The Molecular Basis for Telomerase Trafficking in Human Cancer Cells

By

Eladio Abreu

Under the Direction of Michael Terns, PhD, and Rebecca Terns, PhD

ABSTRACT

Telomerase is the enzyme responsible for synthesizing the repetitive DNA of telomeres. In most human tissues, telomerase activity is confined to prenatal development. However, in the vast majority of cancers, telomerase is reactivated. This is critical to prolonged tumor maintenance and proliferation. Our lab is primarily concerned with understanding the molecular basis for the intracellular trafficking of the core components of human telomerase: human telomerase RNA (hTR), and human telomerase reverse transcriptase (hTERT). Significant progress has been made in identifying and characterizing the trafficking patterns that telomerase components follow throughout the cell cycle. This dissertation work focuses on understanding the mechanisms responsible for telomerase recruitment to the telomere. We hypothesized that telomerase recruitment is facilitated by dynamic association of the enzyme with core telomere binding proteins. Fluorescence microscopy based *in vivo* localization studies were employed in a variety of assays to assess the intrinsic roles of telomerase components, as well as factors residing at telomeres, in facilitating recruitment to telomere ends. In pursuit of this overall objective, it was determined that the hTERT N-DAT domain, TCAB1 (recently discovered telomerase component that resides at Cajal bodies), and TPP1 (one of six core telomere binding proteins), work together to facilitate telomerase recruitment. We also discovered novel coilin-positive nuclear bodies; we call telomerase induced bodies (TIBs). These TIBs are specific to telomerase components and are found at telomeres. hTERT mutants incapable of inducing TIBs are deficient in the ability to traffic to telomeres. This suggests that TIBs play a critical role in telomerase delivery to telomeres. Collectively, the findings contained within this thesis support a model whereby telomerase recruitment to telomeres involves dynamic interactions between telomerase components and at least one recruitment factor residing at the telomere.

INDEX WORDS: Telomerase, Telomere, Trafficking, Cancer, Cajal Body, Shelterin

THE MOLECULAR BASIS FOR TELOMERASE TRAFFICKING IN HUMAN CANCER CELLS

By

Eladio Abreu

B.S., Morehouse College, 2005

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment

of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2011

THE MOLECULAR BASIS FOR TELOMERASE TRAFFICKING IN HUMAN CANCER CELLS

By

ELADIO ABREU

Major Professors:	Michael and Rebecca Terns
Committee:	Stephen Dalton
	Kojo Mensa-Wilmot
	Michael McEachern

Electronic Version Approved:

Maureen Grasso

Dean of the Graduate School

The University of Georgia

May 2011

Dedication

I would like to dedicate this thesis to my Family. My parents have been the most inspirational source of guidance in my life. They have taught me with hard work and perseverance no goal is unobtainable. This lesson has given me the tenacity and ambition to achieve. My wife has supported me in the pursuit of my dreams for as long as we have known each other. Her love and faith reassures me of my path everyday. Most importantly, this achievement is for my Son and my Sister. I know that this milestone in my life will pale in comparison to the many achievements they will make as they reach for their true potential.

Acknowledgments

I would like to thank my advisors, Michael and Rebecca Terns. They have believed in me since the first day I set foot in the lab. They have pushed me to reach for my true potential, even when my ego had convinced me that I was already there. They have taught me to demand the best of myself in everything I do. Last, and most importantly, they taught me how to think like a scientist. I want to thank my committee members: Kojo Mensa-Wilmot, Stephen Dalton, and Michael McEachern. Their input, suggestions, and wisdom, have helped me grow professionally, scientifically, and personally. I want to thank everyone who worked on this project with me. This includes an amazing team of graduate students, undergraduate students, technicians, and postdoctoral fellows. Without Rebecca Tomlinson, Zhu-Hong Li, Jian Li, Brad Culp, Elena Aritonovska, Emem Adolph, Tania Zeigler, Teerawit Supakorndej, Mathew Belcher, William Nije, and Garrett Casale, none of this would have been possible. Thank you to all of my colleagues in the Terns lab past and present. I also would like to thank all of our collaborators, and contributors. A special thanks goes to Joachim Lingner's group in Switzerland. I would like to thank everyone at the University of Georgia Department of Biochemistry & Molecular Biology. Last but not least, I want to thank Jonathon Arnold and Theodore Logan for giving me my first opportunity to work behind the bench.

Table of Contents

Chapter 1:
Introduction

Telomerase

Telomeres are DNA-protein complexes that protect the ends of chromosomes from degradation and end-to-end fusions (14, 41). Telomeres are composed of numerous DNA repeat sequences coated with a complex of core telomere binding proteins (41). Telomerase is the enzyme responsible for adding the short DNA repeats (TTAGGG) of the telomere to the 3' end of the chromosome (20). Human telomerase is a ribonucleoprotein (RNP) enzyme consisting of two main functional subunits: telomerase reverse transcriptase (hTERT) and telomerase RNA (hTR) (7, 17, 25). The reverse transcriptase component of telomerase uses a portion of hTR as a template to add telomeric repeats to the chromosome end (7, 10). Both hTERT and hTR are necessary for the telomerase enzyme to function (7). hTR and hTERT are considered to be the minimum components required for telomerase activity. These two components are sufficient to reproduce telomerase activity *in vitro* (7). Although hTR is expressed ubiquitously, hTERT it is not expressed at detectable levels in adult somatic tissues of long living mammals such as humans (16, 46). This makes hTERT the limiting component in the formation of the active enzyme.

Telomerase involvement in proliferation and disease

Absence of telomerase associated telomere maintenance leads to gradual telomere length shortening in adults (31). Without active telomerase, cells can only survive so many divisions before their telomeres reach a critically short length (44). Critically short telomeres can lead to cellular senescence, apoptosis and/or crisis (44). In this way, loss of telomerase activity gives a cell line a limited lifespan. Telomerase-positive cancers, however, do not have this problem (29). In the majority of cancers, reactivated telomerase activity maintains the shortened telomere after each replication (29). As one would expect, most immortalized or malignant tumor cells do not display net loss of telomere length as a result of successive mitotic divisions (11). Unlike their non-cancerous counterparts, these cells are immortal, having no set lifespan because of constitutive telomere maintenance by telomerase (29). This re-activation of telomerase is

critical to prolonged tumor maintenance and proliferation (19, 24). Furthermore, while expression of oncogenes in normal cells is sufficient to bypass senescence and apoptosis, hTERT expression is still limiting for cellular immortality (22). Last, normal cells are immortalized following expression of hTERT alone (8, 22, 38). Together, these observations confirm that reactivation of telomerase is a critical and essential step in tumorogenesis.

The critical function in cancer development and maintenance makes telomerase a promising target in anti-cancer research. A better understanding of how this RNP and its primary components are recruited from their respective sites of biogenesis and storage to their site of action at the telomere end may prove critical in developing new methods of fighting cancer. If telomerase regulation through nuclear trafficking becomes well understood, the pharmaceutical industry could strategically develop drugs to inhibit this pathway and limit telomerase activity. As an enzyme that is dispensable for the survival of most adult tissues, telomerase represents a target to fight cancer with minimal detrimental side effects.

While cancer relies on illegitimate telomerase activity, other diseases are actually based in a loss of sufficient telomerase activity. Dyskeratosis congenita (DC) is a disease linked to abnormally short telomeres (35). These short telomeres are the result of attenuated telomerase activity in developing, prenatal, and highly proliferative adult tissues (23, 35). DC is a debilitating bone marrow failure syndrome characterized by multiple mucocutaneous problems. These include abnormal skin pigmentation, leukoplakia, and nail/skin dystrophy (23, 35). DC follows both autosomal dominant (AD) and x-linked modes of inheritance (23, 35). The AD form of the disease relies on several heterogeneous mutations in hTR (23). Some of the these hTR mutations result in impaired RNA accumulation or stability, while others have been found to inhibit the catalytic activity of the actual telomerase holoenzyme (35). The defects in telomerase activity exhibited in DC may actually be based on problems in proper telomerase recruitment and/or trafficking. At least one of these mutations occurs at the CR7 domain of hTR, just 3 base pairs from the CAB box (required for telomerase trafficking) (23, 35). This information suggests that at least one DC disease mutant may be capable of disrupting a step in cell cycle regulated trafficking of hTR. The fact that other AD-DC

mutants retain their *in vitro* activity suggests that other disease mutants may lead to trafficking/recruitment deficiencies as well (23, 35).

Human Telomerase RNA (hTR)

Human telomerase RNA is a 451 nucleotide small Cajal body RNA (scaRNA) (9). It forms a distinctive structure with several highly conserved domains and motifs (Fig. 1) (9). These highly conserved domains are critical to telomerase biogenesis and function. The pseudoknot domain contains the template region used for telomere repeat addition as well as motifs required for hTERT binding. The CAB box is a short nucleotide sequence (UGAG) in the CR7 loop of hTR's H/ACA domain (27). The CAB box is critical for *in vivo* localization to Cajal bodies, efficient telomerase recruitment to telomeres, and subsequent telomere maintenance (12, 27).

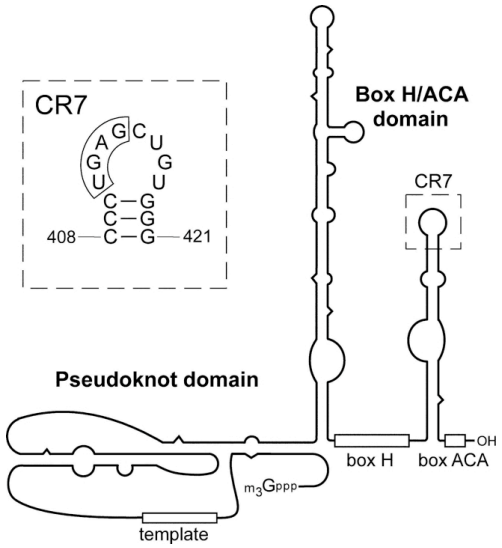


FIGURE 1: The structure of human telomerase RNA. hTR forms a unique secondary structure. The pseudoknot and box H/ACA domains contain motifs required for catalytic activity, enzyme assembly, and telomerase trafficking. Adapted from. The UGAG sequence of the CAB box, within the CR7 loop, is required for hTR localization to Cajal bodies and telomeres (27).

hTR Associates with several secondary telomerase components.

The H/ACA domain of hTR interacts with a complex of secondary telomerase components. This complex of proteins is called the dyskerin complex and includes the following proteins: dyskerin, Nhp2, Nop10, and GAR1 (15, 36, 42). The hTR H/ACA motif and the aforementioned components of the dyskerin complex are required for hTR stabilization and accumulation *in vivo* (18). These functions make these secondary telomerase components vital to telomerase activity and telomere maintenance (18). Various DC patient mutations effect relative levels of functional dyskerin protein (36). Other DC mutations affect the ability of hTR to interact with dyskerin and/or TCAB1, a more recently identified telomerase component (23, 35, 48, 54). Resulting hTR stability and trafficking deficiencies contribute to the pathology of DC (54). This work will further explore the critical role of TCAB1 in telomerase trafficking and telomere maintenance.

Human Telomerase Reverse Transcriptase (hTERT)

Human telomerase reverse transcriptase is a ~127 kDa catalytic protein (25). The general domain structure of hTERT consists of two main parts: an N-terminal half, and a C-terminal half. Each of these segments encompasses sub-domains with a subset of functions (Fig. 2) (4). The N-terminal half of the protein is home to the telomerase essential N-terminal (TEN) domain telomerase RNA binding (TRBD, binds hTR and required for telomerase assembly) domain. The TEN domain encompasses critical domains required for interactions with telomeric DNA (26, 43). The TEN domain is also home to the N-terminal dissociation of activity of telomerase (N-DAT) domain (2). The N-DAT is thought to be involved in telomerase recruitment or binding to telomeric DNA (2, 30). The RT domain is found at the beginning of the C-terminal half the hTERT peptide sequence (4). RT domains are conserved in all reverse transcriptases. The hTERT RT domain is considered the catalytic portion of the telomerase enzyme (4). The RT domain encompasses the following 7 sub-domains: 1, 2, A, B',C ,D, and E (also known as RT 1-7) (4). The C-terminal domain (CTD) extends from the RT domain to the

C-terminal end of hTERT (4). This domain includes a C-terminal disassociation of activity of telomerase (C-DAT) domain (5). For some time, it was thought that the C-terminal domain and C-DAT were involved in telomerase recruitment and regulation (2, 3, 5).

