucial for its unique repeat addition processivity. Several studies have demonstrated that a telomerase anchor region is physically and functionally mapped to the TERT N-terminus in various organisms [23-30]. During repeat addition, the telomerase anchor site remains associated with the DNA upstream of the DNA-RNA hybrid, preventing telomerase from dissociating from the DNA primer. The crystal structure of the Tetrahymena TEN domain revealed that, among the three phylogenetically conserved residues, only the invariant Q168 is exposed on the surface of the TEN domain. This residue forms the floor of the ssDNA-binding groove, along with other conserved residues [31]. The conserved G171, although not on the surface, also contributes to the local structure of the ssDNA-binding groove [31]. The third conserved residue, G144, is located between two helices and is involved in the folding of the TEN domain [31]. In this study, we investigated the corresponding residues in hTERT: G145, Q169, and G172. We predicted the putative structure of hTERT with AlphaFold prediction [41, 42] since the crystal structures of the full-length hTERT or part of hTERT protein are not currently available. The green square is the RID1 domain with the three mutations (G145, Q169, and G172) we studied in the manuscript. We also need to notice that the AlphaFold prediction is for theoretical modeling only and caution should be exercised in its use and explanation since no warranty is given that the use of the information shall not infringe the rights of any third party. As indicated on the website, AlphaFold prediction information is not intended to be a substitute for professional medical advice, diagnosis, or treatment, and does not constitute medical or other professional advice since the AlphaFold Data have not been validated for, and are not approved for any clinical use.

We first tested the previously characterized Q169 mutant in our cell culture system. Mutation of tTERT Q168 significantly decreased telomerase activity without affecting repeat addition processivity [28, 31]. The corresponding mutation in Est2p and hTERT severely impaired telomerase activity *in vitro* and *in vivo* [29, 32, 43]. In addition, the hTERT Q169 mutant was not able to maintain telomeres and immortalize cells [29, 32]. Using an *in vitro* primer binding assay, the mutations of Q169 did not affect interactions with ssDNA or TERC [29, 32]. Furthermore, the Q169 mutant altered the relative strength

of hTERT-ssDNA interaction, suggesting Q169 is required for optimal primer binding [32]. In this work, consistent with previous observations, we showed that hTERT Q169A had severely decreased telomerase activity *in vitro* and *in vivo*, and failed to maintain telomere length and to immortalize cells. However, we also observed that hTERT Q169A could not interact with telomere using a quantitative telomere ChIP assay. The discrepancy between our result and previously reported results could be due to two different systems (*in vitro* vs in cells). In this study, we measured the direct interaction between hTERT and telomeres in cells, whereas other groups have used *in vitro* primer binding assays. Although the hTERT Q169 mutations retained telomeric ssDNA-binding activity *in vitro*, the strength of the interaction between the hTERT Q169 mutants and telomeric primers was variable, depending on both the length of the primer and the specific hTERT variant [32]. Unlike the *in vitro* primer binding assay, our method is independent of both the length of the DNA primer and hTERT. It is possible that optimal primer binding is not required for the hTERT-DNA primer interaction in the *in vitro* system but is crucial for the interaction between hTERT and telomeres *in vivo*. Therefore, while previous studies have shown that hTERT Q169 mutants can interact with DNA primers *in vitro*, our results indicate that hTERT Q169A loses telomere-binding activity *in vivo*. This suggests that Q169 plays a role in binding to telomeres and that this protein-DNA interaction is essential for telomerase activity.

We next characterized the other two conserved glycine residues, G145 and G172. Alanine substitution of the corresponding residues in yeast TERT (Est2p), G123, and G141, significantly decreased telomerase enzymatic activity [23]. Moreover, the G141A mutant did not exhibit any growth defects but showed telomere shortening of at least 150 to 200 bp. In contrast, the G123 mutation caused pronounced growth defects and resulted in even shorter telomeres [23]. In this study, we demonstrated that human telomerase reconstituted with hTERT G145A did not show any reduction in telomerase enzymatic activity, while the G172A mutant caused a significant decrease in telomerase activity both *in vitro* and *in vivo*. For both the hTERT G145A and G172A mutants, there was a strong correlation between DNA binding activity and telomerase enzymatic activity, suggesting that the interaction between hTERT and telomeres is essential for telomerase function. Since the corresponding residue in tTERT, G171, contributes to the local structure of the ssDNA binding groove, it is possible that mutation of G172 altered the local structure of this groove, thereby disrupting the direct interaction between hTERT and telomeres.

We next tested whether these two conserved glycine residues were important for telomere maintenance and cell immortalization. The hTERT G172A mutant lost the ability to maintain telomere length and immortalize primary human foreskin fibroblasts. As noted earlier, the Est2p G141A mutant did not cause any growth defects despite severely reduced telomerase activity and shortened telomeres. The discrepancy between Est2p G141A and hTERT G172A in terms of cell immortalization may reflect species-specific differences in the function of this conserved residue. It is possible that a single amino acid mutation in Est2p is insufficient to induce senescence in yeast. Surprisingly, although hTERT G145A retained telomerase activity and could still bind to telomeres, cells transduced with this hTERT mutant exhibited shortened telomeres and entered senescence.

Previous studies have indicated that the hTERT RID1 domain is a major accessory domain that interacts with TERC and is crucial for repeat addition processivity [27, 44]. Therefore, the hTERT G145A mutant may lose its ability to interact with TERC and add telomeric repeats, but it remains capable of binding to telomeres. Although hTERT G145A maintained telomerase enzymatic activity in our TRAP assay, it is important to note that the TRAP assay is an *in vitro* system, which may not fully reflect the events occurring in cells. This discrepancy could be due to the fact that RID1 is not the primary TERC binding domain of hTERT [27]. The single amino acid mutation at G145 could still maintain enzymatic activity but was less efficient in adding telomeric repeats compared to wild-type hTERT. Based on the crystal structure of tTERT, G145 likely plays a role in the folding of the N-terminus of hTERT. Therefore, the G145A mutation may disrupt the local structure of the hTERT N-terminus, potentially impairing the interaction between hTERT and other molecular chaperones in the telomerase complex.

Future studies are needed to further elucidate the precise mechanism of this conserved Gly residue. Collectively, our data show that the interaction between hTERT and telomeres is essential for maintaining telomerase enzymatic activity. However, while this interaction is necessary for telomere elongation

and immortalization, it is not sufficient on its own to accomplish these processes in human primary foreskin fibroblasts.

Conclusion

In summary, our study demonstrates that the GQ motif within the RID1 domain of the N-terminal region of hTERT is essential for telomerase function and cell immortalization. While Q169 and G172 are critical for telomerase activity and telomere binding, G145 appears to play a distinct role in telomerase function beyond catalytic activity and telomere association. Further investigation of these residues will provide a deeper understanding of the mechanisms underlying telomerase-mediated cellular immortalization and may lead to the development of novel therapeutic strategies for cancer and aging-related diseases.

Authors' contribution

Renxiang Chen, Guangzhao Li, Aleksandra Dakic: performing experiments, collecting and analyzing data. Yun-Ling Zheng, Jenny Li: interpreting and analyzing data, review, and editing; Xuefeng Liu: conceptual design, writing and editing, and final review.

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Competing Interest declaration

The authors have nothing to report.

Data Availability Statement

The authors have nothing to report.

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