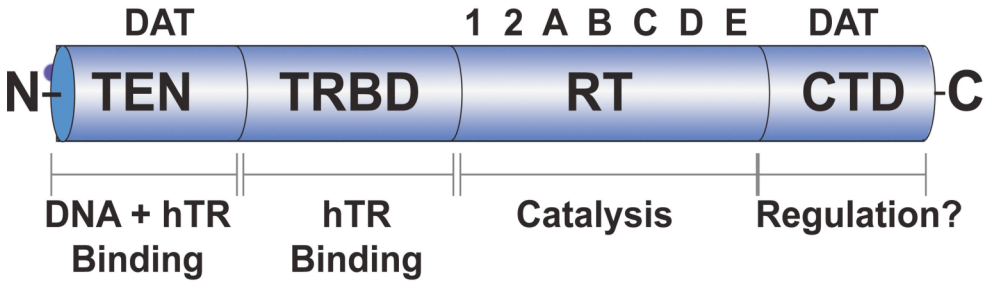


FIGURE 2: The domain structure of hTERT. See text for explicit description of hTERT structure/function relationships.

Six core telomere binding proteins comprise the shelterin complex

Shelterin (also known as the telosome), is a complex of six core telomere binding proteins found on mammalian telomere ends throughout the cell cycle (13, 32) (Fig.3). The constituents of the shelterin complex play important roles in telomere length regulation, protection, and end structure (13). These core telomere binding proteins fit several criteria. First, shelterin components are abundant at telomere ends but do not accumulate elsewhere in the cell. Second, shelterin components are present at telomere ends throughout the cell cycle. Finally, their known function is limited to telomeres (13). Six core telomere binding proteins make up the shelterin/telosome complex: TRF1, TRF2, RAP1, TIN2, TPP1, and POT1 (telomere repeat binding factors 1 & 2, repressor-activator protein 1, TRF1-interacting nuclear protein 2, TIN2-POT1 interacting protein 1, and protection of telomeres 1, respectively.) (13, 32) (Fig. 3).

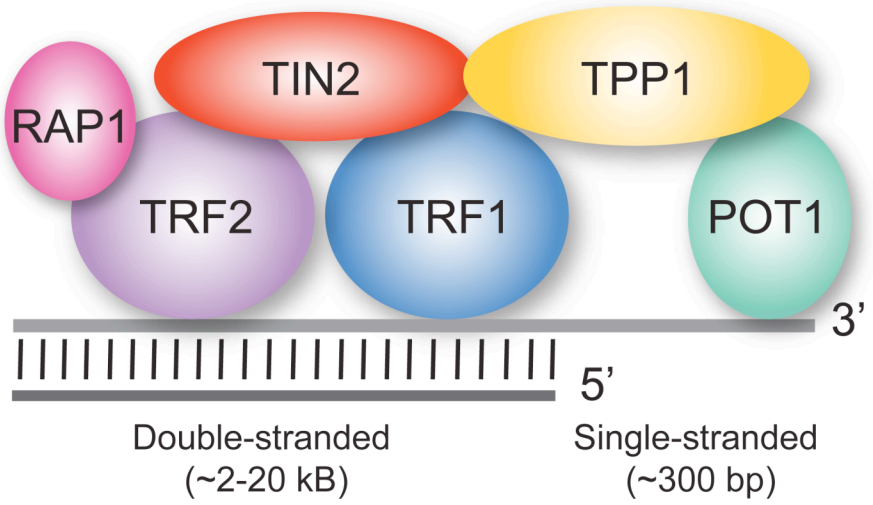


FIGURE 3: The shelterin complex. The shelterin complex consists of six core telomere binding proteins that regulate telomere end-structure.

Shelterin serves in the protection and regulation of the telomere end (13). This is most likely accomplished by regulating telomere end structure. Telomeres display a single stranded overhang at their 3' end proposed to invade double stranded portions of the telomere upstream, displacing the g-rich strand and base pairing with the c-rich strand, thus, forming various lariat structures such as the T-loop (13, 21). This so called "capped" end serves several protective functions. These include, limiting telomerase access to the telomere end outside of S-phase (13). Although, it is very likely that the replication fork destabilizes the T-loop during DNA replication, switching the telomere to an uncapped/open form receptive to telomerase action, there has been no solid evidence to definitively support this. However, the individual components of shelterin may respond to some kind cell cycle related stimulus. This may result in the complex undergoing some conformational changes to facilitate the recruitment of telomerase.

TRF1 and TRF2 are similar double stranded DNA binding proteins that form homodimers on telomeric DNA (see Fig. 3) (13, 32). These proteins serve as the base of the shelterin complex (13, 32). Rap1 binds TRF2 while TIN2 bridges the TRF1 and TRF2 homodimers in a saw horse like manner (see Fig. 3) (13, 32). TIN2 also binds TPP1 and stabilizes its attachment to the complex (see Fig. 3) (13, 32). TPP1 forms a

hetero-dimer with POT1, which has its own single stranded DNA binding activity (see Fig. 3) (13, 32, 49, 51). There is evidence that POT1 is dependent on dimerization with TPP1 to bind the telomere, and that this dimer is the true functional subunit of shelterin, possibly regulating recruitment of the telomerase enzyme itself (44, 45, 47)

The TPP1/ POT1 dimer has a role in telomerase regulation

Data presented in recent studies suggest that TPP1 can bind hTERT *in vitro (51)*. It is thought that this interaction takes place *in vivo* and is integral to the recruitment of telomerase to the telomere end, facilitating positive regulation of telomerase activity (45, 51, 53). In addition to the proposed ability to bind hTERT, the TPP1/POT1 dimer has also been found to increase *in vitro* telomerase processivity and subsequent elongation of telomere repeat primers (49, 53). These positive effects on telomerase activity came as a surprise. Until recently, shelterin has been considered a telomerase-inhibiting complex (13). Furthermore, there is evidence that TPP1 and POT1 present independent inhibitory activity (33, 34, 52). In light of the recent studies, it has been proposed that the TPP1/POT dimer can switch from a negative to positive regulator of telomerase activity (49). The TPP1/POT1 dimer primarily displays positive regulation *In vitro*, while its negative regulatory effects are primarily observed *in vivo (49)*. This suggests that the negative effect of TPP1/POT1on telomerase activity is based on interactions with the rest of the shelterin complex (49).

TEBP-α/β is a ciliate protein responsible for the protection of single stranded chromosome ends in *Oxytricha nova (6)*. Homology studies suggest that the β subunit in TEBP-α/β is actually the homolog of TPP1(51). POT1 is a binding partner of TPP1 and has a structure and ssDNA binding ability that mirrors that of TEBP-α (6, 51). The discovery of striking similarities between TPP1 and TEBP-β as well as POT1 and TEBP-α suggests that the TPP1/POT1 dimer is actually the human equivalent of *O. nova'*s TEBP-α/β protein (6). More recent studies in ciliates have shown that TEBP-β follows cell cycle specific patterns of phosphorylation and dephosphorylation (40). This phosphorylation is linked to the release of protective G-quadruplex structures thought to inhibit telomerase activity at the telomere end (40). The same studies provided evidence

that CDK2 was responsible for phosphorylating TEBP-β (40). These events connect the ciliate homolog of TPP1 to cell cycle mediated regulation of telomerase access to telomeres. This suggests that TPP1 may be involved in similar activities in humans.

Cajal Bodies

Cajal bodies are dynamic nuclear structures involved in RNP assembly, and non-coding RNA maturation (37, 39). The Cajal body (CB) was identified in 1903 by Ramon y Cajal, a renowned neuroanatomist and nobel prize laureate (39). Cajal discovered these small round bodies within the nuclei of various nerve cells using a silver impregnation technique (45). He called these structures accessory bodies (39). Over 100 years later, the exact function of the Cajal body is not well understood. However, the contents of CBs provide clues to their function. CBs contain various proteins and RNAs that take part in RNA processing, particularly those required in splicing snRNAs (37, 39). Cajal bodies also contain a unique class of RNAs called small Cajal body RNAs (scaRNAs) (37, 39). These scaRNAs share structural/functional similarities with small nucleolar RNAs (snoRNAs) (37, 39). Both types of RNAs share box C/D and box H/ACA motifs and play critical roles in posttranscriptional modifications of other RNAs (37, 39). The inclusion of snRNP and snoRNP components support a role for Cajal bodies in RNP biogenesis (37, 39). There is a consensus that Cajal bodies increase the efficiency of RNP activity by catalyzing RNP assembly and bringing assembled active enzyme together with RNA substrates to facilitate posttranscriptional processing and modifications (37, 39). This work will explore the role of Cajal bodies in telomerase biogenesis and trafficking.

Telomerase Trafficking to telomeres in cancer cells

The primary focus of this dissertation project has been to investigate the molecular basis for telomerase recruitment to telomeres. We have successfully identified the nuclear trafficking patterns primary telomerase components follow throughout the cell cycle. Our lab was one of the first in the telomerase field to develop an accurate means of monitoring telomerase localization through fluorescence *in situ* hybridization

(FISH) (55). Immunofluorescence (IF) was used to label several protein markers in order to determine the position of hTR relative to other nuclear components (55). In early studies, our lab established that hTR forms foci at Cajal bodies throughout most of the cell cycle (55). This localization is observed in a variety of telomerase positive cancer cell lines (55). The association of hTR, and later, hTERT with these nuclear structures lead to the proposed roles of the Cajal body in telomerase assembly and delivery (12, 28, 47, 55). Additionally, hTR foci are found to associate with a specific subset of telomeres during mid S-phase of the cell cycle (28, 47). However, hTR does not localize to any nuclear foci in telomerase negative primary cell lines (55). These foci are also absent in ALT cell lines (55). ALT cells are immortalized cell lines that use a recombinant method of elongating their telomeres, independent of telomerase (50). Despite the insight gained from these previous studies, relatively little is known about the molecular basis of these recruitment and trafficking events.

The focus of this work is to achieve an understanding of the process that facilitates telomerase recruitment to the telomere. The studies detailed here were conceived to reveal the molecular mechanisms governing telomerase recruitment to telomeres in human cancers. We hypothesize that the recruitment of telomerase to telomeres is facilitated by the dynamic association of the enzyme with core telomere binding proteins. We postulate that this recruitment process plays an important role in telomerase regulation. This would allow for recruitment of telomerase to accessible telomere ends immediately following replication, rather than double stranded breaks throughout the cell cycle. *In vivo* localization studies were employed in a variety of screens to assess the intrinsic roles of telomerase components and core telomere binding proteins, in facilitating telomerase trafficking. We have established the functional importance of key aspects of telomerase RNA structure in trafficking dynamics (12). The primary aims at the outset of this thesis work were to dissect the role of hTERT and core telomere binding proteins in mediating the recruitment of telomerase to telomeres.

The studies detailed here will describe the roles of domains intrinsic to a core telomere binding protein and telomerase in facilitating telomerase recruitment. We will show that the oligonucleotide binding domain of TPP1 (one of six core telomere binding proteins) is required for telomerase recruitment to telomeres (1) (see chapter 2). Past

studies have shown that hTERT expression is required to induce hTR localization to telomeres and Cajal bodies, suggesting assembly of hTR and hTERT is a prerequisite of the trafficking process (see supplemental chapter I). Here, we will also show that the hTERT N-terminal disassociation of activity of telomerase (N-DAT) domain is required to facilitate telomerase recruitment to telomeres (see chapter 3). Additional, supplemental studies have also revealed a role for TCAB1 (newly discovered telomerase component) in the recruitment process (48) (see supplemental chapter II).

Our group has presented evidence that Cajal bodies play a prominent role in telomerase recruitment, and subsequent telomeres maintenance (12). Here, we also provide evidence of a novel coilin-positive telomerase delivery body (see chapter 3). These Cajal body like structures are specific to telomerase components and show a strong correlation to telomerase delivery to telomeres.

Last, we present evidence that under conditions of steady-state homeostatic telomere maintenance, telomerase acts on individual telomeres in a processive manner (a single enzyme adding repeats processively along a single telomere) (see supplemental chapter III). However, when telomeres are being elongated under non-equilibrium conditions, telomerase acts distributively (multiple enzymes acting on each telomere) (see supplemental chapter III). Data from these studies also imply that Cajal body processing is linked to the processivity of telomerase molecules present in the nucleus.

Taken together, our group's body of work establishes that telomerase recruitment is facilitated by factors intrinsic to the telomerase enzyme, and the shelterin complex. These findings contribute significantly to our understanding of the dynamic mechanism of telomerase regulation through recruitment and trafficking.

References

1. Abreu, E., E. Aritonovska, P. Reichenbach, G. Cristofari, B. Culp, R. M. Terns, J. Lingner, and M. P. Terns. 2010. TIN2-tethered TPP1 recruits human telomerase to telomeres in vivo. Mol Cell Biol 30:2971-2982.

2. Armbruster, B. N., S. S. Banik, C. Guo, A. C. Smith, and C. M. Counter. 2001. N-terminal domains of the human telomerase catalytic subunit required for enzyme activity in vivo. Mol Cell Biol 21:7775-7786.
3. Armbruster, B. N., K. T. Etheridge, D. Broccoli, and C. M. Counter. 2003. Putative telomere-recruiting domain in the catalytic subunit of human telomerase. Mol Cell Biol 23:3237-3246.
4. Autexier, C., and N. F. Lue. 2006. The structure and function of telomerase reverse transcriptase. Annu Rev Biochem 75:493-517.
5. Banik, S. S., C. Guo, A. C. Smith, S. S. Margolis, D. A. Richardson, C. A. Tirado, and C. M. Counter. 2002. C-terminal regions of the human telomerase catalytic subunit essential for in vivo enzyme activity. Mol Cell Biol 22:6234-6246.
6. Baumann, P., and T. R. Cech. 2001. Pot1, the putative telomere end-binding protein in fission yeast and humans. Science 292:1171-1175.
7. Beattie, T. L., W. Zhou, M. O. Robinson, and L. Harrington. 1998. Reconstitution of human telomerase activity in vitro. Curr Biol 8:177-180.
8. Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science 279:349-352.
9. Chen, J. L., M. A. Blasco, and C. W. Greider. 2000. Secondary structure of vertebrate telomerase RNA. Cell 100:503-514.
10. Cong, Y. S., J. Wen, and S. Bacchetti. 1999. The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. Hum Mol Genet 8:137-142.
11. Counter, C. M., A. A. Avilion, C. E. LeFeuvre, N. G. Stewart, C. W. Greider, C. B. Harley, and S. Bacchetti. 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 11:1921-1929.
12. Cristofari, G., E. Adolf, P. Reichenbach, K. Sikora, R. M. Terns, M. P. Terns, and J. Lingner. 2007. Human telomerase RNA accumulation in Cajal bodies facilitates

telomerase recruitment to telomeres and telomere elongation. Mol Cell 27:882-889.

13. de Lange, T. 2005. Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev 19:2100-2110.
14. Donate, L. E., and M. A. Blasco. 2011. Telomeres in cancer and ageing. Philos Trans R Soc Lond B Biol Sci 366:76-84.
15. Dragon, F., V. Pogacic, and W. Filipowicz. 2000. In vitro assembly of human H/ACA small nucleolar RNPs reveals unique features of U17 and telomerase RNAs. Mol Cell Biol 20:3037-3048.
16. Dwyer, J., H. Li, D. Xu, and J. P. Liu. 2007. Transcriptional regulation of telomerase activity: roles of the the Ets transcription factor family. Ann N Y Acad Sci 1114:36-47.
17. Feng, J., W. D. Funk, S. S. Wang, S. L. Weinrich, A. A. Avilion, C. P. Chiu, R. R. Adams, E. Chang, R. C. Allsopp, J. Yu, and et al. 1995. The RNA component of human telomerase. Science 269:1236-1241.
18. Fu, D., and K. Collins. 2003. Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. Mol Cell 11:1361-1372.
19. Greider, C. W. 1998. Telomerase activity, cell proliferation, and cancer. Proc Natl Acad Sci U S A 95:90-92.
20. Greider, C. W., and E. H. Blackburn. 1985. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 43:405-413.
21. Griffith, J. D., L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss, and T. de Lange. 1999. Mammalian telomeres end in a large duplex loop. Cell 97:503-514.
22. Hahn, W. C., C. M. Counter, A. S. Lundberg, R. L. Beijersbergen, M. W. Brooks, and R. A. Weinberg. 1999. Creation of human tumour cells with defined genetic elements. Nature 400:464-468.
23. Handley, T. P., J. A. McCaul, and G. R. Ogden. 2006. Dyskeratosis congenita. Oral Oncol 42:331-336.
24. Harley, C. B., and N. W. Kim. 1996. Telomerase and cancer. Important Adv Oncol:57-67.

25. Harrington, L., W. Zhou, T. McPhail, R. Oulton, D. S. Yeung, V. Mar, M. B. Bass, and M. O. Robinson. 1997. Human telomerase contains evolutionarily conserved catalytic and structural subunits. Genes Dev 11:3109-3115.
26. Jacobs, S. A., E. R. Podell, and T. R. Cech. 2006. Crystal structure of the essential N-terminal domain of telomerase reverse transcriptase. Nat Struct Mol Biol 13:218-225.
27. Jady, B. E., E. Bertrand, and T. Kiss. 2004. Human telomerase RNA and box H/ACA scaRNAs share a common Cajal body-specific localization signal. J Cell Biol 164:647-652.
28. Jady, B. E., P. Richard, E. Bertrand, and T. Kiss. 2006. Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. Mol Biol Cell 17:944-954.
29. Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011-2015.
30. Lee, S. R., J. M. Wong, and K. Collins. 2003. Human telomerase reverse transcriptase motifs required for elongation of a telomeric substrate. J Biol Chem 278:52531-52536.
31. Lindsey, J., N. I. McGill, L. A. Lindsey, D. K. Green, and H. J. Cooke. 1991. In vivo loss of telomeric repeats with age in humans. Mutat Res 256:45-48.
32. Liu, D., M. S. O'Connor, J. Qin, and Z. Songyang. 2004. Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. J Biol Chem 279:51338-51342.
33. Liu, D., A. Safari, M. S. O'Connor, D. W. Chan, A. Laegeler, J. Qin, and Z. Songyang. 2004. PTOP interacts with POT1 and regulates its localization to telomeres. Nat Cell Biol 6:673-680.
34. Loayza, D., and T. De Lange. 2003. POT1 as a terminal transducer of TRF1 telomere length control. Nature 423:1013-1018.
35. Marrone, A., A. Walne, and I. Dokal. 2005. Dyskeratosis congenita: telomerase, telomeres and anticipation. Curr Opin Genet Dev 15:249-257.

36. Mitchell, J. R., E. Wood, and K. Collins. 1999. A telomerase component is defective in the human disease dyskeratosis congenita. Nature 402:551-555.
37. Morris, G. E. 2008. The Cajal body. Biochim Biophys Acta 1783:2108-2115.
38. Nakayama, J., H. Tahara, E. Tahara, M. Saito, K. Ito, H. Nakamura, T. Nakanishi, T. Ide, and F. Ishikawa. 1998. Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. Nat Genet 18:65-68.
39. Nizami, Z., S. Deryusheva, and J. G. Gall. 2010. The Cajal body and histone locus body. Cold Spring Harb Perspect Biol 2:a000653.
40. Paeschke, K., T. Simonsson, J. Postberg, D. Rhodes, and H. J. Lipps. 2005. Telomere end-binding proteins control the formation of G-quadruplex DNA structures in vivo. Nat Struct Mol Biol 12:847-854.
41. Pinto, A. R., H. Li, C. Nicholls, and J. P. Liu. 2011. Telomere protein complexes and interactions with telomerase in telomere maintenance. Front Biosci 16:187-207.
42. Pogacic, V., F. Dragon, and W. Filipowicz. 2000. Human H/ACA small nucleolar RNPs and telomerase share evolutionarily conserved proteins NHP2 and NOP10. Mol Cell Biol 20:9028-9040.
43. Rouda, S., and E. Skordalakes. 2007. Structure of the RNA-binding domain of telomerase: implications for RNA recognition and binding. Structure 15:1403-1412.
44. Shay, J. W., and W. E. Wright. 2000. Hayflick, his limit, and cellular ageing. Nat Rev Mol Cell Biol 1:72-76.
45. Tejera, A. M., M. Stagno d'Alcontres, M. Thanasoula, R. M. Marion, P. Martinez, C. Liao, J. M. Flores, M. Tarsounas, and M. A. Blasco. 2010. TPP1 is required for TERT recruitment, telomere elongation during nuclear reprogramming, and normal skin development in mice. Dev Cell 18:775-789.
46. Tesmer, V. M., L. P. Ford, S. E. Holt, B. C. Frank, X. Yi, D. L. Aisner, M. Ouellette, J. W. Shay, and W. E. Wright. 1999. Two inactive fragments of the integral RNA cooperate to assemble active telomerase with the human protein catalytic subunit (hTERT) in vitro. Mol Cell Biol 19:6207-6216.

47. Tomlinson, R. L., T. D. Ziegler, T. Supakorndej, R. M. Terns, and M. P. Terns. 2006. Cell cycle-regulated trafficking of human telomerase to telomeres. Mol Biol Cell 17:955-965.

48. Venteicher, A. S., E. B. Abreu, Z. Meng, K. E. McCann, R. M. Terns, T. D. Veenstra, M. P. Terns, and S. E. Artandi. 2009. A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. Science 323:644-648.

49. Wang, F., E. R. Podell, A. J. Zaug, Y. Yang, P. Baciu, T. R. Cech, and M. Lei. 2007. The POT1-TPP1 telomere complex is a telomerase processivity factor. Nature 445:506-510.

50. Wen, J., Y. S. Cong, and S. Bacchetti. 1998. Reconstitution of wild-type or mutant telomerase activity in telomerase-negative immortal human cells. Hum Mol Genet 7:1137-1141.

51. Xin, H., D. Liu, M. Wan, A. Safari, H. Kim, W. Sun, M. S. O'Connor, and Z. Songyang. 2007. TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. Nature 445:559-562.

52. Ye, J. Z., D. Hockemeyer, A. N. Krutchinsky, D. Loayza, S. M. Hooper, B. T. Chait, and T. de Lange. 2004. POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. Genes Dev 18:1649-1654.

53. Zaug, A. J., E. R. Podell, J. Nandakumar, and T. R. Cech. 2010. Functional interaction between telomere protein TPP1 and telomerase. Genes Dev 24:613-622.

54. Zhong, F., S. A. Savage, M. Shkreli, N. Giri, L. Jessop, T. Myers, R. Chen, B. P. Alter, and S. E. Artandi. 2011. Disruption of telomerase trafficking by TCAB1 mutation causes dyskeratosis congenita. Genes Dev 25:11-16.

55. Zhu, Y., R. L. Tomlinson, A. A. Lukowiak, R. M. Terns, and M. P. Terns. 2004. Telomerase RNA accumulates in Cajal bodies in human cancer cells. Mol Biol Cell 15:81-90.

Chapter 2:

TIN2-Tethered

TPP1 Recruits Human Telomerase to Telomeres In Vivo

Eladio Abreu,[1] Elena Aritonovska,[2] Patrick Reichenbach,[2] Gaël Cristofari,[2] Brad Culp,[1] Rebecca M. Terns,[1] Joachim Lingner,[2] and Michael P. Terns[1]

Published Mol Cell Biol. (12):2971-82 2010 June 30. Reprinted here with permission of publisher

Abstract

Recruitment to telomeres is a pivotal step in the function and regulation of human telomerase; however, the molecular basis for recruitment is not known. Here, we have directly investigated the process of telomerase recruitment via fluorescence in situ hybridization (FISH) and chromatin immunoprecipitation (ChIP).We find that depletion of two components of the shelterin complex that is found at telomeres—TPP1 and the protein that tethers TPP1 to the complex, TIN2—results in a loss of telomerase recruitment. On the other hand, we find that the majority of the observed telomerase association with telomeres does not require POT1, the shelterin protein that links TPP1 to the single-stranded region of the telomere. Deletion of the oligonucleotide/oligosaccharide binding fold (OB-fold) of TPP1 disrupts telomerase recruitment. In addition, while loss of TPP1 results in the appearance of DNA damage factors at telomeres, the DNA damage response per se does not account for the telomerase recruitment defect observed in the absence of TPP1. Our findings indicate that TIN2-anchored TPP1 plays a major role in the recruitment of telomerase to telomeres in human cells and that recruitment does not depend on POT1 or interaction of the shelterin complex with the single-stranded region of the telomere.

Introduction

The physical ends of eukaryotic chromosomes, termed telomeres, are maintained by the cellular reverse transcriptase telomerase. Telomerase uses an internal RNA moiety as a template to add short telomeric repeats to the 3′ ends of chromosomes (18, 32). Telomeres protect chromosomes from nucleolytic degradation and inappropriate DNA repair reactions (38). In humans, telomerase is developmentally regulated and is expressed primarily during the first weeks of embryogenesis (10). Later in life, most normal human somatic cells express only very low levels of telomerase, and telomeres shorten with continuous cell division cycles due to the end replication problem and nucleolytic processing of chromosome ends. Upon reaching a critical length, short telomeres activate a DNA damage response that leads to a permanent cell cycle arrest or apoptosis (15). Reactivation of telomerase is a key requisite for human cancer cells to

attain unlimited proliferation potential (7). Telomere shortening suppresses tumor formation, but at the same time, the telomere reserve must be long enough to allow tissue renewal by healthy cells during the entire life span (27). Indeed, accelerated telomere shortening causes dyskeratosis congenita, a bone marrow failure syndrome that leads to premature death due to aplastic anemia (47). Telomere dysfunction has also been linked to the pathogenesis of idiopathic pulmonary fibrosis (2), ICF syndrome (55), and Werner syndrome (11).

The maturation and activity of telomerase depend on subcellular trafficking. A minimal, catalytically active telomerase enzyme (that can add telomeric repeats to the ends of DNA oligonucleotide substrates in vitro) can be formed by the telomerase reverse transcriptase (TERT) and the telomerase RNA moiety (TR) (3, 49). However, the human TERT (hTERT)-hTR core complex is not competent for telomere elongation in vivo. Within cells, hTR accumulates in Cajal bodies (CBs), subnuclear structures that also contain the subset of box H/ACA pseudouridylation guide RNAs termed small CB-specific RNAs (scaRNAs), which modify snRNAs (22, 56). hTR accumulation in Cajal bodies is not needed for assembly of the catalytic core of telomerase but is required to render telomerase competent for telomere association and extension in vivo (12, 46). The telomerase holoenzyme subunit TCAB1 (telomerase Cajal body factor 1) mediates the essential CB localization step (46). Telomere synthesis occurs during S phase, and hTR localizes to telomeres specifically during this phase of the cell cycle (23, 44). However, factors that function in the recruitment of telomerase to telomeres are not known.
The six-component telomere capping complex termed shelterin is important for telomere length control in vivo (38, 51), suggesting potential roles for the complex in the regulation of telomerase access to telomeres. Interestingly (and perplexingly), current evidence suggests that shelterin components can both inhibit and stimulate telomere elongation. The six shelterin components are TRF1, TRF2, RAP1, TIN2, POT1, and TPP1 (16, 51) (Fig. 4A). The shelterin complex associates with the double-stranded region of the telomere through direct interactions of TRF1 and TRF2 with the DNA (8, 16). POT1 binds the single-stranded region of the telomere (6, 29). Depletion of TRF1 leads to telomere elongation, and overexpression of TRF1 causes telomere shortening in human telomerase-positive cells without affecting in vitro-assayed telomerase activity

(41), suggesting that reinforcement of the shelterin complex inhibits telomerase function. Similarly, depletion of TPP1 by RNA interference (RNAi) or disruption of the TPP1-POT1 interaction (which are both accompanied by loss of the POT1 signal at telomeres) also results in telomere lengthening (33, 54). At the same time, however, several findings support positive roles of shelterin in telomere length regulation. In particular, TPP1 together with POT1 has been shown to improve telomerase activity and processivity in vitro (48) by slowing primer dissociation and aiding telomerase translocation (28). Dissection of the apparently opposing roles of the shelterin complex components in telomerase function awaits further investigation.

TPP1 has been hypothesized to play a role specifically in the recruitment of telomerase to telomeres based on its association with telomerase (52). Xin et al. demonstrated that tandem affinity purification (TAP)-tagged hTERT and glutathione S-transferase (GST)-tagged TPP1 copurify when fractionated from cellular extracts derived from cells coexpressing the tagged proteins (52). In addition, both GST-tagged TPP1 and the oligonucleotide/oligosaccharide binding fold (OB-fold) of TPP1 pull down in vitro-translated hemagglutinin (HA)-tagged TERT and telomerase activity (52), indicating that the TPP1 OB-fold is important for association of TPP1 with telomerase. It is not clear whether the interaction between TPP1 and hTERT is direct. These studies did not examine recruitment of telomerase to telomeres. However, based on their findings, the authors speculated that TPP1 together with POT1 could play a role in positively (and negatively) regulating access of telomerase to telomeres (52).

In this work, we have directly investigated the process of telomerase recruitment using fluorescence in situ hybridization (FISH) and immunofluorescence (IF) in parallel with quantitative chromatin immunoprecipitation (ChIP) to monitor the association of hTR and hTERT with telomeres. We find that short hairpin RNA (shRNA)-mediated depletion of TPP1 and TIN2, but not POT1, significantly reduces the presence of telomerase at telomeres. Our findings reveal that TPP1 bound to telomeres via TIN2 plays a key role in the accumulation of telomerase at telomeres.

Materials and Methods

Plasmids

shRNA vectors were prepared by cloning double-stranded DNA oligonucleotides into pSuper-Puro (4). The target sequences were as follows: TPP1, 5'-GACUUAGAUGUUCAGAAAA-3'; TIN2, 5'-GTGGAACATTTTCCGCGAGTACTGGAGT-3' (53); and POT1, 5'-GTACTAGAAGCCTATCTCA-3' (shRNA 1) and 5'-GGGTGGTACAATTGTCAAT-3' (shRNA 2). Full-length TPP1, TPP1 lacking the OB-fold (TPP1ΔOB) (52), and full-length TPP1 bearing two silent mutations in the shRNA target site (1575 A→G and 1581 T→A) were eptitope tagged (N-terminal and C-terminal 3xFlag from Sigma) and expressed from pcDNA6 (Invitrogen).

Cell culture and transfection.

Super-telomerase HeLa cells were generated as described previously (13). Cells were transfected using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Puromycin (1 μg/ml) (InvivoGen) was added to the medium 24 h after transfection of pSuper-Puro derivatives. Puromycin selection was maintained until mock-transfected cells were dead (approximately 3 days). For TPP1 shRNA- and TIN2 shRNA-treated cells, ChIP analysis was performed 4 days posttransfection. POT1 shRNA-treated cells were analyzed 6 days posttransfection. For FISH and IF, HeLa cells and super-telomerase HeLa (13) cells were grown on coverslips in Dulbecco modified Eagle medium (DMEM) (Fisher Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific). All cells were cultured at 37°C with 5% CO_2. Transfections were carried out using Lipofectamine 2000 transfection reagent, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were selected in 1 μg/ml puromycin (Sigma-Aldrich) for 48 h following transfection. In some cases, cells were synchronized to mid-S phase using double thymidine block as previously described (44) except that 18-h thymidine treatments were used. (Cells were released for 9 h between thymidine treatments.) Bromodeoxyuridine (BrdU) labeling was performed as described previously (44) to confirm S-phase synchronization.

Fluorescence in situ hybridization (FISH) and immunofluorescence (IF)

Three DNA probes (probes 1, 2, and 3), complementary to different regions of telomerase RNA, were used in hTR FISH (44). A fourth DNA probe, complementary to the G-rich strand of the telomere (CT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*A [T* indicates aminoallyl-modified thymidines]), was synthesized by Qiagen (Valencia, CA) and used to detect telomeres. Probes were conjugated with Cy3 or Oregon green monofunctional reactive dye according to the manufacturer's protocol (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom; Invitrogen). A 25-ng portion of each Cy3-labeled hTR FISH probe and/or 0.2 ng of telomere FISH probe was used per coverslip. FISH was performed essentially as described previously (44). However, when hTR FISH was performed in combination with BrdU or telomere FISH, the cells were subjected to a 10-min denaturation at 85°C in 70% formamide, 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) prior to FISH.

Following FISH, cells were analyzed by IF as described previously (44). Cells were washed three times with 1x phosphate-buffered saline (PBS) and blocked for 1 h in 0.05% Tween-20 in PBS (PBS-T) or 3% bovine serum albumin (BSA) in PBS. Next, cells were incubated with one of several combinations of the following primary antibodies at the indicated dilution for 1 h at room temperature: mouse anti-p80 coilin (1:5,000, Π) (1), mouse anti-TRF2 (1:1,000; Imgenex Corp., San Diego, CA), rabbit anti-hTERT (1:400; Rockland, Gilbertsville, PA), mouse anti-FLAG (1:500; Sigma-Aldrich, St. Louis, MO), rabbit anti-RAP1(1:2,000; Novus Biologicals, Littleton, CO), and rabbit anti-53BP1(1:500; Bethyl, Montgomery, TX). Cells were washed three times in 1x PBS and then incubated with secondary antibody (1:100 Cy2-conjugated goat anti-rabbit IgG [H+L], 1:100 Cy2-conjugated goat anti-mouse IgG [H+L], 1:100 Cy5-conjugated goat anti-mouse IgGγ, 1:100 Cy5-conjugated goat anti-rabbit IgG [H+L], 1:100 7-amino-4-methylcoumarin-3-acetic acid [AMCA]-conjugated goat anti-mouse IgG [H+L], or 1:100 AMCA-conjugated goat anti-rabbit IgG [H+L]) (all from Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. Primary antibodies were diluted in PBS-T or 3% BSA in PBS, while secondary antibodies were diluted in PBS-T

only. Cells were subjected to three final 1x PBS washes and mounted in Prolong Gold (Invitrogen).

Microscopy

Slides were analyzed using a Zeiss Axioskop 2 Mot Plus fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were acquired at 63x (Plan Apochromat objectives; numerical aperture, 1.4) using a cooled charge-coupled-device ORCA-ER digital camera (Hamamatsu Photonics, Bridgewater, NJ) and IPLab Spectrum software (BioVision Technologies, Inc., Exton, PA). Linear image adjustments were made when necessary using Adobe Photoshop and applied simultaneously to image groups. The colors depicted in the figures do not necessarily correspond to the "colors" of the fluorescent labels used in the experiment. All data are collected in gray scale and converted to the indicated colors using IPLab Spectrum and/or Adobe Photoshop software. Representative cells are shown in all microscopy figure panels. For quantitation of 53BP1 and POT1 IF data, images from treatment groups were normalized (to the same maximum) before analysis. Plots of average numbers of colocalizations observed per cell (one focal plane) show data obtained from 8 to 12 fields of cells for each treatment group processed in parallel on the same day. Error bars indicate standard errors calculated with N equal to the number of fields quantitated.

Chromatin immunoprecipitation.

ChIP assays were performed as described previously (13), with the following modifications. For immunoprecipitations, 25 µl of hTERT R484 rabbit serum (50), 2.5 µg mouse monoclonal TPP1 antibody (ACD; Abnova H00065057-M02), or 2 µg mouse monoclonal γH2AX antibody (Millipore 05-636) was used, and the mixtures were incubated for 6 h at 4°C with 50 µl of a 50% slurry of protein A/G-Sepharose beads (GE Healthcare). Telomeric DNA was detected as described previously (5). For detection of Alu sequences, a 5' ^{32}P-labeled oligonucleotide probe (5'-GTGATCCGCCCGCCTCGGCCTCCCAAAGTG-3') was used.

qRT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen). The isolated RNA fraction was treated with RNase-free DNase (Qiagen) and repurified with the TRIzol LS reagent (Invitrogen). For quantitative reverse transcriptase PCR (qRT-PCR), cDNA was prepared from 2 μg total RNA, using random primers and SuperScript III reverse transcription (Invitrogen) followed by qPCR on a 7900HT fast real-time PCR system (Applied Biosystems), using the PowerSYBR Green PCR master mix (Applied Biosystems). For PCR amplification of TIN2 cDNA, forward and reverse primers were 5'-GTCAGAGGCTCCTGTGGATT-3' and 5'-CAGTGCTTTCTCCAGCTGAC-3', respectively; POT1 cDNA was amplified with previously described primers (26). Serial dilutions of TIN2 and POT1 cDNAs were used to determine amplification efficiencies. TIN2 and POT1 quantities were normalized to the level of β-actin cDNA.

Immunoblots. A total of 5 x 10^4 cells (or 1 x 10^4 cells for FLAG immunoblots) were boiled for 5 min in Laemmli loading buffer and fractionated on 4 to 20% SDS-polyacrylamide gradient gels (Lonza) except for TIN2 (10% polyacrylamide gel) and POT1 (8% polyacrylamide gel). Standard immunoblot protocols were used with the following antibodies: mouse monoclonal TPP1 antibody, ACD Abnova H00065057-M02 (1:1,000); rabbit polyclonal TIN2C 701 antibody, a kind gift from S. Smith (1:1,000); rabbit polyclonal hPOT1 978 antibody, a kind gift from T. de Lange (1:1,000); rabbit polyclonal hTERT antibody, Rockland 600-401-252 (1:2,500); rabbit polyclonal CENP-A antibody, Upstate 07-240 (1:2,000); mouse monoclonal γ-H2AX antibody, Millipore 05-636 (1:2,000); and mouse monoclonal FLAG M2 antibody, Sigma F3165 (1:5,000). For POT1 immunoblots, guanidine renaturation was performed as described previously (34). Secondary horseradish peroxidase-conjugated goat antibodies against rabbit or mouse IgG (1:3,000; Promega) were used to reveal the primary antibodies. The AlphaInnotech chemiluminescence substrate and imaging system was used for signal detection and quantification.

Coimmunoprecipitation

Coimmunoprecipitation of endogenous TPP1 and POT1 proteins was performed in super-telomerase HeLa cells transfected with two different shRNAs against POT1. Precleared

cell lysates from 10^6 cells were prepared as previously described (34). Lysates were immunoprecipitated with 10 μg mouse monoclonal TPP1 antibody ACD Abnova H00065057-M02, and immune complexes were bound to a 50% slurry of protein G-Sepharose beads. After an overnight incubation at 4°C, beads were washed four times with lysis buffer (34) and proteins were eluted with Laemmli loading buffer for analysis by 8% SDS-PAGE.

Real-time quantitative telomeric repeat amplification protocol (RQ-TRAP).

Telomerase activity was measured as previously described (13), with the following modifications. Reaction mixtures containing the PowerSYBR Green PCR master mix (Applied Biosystems), 1.8 μg undiluted or 3-fold-diluted cell extracts, 1 μM telomerase primer TS, 0.3 μM reverse primer ACX, and 0.5 mM $MgCl_2$ were incubated for 30 min at 30°C and for 10 min at 95°C. Using the 7900HT fast real-time PCR System (Applied Biosystems), samples were amplified in 40 PCR cycles for 15 s at 95°C and 1 min at 60°C. Threefold serial dilutions of the empty-vector-transfected samples were used to obtain a standard curve of the form $\log_{10}$ (protein quantity) = $aC_T + b$, where C_T is the threshold constant, a is the slope of the curve, and b is the y intercept. Telomerase activity was expressed relative to this standard as the quantity of standard sample extract giving the same C_T value. All samples were serially diluted to verify the linearity of the RQ-TRAP reaction and heat inactivated to verify what the amplification product was.

Results

TPP1 depletion results in loss of association of telomerase with telomeres.

The recruitment of telomerase to telomeres is essential for telomere maintenance; however, the mechanism of recruitment is not known. Association of telomerase with telomeres can be observed in cancer cells during S phase by FISH with oligonucleotide probes complementary to hTR or IF with hTERT antibodies (12, 22, 23, 43, 44, 46, 56).

Analysis of telomerase recruitment is facilitated by the use of so-called super-telomerase cells, which concomitantly overexpress hTERT and hTR, allowing detection of telomerase association with telomeres by ChIP as well as by microscopy in all phases of the cell cycle (12, 13). In order to identify proteins that are necessary for recruitment of telomerase to telomeres, we depleted candidate recruitment factors using shRNAs in super-telomerase HeLa cells and examined the localization of telomerase by FISH and ChIP analysis. We found that shRNA-induced depletion of the shelterin component TPP1 (Fig. 5A) caused a striking loss of telomerase localization to telomeres as assessed by FISH (Fig. 4) and ChIP (Fig. 6)

In untreated super-telomerase cells, hTR (visualized by FISH) is found at the Cajal bodies (visualized via the Cajal body marker protein coilin) and numerous telomeres (visualized via the telomere-binding protein TRF2) (Fig. 4B, −shTPP1; a subset of the hTR-telomere colocalizations is indicated with arrowheads). Following TPP1 depletion, hTR remains at Cajal bodies but is found only at very few telomeres (Fig. 4B and C, +shTPP1). TPP1 depletion reduced the number of observed hTR-telomere colocalizations by 77%, from a mean of 3.5 ± 0.3 (standard error of the mean [SEM]) colocalizations per cell (one focal plane) in parental super-telomerase cells to 0.8 ± 0.1 (SEM) after TPP1 depletion (Fig. 4C). hTERT localization to telomeres was also noticeably reduced by depletion of TPP1 (Fig. 4B), lower panels; a subset of hTR-hTERT-telomere colocalizations is indicated with arrowheads). At the same time, progression through the cell cycle (assessed by percentage of cells found in S phase), cellular telomerase levels (assayed in vitro by RQ-TRAP), and hTERT protein levels (assayed by immunoblot analysis) were not affected by TPP1 depletion (Fig. 5A, data not shown). These results suggest that TPP1 is necessary for the recruitment of telomerase from Cajal bodies to telomeres in super-telomerase cells. Importantly, TPP1 depletion results in a similar loss of hTR localization to telomeres during S phase in standard HeLa cells (not expressing exogenous telomerase) (Fig. 4D and E; telomeres are detected with a DNA probe; a subset of hTR-telomere colocalizations is indicated with arrowheads). TPP1 depletion reduced the number of hTR-telomere colocalizations by 68%, from a mean of 0.41 ± 0.16 (SEM) per cell to 0.13 ± 0.08 (SEM) after TPP1 depletion (Fig. 4E).

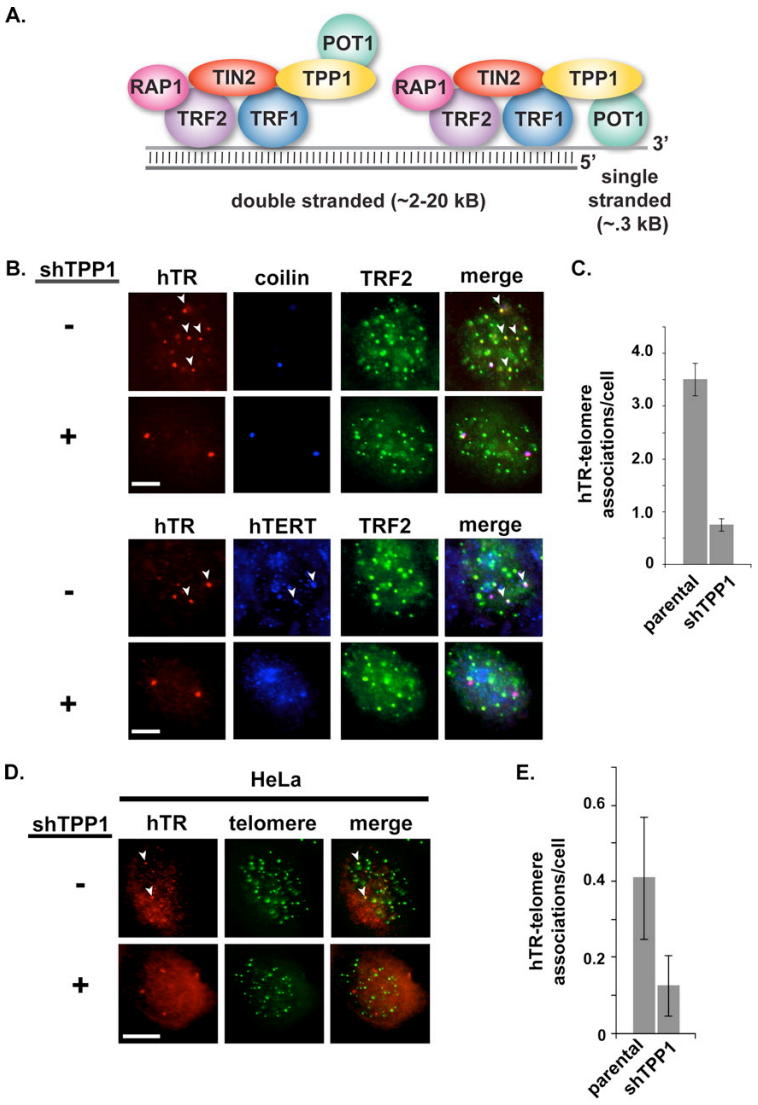


FIGURE 4. TPP1 depletion results in loss of localization of telomerase to telomeres (assessed by FISH). (A) Mammalian chromosome end structure is regulated by a complex of six core telomere-associated proteins (indicated) that make up the shelterin complex (16, 51). TPP1 is associated with the double-stranded and single-stranded portion of telomeres via direct interactions with TIN2 and POT1, respectively (16, 51). (B) Fluorescence *in situ* hybridization (FISH) was used to detect hTR (red), and immunofluorescence (IF) was used to detect TRF2 (telomere marker, green) and hTERT (blue) or coilin (Cajal body marker, blue) in parental (–) and TPP1-depleted (+) super-telomerase HeLa cells. Cells were imaged by

fluorescence microscopy. Merge panels in all microscopy figures show superimposition of the individual panels. A subset of hTR (hTERT) colocalizations with telomeres is indicated with arrowheads. Scale bars in all microscopy panels represent 10 µm. (C) The average number of hTR-telomere associations per cell (one focal plane) detected by FISH/IF in parental and TPP1-depleted cells represented in panel B is shown. Error bars in plots of localization data in all figures indicate standard errors (see Materials and Methods). (D) Parental (–) and TPP1-depleted (+) HeLa cells (no exogenous telomerase expression) were synchronized to mid-S phase during drug selection. hTR (red) and telomeres (green) were detected by FISH. hTR colocalizations with telomeres are indicated with arrowheads. (E) The average number of hTR-telomere associations per cell (one focal plane) detected by FISH/IF in parental and TPP1-depleted cells represented in panel D is shown.

We also examined telomerase recruitment to telomeric DNA by ChIP in super-telomerase HeLa cells (Fig. 6). ChIP relies on formaldehyde-mediated covalent linkage of proteins to DNA and therefore reflects close physical as well as spatial associations of proteins and DNA. TPP1 was depleted (Fig. 5), and the association of telomerase with telomeres (and Alu repeats) was assessed by IP with hTERT antibodies. As expected, little telomeric DNA was immunoprecipitated with TPP1 antibodies upon depletion of TPP1 (Fig. 6A). In addition, however, immunoprecipitation of telomeric DNA with hTERT antibodies was reduced by 36% when cellular TPP1 was depleted (Fig.5B). The small degree of association of hTERT observed with Alu-repeat DNA, which served as a negative control, was not affected by TPP1 depletion. Thus, both FISH and ChIP analyses implicate TPP1 as a telomerase recruitment factor.

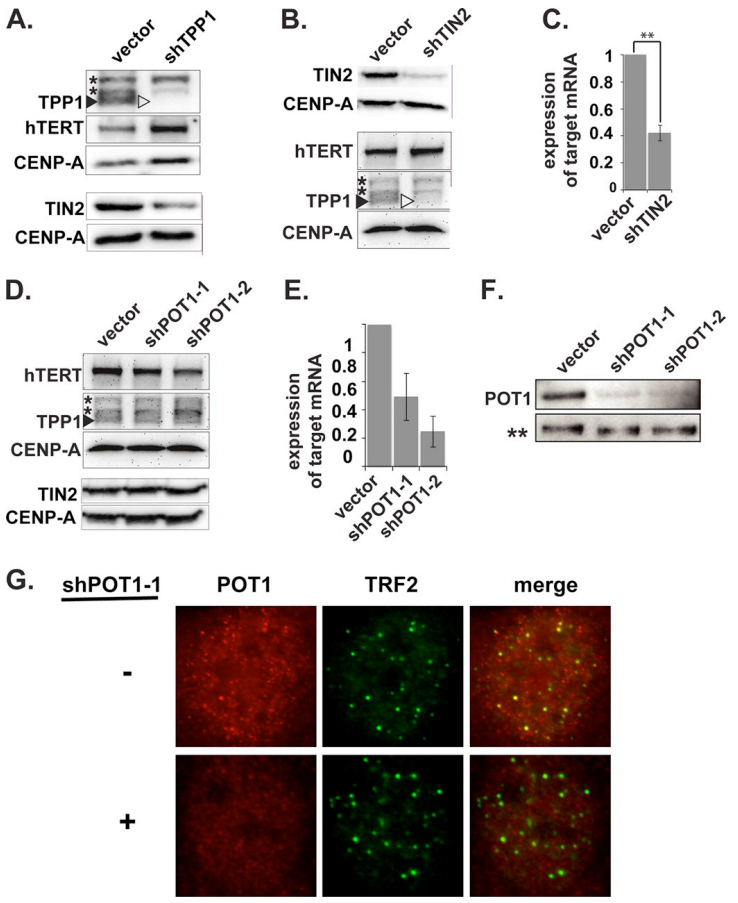


FIGURE 5. Depletion of TPP1, TIN2, and POT1. (A) Immunoblot analysis of TPP1-depleted cells. Super-telomerase HeLa cells were transfected with pSuper-Puro (vector) or pSuper-Puro-TPP1 shRNA vector (shTPP1). Four days after transfection, TPP1, hTERT, TIN2, and CENP-A expression was analyzed by immunoblotting. The arrowheads indicate the position of endogenous TPP1 protein. Asterisks indicate nonspecific bands. CENP-A was used as a loading control. (B) Immunoblot analysis of TIN2-depleted cells. Four days after transfection, protein expression was analyzed as described for panel A. (C) qRT-PCR analysis of TIN2-depleted cells. qRT-PCR detection of mRNA levels of TIN2 in super-telomerase HeLa cells, 4 days after transfection with TIN2-shRNA, relative to empty-vector control. Error bars correspond to standard deviations of results of three independent experiments. Statistical analyses were done using a two-tailed Student's *t* test (, $P < 0.01$). (D) Immunoblot analysis of POT1-depleted cells. Six days after**

transfection, protein expression was analyzed as described for panel A. (E) qRT-PCR analysis of POT1-depleted cells. qRT-PCR showing mRNA levels of POT1 in super-telomerase HeLa cells, 6 days after transfection with two different POT1-shRNAs, relative to empty-vector control. Error bars correspond to standard deviations of results of two independent experiments. (F) IP/immunoblot analysis of POT1-depleted cells. Coimmunoprecipitation of endogenous POT1 with TPP1 in POT1-depleted cells. TPP1-immunoprecipitated complexes from super-telomerase HeLa cells transfected with the indicated plasmids were resolved by 8% SDS-PAGE. Immunoblot antibodies are indicated on the left. No detection of TPP1 was observed in the supernatant fraction after IP (data not shown). A nonspecific band recognized by the TPP1 antibody in the IP fraction (**) served as a loading control. (G) Direct IF analysis of POT1-depleted cells. POT1 (red) and TRF2 (green) were detected by IF in parental (–) and POT1-depleted cells.

POT1 is not required for association of telomerase with telomeres, but depletion of TIN2 results in reduced association.

TPP1 interacts with telomeres via two proteins: TIN2, which binds to the double-stranded telomere-bound TRF1 and TRF2 proteins, and POT1, which mediates interaction with the single-stranded region of the telomere (16, 51) (see Fig. 4A). We found that TPP1 depletion reduced TIN2 protein levels (Fig. 5A), suggesting that the recruitment defect that we observed in TPP1-depleted cells may require TIN2. At the same time, a current model suggests that TPP1 functions with POT1 to recruit telomerase to telomeres (52). To further investigate the mechanism of TPP1-mediated telomerase recruitment, we depleted TIN2 or POT1 using shRNAs (Fig. 5B to G). ChIP analysis indicated that association of hTERT with telomeres was reduced 43% upon depletion of TIN2 (Fig. 7A and B), similar to the reduction observed with TPP1 depletion (Fig. 6). However, immunoblot analysis revealed that depletion of TIN2 also resulted in a reduction in TPP1 protein levels (but not telomerase activity or hTERT protein levels) (Fig. 5B and data not shown). On the other hand, POT1 depletion did not detectably change TPP1 levels (Fig. 5D). In addition, POT1 depletion did not disrupt hTERT association with telomeres in ChIP analysis (Fig. 7A and B).

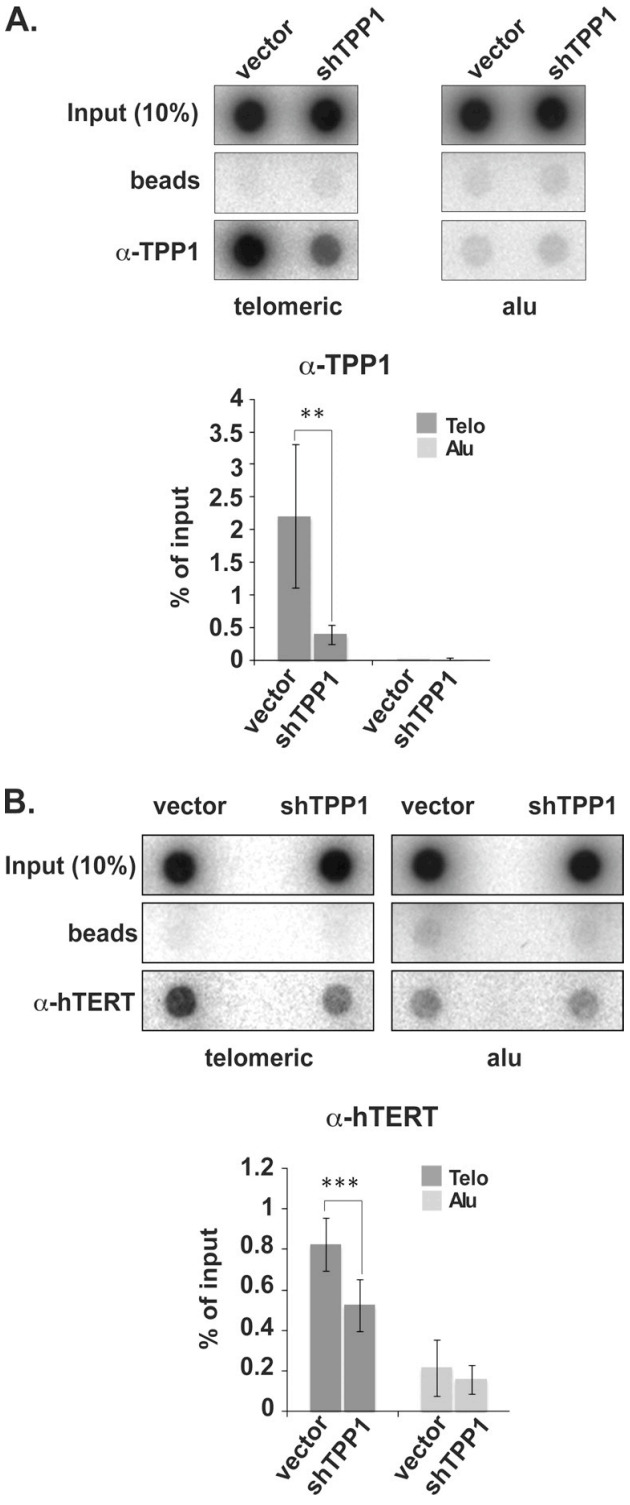


FIGURE 6: depletion results in loss of physical association of hTERT with telomeres (assessed by ChIP). (A) ChIP of telomeric and Alu DNA with TPP1-

specific (A) and hTERT-specific (B) antibodies in super-telomerase HeLa cells. The percentage of telomeric and Alu DNA recovered in each ChIP is indicated. Error bars correspond to standard deviations of results of three (hTERT ChIP) and five (TPP1 ChIP) independent experiments. Statistical analyses were done using a two-tailed Student's *t* test (*, $P < 0.001$; **, $P < 0.01$).**

FISH analysis of hTR also showed a marked difference in the effects of TIN2 and POT1 depletion. hTR-telomere colocalization with TIN2 depletion was reduced to an extent similar to that observed with TPP1 depletion (Fig. 7C and D). Following TIN2 depletion, hTR-telomere colocalizations decreased by 69%, from a mean of 3.5 ± 0.3 (SEM) per cell to 1.1 ± 0.3 (SEM) per cell (Fig. 7D). However, POT1 depletion did not reduce the colocalization of hTR with telomeres (Fig. 7C and E). The results indicate that POT1 and association with the single-stranded region of the telomere (see Fig. 4A) are not required for the association of the majority of telomerase with telomeres and suggest a role for TIN2-tethered TPP1 in recruitment.

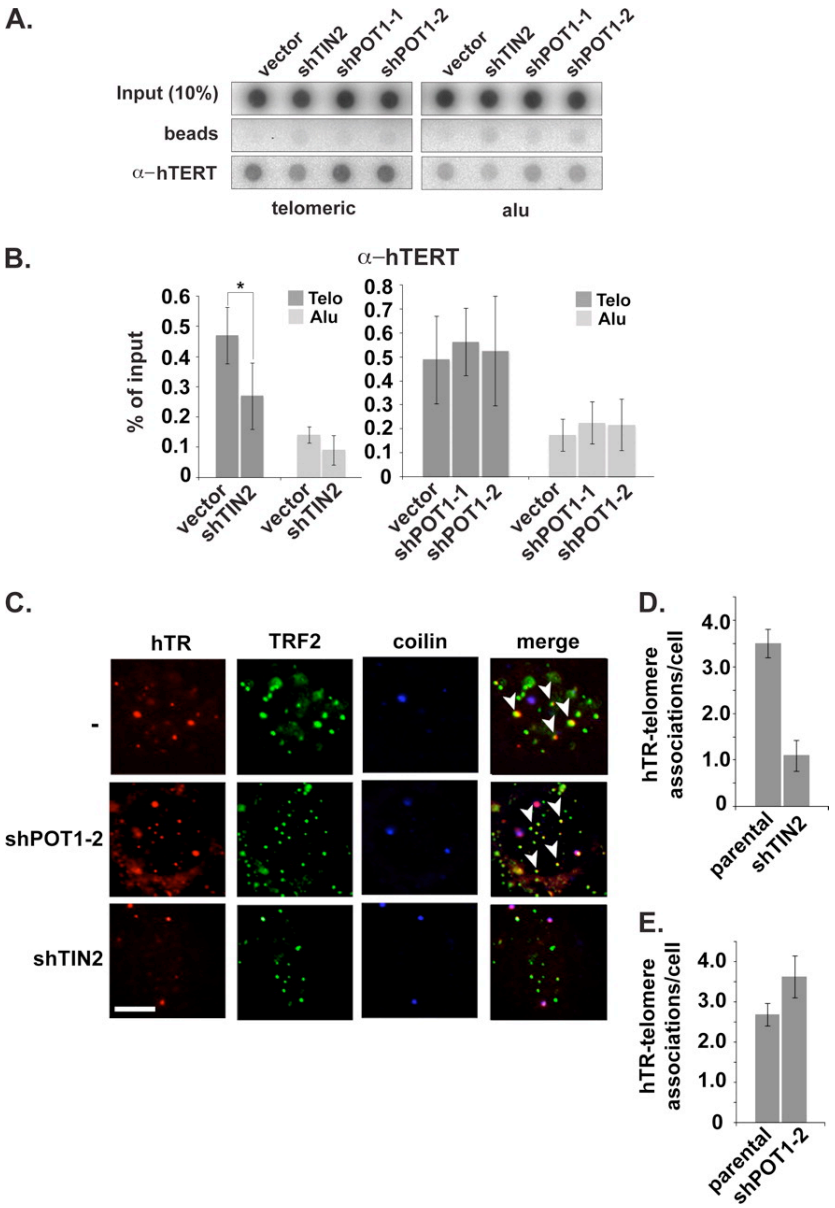


FIGURE 7. Telomerase recruitment depends on TIN2 but not POT1. (A) ChIP of telomeric and Alu DNA with hTERT antibody. (B) Quantification of ChIP data shown in panel A. Error bars correspond to standard deviations of results of three (TIN2 data) and two (POT1 data) independent experiments. Statistical analyses were done using a one-tailed Student's *t* test (*, $P < 0.05$). (C) hTR (red) was detected by FISH and TRF2 (green) and coilin (blue) were detected by IF in parental (–) and POT1- or TIN2-depleted cells. hTR colocalizations with telomeres are indicated with arrowheads. (D and E) The average number of hTR-telomere

associations per cell (one focal plane) detected by FISH/IF in parental and POT1- or TIN2-depleted cells represented in panel C is shown.

The OB-fold of TPP1 is required for association of telomerase with telomeres.

In order to further investigate the function of TPP1 in telomerase recruitment, we rescued the shRNA-mediated depletion of endogenous TPP1 by expression of FLAG epitope-tagged TPP1. Control (empty vector) and rescue (TPP1) plasmids were cotransfected along with the TPP1 shRNA-encoding plasmid. Expression of endogenous and exogenous TPP1 was assessed by immunoblot analysis with TPP1 and FLAG antibodies 4 days following transfection (Fig. 8A). The reduction in association of telomerase with telomeres observed with TPP1 depletion by ChIP analysis was not rescued by expression of an shRNA-sensitive TPP1 gene (Fig. 8B and C); in both cases, the telomeric DNA precipitated with hTERT antibody was approximately 50% of the shRNA-negative control. However, expression of an shRNA-resistant TPP1 gene (TPP1*, shRNA mRNA recognition site destroyed without altering the protein sequence) encoding full-length FLAG-tagged TPP1 fully restored telomerase association, indicating that the phenotype is related to TPP1 depletion. In order to test the potential role of the N-terminal OB-fold domain of TPP1 (required for coprecipitation of telomerase and TPP1 in pulldown experiments) (52) in recruitment to telomeres, we introduced an shRNA-resistant truncated version of TPP1 (TPP1ΔOB*). TPP1 lacking the OB-fold did not rescue telomerase recruitment assessed by ChIP (Fig. 8B and C).

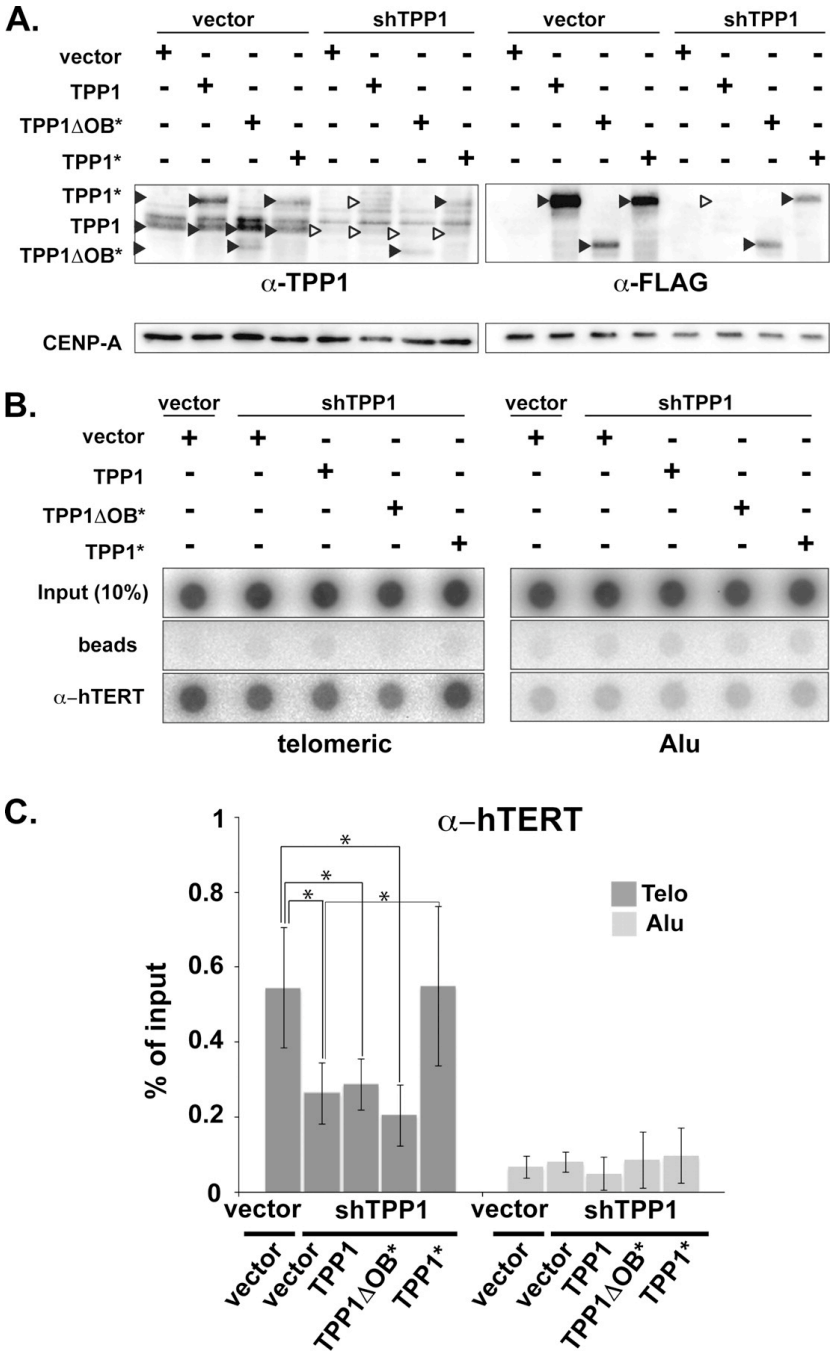


FIGURE 8. Human telomerase is recruited to telomeres via the OB-fold of TPP1. (A) Immunoblot analysis of ectopically expressed FLAG epitope-tagged full-length TPP1 (TPP1), FLAG-tagged, shRNA-resistant full-length TPP1 (TPP1*), and FLAG-tagged, shRNA-resistant TPP1 lacking the OB-fold (TPP1ΔOB*). Super-telomerase HeLa cells were cotransfected with the indicated plasmids, and protein

expression was analyzed 4 days after transfection. Black arrowheads indicate the presence of the respective TPP1 proteins, and white arrowheads indicate the lack of expression. Immunoblots were probed with anti-TPP1, anti-FLAG and CENP-A antibodies as indicated. (B) ChIP of telomeric and Alu DNA with hTERT antibody from cells in panel A. (C) Quantification of data in panel B representing percentage of telomeric and Alu DNA recovered in hTERT ChIP. Error bars correspond to standard deviations of results of four independent experiments. Statistical analyses were done using a two-tailed Student's *t* test (*, $P < 0.05$).

At the cellular level, we also observed that cotransfection of shRNA-resistant full-length TPP1 (TPP1*) restored localization of hTR to telomeres (localized via telomere-binding protein TRF2 or RAP1) (Fig. 9A). The FLAG-tagged TPP1 localized to telomeres, including those where hTR was found (Fig. 9A; see arrowheads, TPP1*). Expression of the shRNA-resistant TPP1 increased the number of hTR-telomere colocalizations from a mean of 0.7 ± 0.1 (SEM) per cell, observed in TPP1-depleted cells, to 2.4 ± 0.4 (SEM) per cell, similar to the 2.1 ± 0.3 (SEM) colocalizations per cell observed in parental super-telomerase cells in this experiment (Fig. 9B). On the other hand, TPP1 lacking the OB-fold domain was unable to rescue telomerase recruitment (0.3 ± 0.1 [SEM] hTR-telomere colocalizations per cell) (Fig. 9B), despite the fact that the TPP1ΔOB protein localized to telomeres (see FLAG and RAP1 in Fig. 9A). These results suggest that the association of TPP1 and telomerase, which is mediated by the OB-fold of TPP1 (52), functions in recruitment of telomerase to telomeres.

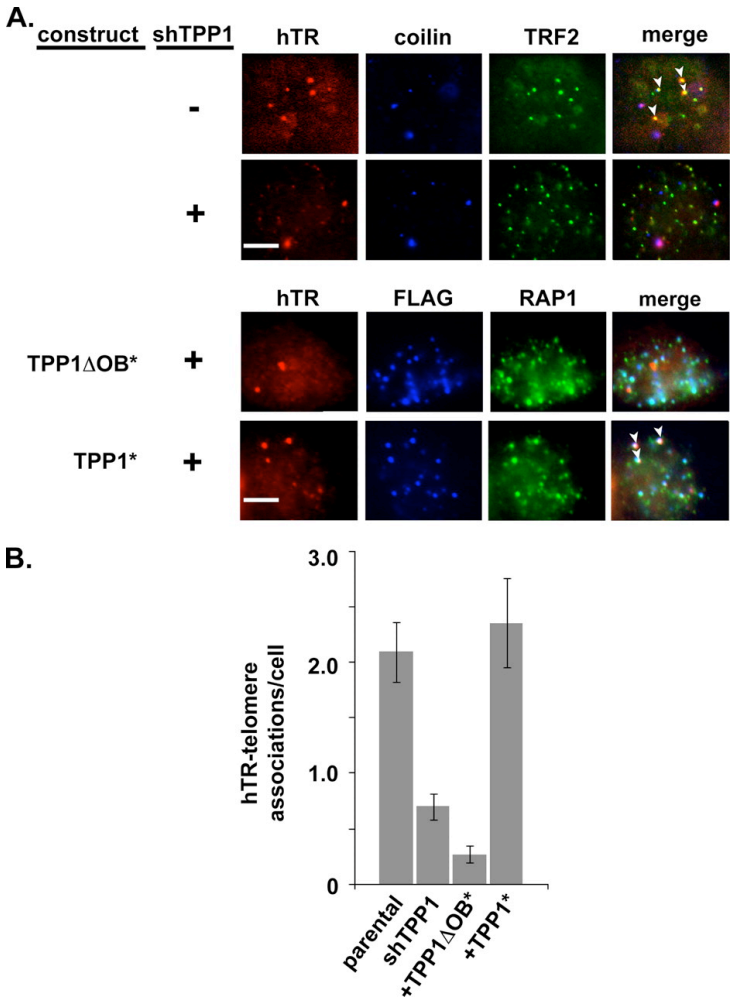


FIGURE 9. The TPP1 OB-fold is required to rescue telomerase recruitment to telomeres. An shRNA-resistant form of TPP1 is able to restore hTR localization to telomeres in TPP1-depleted cells. However, an shRNA-resistant form of TPP1 lacking the OB-fold cannot restore localization. (A) Parental and TPP1-depleted super-telomerase HeLa cells were subjected to FISH and IF to detect hTR (red), coilin (blue), and TRF2 (green). Merge panels show superimposition of hTR, coilin, and TRF2. Next, parental cells were cotransfected with shTPP1 and either TPP1* or TPP1ΔOB*. Treated cells were subjected to FISH and IF to detect hTR (red), FLAG (blue), and RAP1 (telomere marker, green). Merge panels show superimposition of hTR, FLAG, and RAP1. (B) Plot of the average number of telomere-associated hTR foci per cell in the parental cells and each experimental

group. Error bars indicate standard errors calculated with *N* equal to the number of samples quantitated.

TIF formation does not impair association of telomerase with telomeres.

Previous studies demonstrated that depletion of TPP1 activates a DNA damage response marked by the formation of telomere dysfunction-induced foci (TIFs) at telomeres (19, 20, 52). TIF formation can be detected via the association of DNA damage proteins, such as γ-H2AX and 53BP1, with telomeres (42). Accordingly, our ChIP analysis of telomeric DNA with antibodies against the DNA damage marker γ-H2AX revealed a 7-fold increase in γ-H2AX at telomeres when TPP1 levels are reduced (Fig. 10A and B) (without a detectable change in cellular γ-H2AX levels; Fig. 10C). In addition, we found the DNA damage marker 53BP1 at telomeres in 29% ± 8% (SEM) of cells by IF when TPP1 was depleted, relative to 0.4% ± 0.4% (SEM) of untreated cells in our experiments (Fig. 10D) (scoring threshold = 7 or more 53BP1-telomere colocalizations per cell, similar to that used by others [17, 24]). These findings support the previous observations of TIF formation in response to TPP1 depletion and suggest general effects of TPP1 knockdown on telomere composition that could lead to the observed loss of telomerase recruitment. However, additional observations indicate that TIF formation per se does not account for the loss of telomerase recruitment observed in the absence of TPP1.

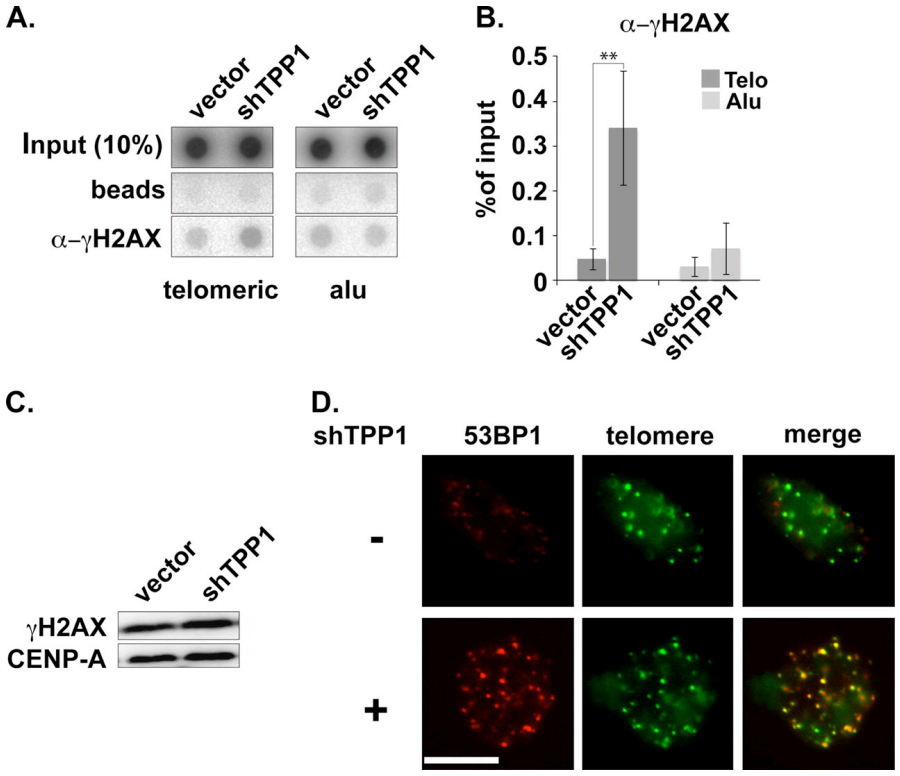


FIGURE 10. DNA damage response at telomeres following TPP1 depletion. (A) ChIP of telomeric and Alu DNA with γ-H2AX antibodies. Transfected plasmids are indicated. (B) The graph represents the quantification of the dot blot indicating the percentages of telomeric and Alu DNA recovered with γ-H2AX antibodies. Error bars correspond to standard deviations of four independent experiments. Statistical analyses were done using a two-tailed Student's *t* test (, $P < 0.01$). (C) Immunoblot was probed with anti-γ-H2AX and CENP-A antibodies following transfection of super-telomerase HeLa cells with empty vector or TPP1 shRNA construct as indicated. (D) TPP1-depleted cells were subjected to FISH and IF to label for telomeres (green) and 53BP1 (TIF marker, red). Merge panels show superimposition of telomeres and 53BP1 (colocalizations are indicated by yellow).**

Depletion of POT1 (and TIN2) also leads to a DNA damage response at telomeres (21, 25), evidenced by an increase in γ-H2AX association with telomeric DNA by ChIP analysis (6-fold increase in TIN2-depleted cells and 8- to 10-fold increase in POT1-

depleted cells) (Fig. 11). However, the DNA damage response associated with POT1 depletion does not disrupt recruitment of telomerase to telomeres (Fig. 7). In addition, we found that TIFs remained in our cells rescued with the wild-type TPP1 construct. ChIP analysis of γ-H2AX suggests that the TIF formation that occurred with TPP1 depletion was only partially rescued by the full-length TPP1 (or by the TPP1ΔOB protein) under

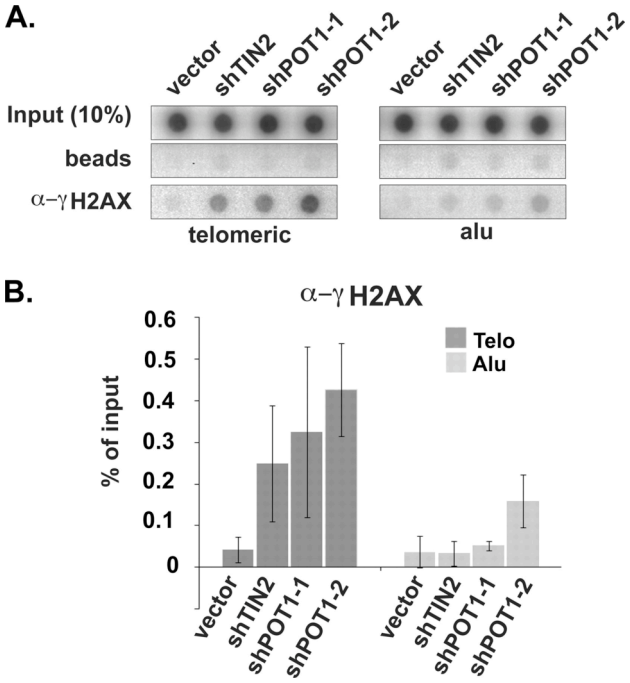


FIGURE. 11. Increased DNA damage response at telomeres upon TIN2 or POT1 depletion. (A) ChIP of telomeric and Alu DNA with γ-H2AX antibodies. (B) The graph represents the quantification of the dot blot. Error bars correspond to standard deviations of results of two independent experiments.

the conditions of the experiment (TPP1*; Fig. 12A and B). (The incomplete suppression of TIFs may reflect lower expression levels of transgenic TPP1 than of endogenous TPP1 (Fig. 8A, lanes 1 and 8) Moreover, in microscopy experiments, 53BP1 was found at telomeres in 19% ± 6% (SEM) of cells rescued with full-length TPP1 (Fig. 12C) where telomerase recruitment was restored (Fig. 8 and 9) (compared to 0.4% ± 0.4% [SEM] of untreated cells), suggesting that the loss of telomerase recruitment is not a result of

secondary effects of TPP1 knockdown on telomere structure. In fact, telomerase was observed at the same telomeres as the TIF marker protein 53BP1 in the rescued cells (Fig. 12C), clearly indicating that TIF formation does not prevent recruitment of telomerase. At the same time, TIFs (indicated by the presence of γ-H2AX or 53BP1 at telomeres) were present in cells depleted of TPP1 (Fig. 10) or expressing the TPP1ΔOB protein (Fig. 12), indicating that the DNA damage response at telomeres also does not stimulate telomerase recruitment in the absence of intact TPP1. The results indicate that the association of telomerase with telomeres depends on TPP1 and, in particular, on the OB-fold domain of TPP1.

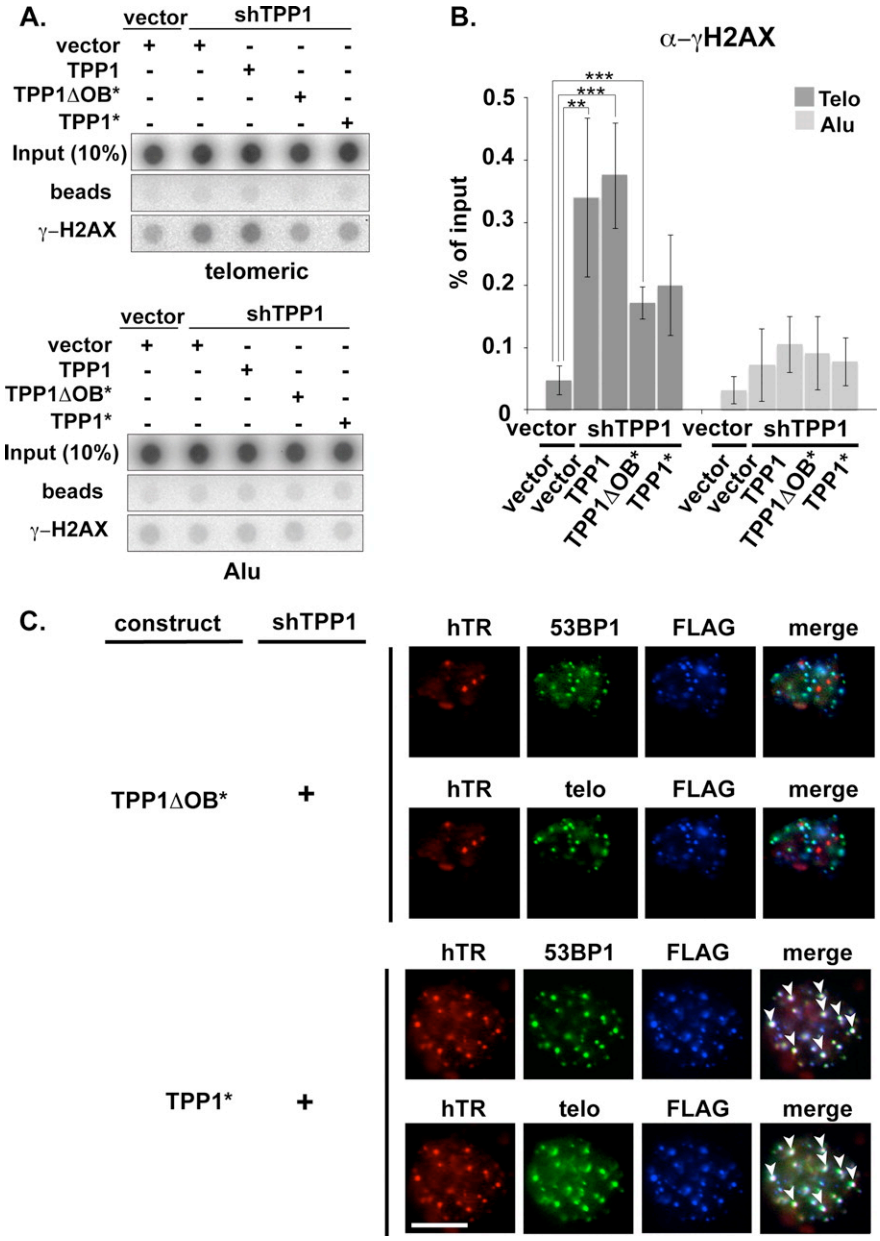


FIGURE 12. The presence of TIFs (telomere dysfunction-induced foci) does not impact the ability of TPP1 to rescue telomerase associations with telomeres. (A) ChIP of telomeric and Alu DNA with γ-H2AX antibodies. Transfected plasmids are indicated. Expression of TPP1* and TPP1ΔOB* partially rescues TIF formation observed in TPP1-depleted cells. (B) The graph represents the quantification of the dot blot indicating the percentages of telomeric and Alu DNA recovered with γ-H2AX antibodies. Error bars correspond to standard deviations of results of four

independent experiments. Statistical analyses were done using a two-tailed Student's *t* test (*, $P < 0.001$; **, $P < 0.01$). (C) Although TIFs were detected in TPP1-depleted cells coexpressing TPP1* or TPP1△OB*, TIFs did not inhibit rescue of hTR recruitment to telomeres by TPP1*. Super-telomerase HeLa cells were cotransfected with shTPP1 and either TPP1* or TPP1△OB*. Treated cells were subjected to FISH and IF to label for hTR (red), FLAG (blue), 53BP1 (green), and telomeres (green). Merge panels show superimposition of hTR, 53BP1, and FLAG or hTR, telomeres, and FLAG.**

Discussion

It is now clear that one primary mechanism for the regulation of telomerase activity is through regulated intracellular trafficking of the enzyme (23, 43, 44). Accumulation of hTR in Cajal bodies is mediated by TCAB1 and is required to render telomerase competent for association with telomeres in the S phase of the cell cycle (12, 46). The factors responsible for the recruitment of telomerase to telomeres have remained unidentified. In this study, using combined FISH and ChIP analysis, we have determined that depletion of shelterin proteins TPP1 and TIN2 (but not POT1) prevents association of telomerase with telomeres (Fig. 4 and 6). These findings indicate that the majority of telomerase is recruited to telomeres by TPP1, which is bound to telomeres via TIN2 in humans and likely other vertebrates (see Fig. 1A).

TPP1 could theoretically function in telomerase recruitment specifically when bound to the single-stranded 3′ region of the telomere via POT1 (14, 16, 38, 51) (Fig. 1A). Indeed, TPP1 has been speculated to function with POT1 to recruit telomerase (52). However, we did not detect significant changes in telomerase recruitment upon depletion of POT1 (Fig. 7), indicating that interaction with POT1 and the single-stranded end of the telomere is not required for recruitment of telomerase to telomeres by TPP1. At the same time, our results do not exclude the possibility that POT1 also plays an important role (positive or negative) in telomerase recruitment. The single-stranded 3′ overhang of the telomere is generally much less extensive (typically 0.1 to 0.3 kb) than the double-

stranded tract (typically 2 to 20 kb), and thus the fraction of telomerase that may be present at the 3′ overhang would be expected to be small relative to that bound to the double-stranded part of the telomere. A specific change in telomerase levels at the single-stranded end may be difficult to detect above the background of telomerase associated with the rest of the telomere. Logically, the association of telomerase with the single-stranded region of the telomere is important for telomere elongation. Recruitment of telomerase to the double-stranded part of the telomere may anticipate its catalytic action at the 3′ end, perhaps by increasing the local concentration of telomerase. Importantly, the previously described S-phase-specific trafficking of telomerase to telomeres (23, 43, 44) is TIN2-TPP1 dependent (Fig. 4D and E), indicating that TIN2-TPP1-mediated recruitment is regulated by the cell cycle. While we cannot formally exclude an independent role for TPP1 and/or TIN2 in telomerase recruitment (since depletion of one is accompanied by reduction of the other (Fig. 5A and B), current knowledge about the organization of the shelterin complex supports cofunction in the form of TIN2-anchored TPP1 (16, 51). The significance of telomerase association with telomeres via TIN2-TPP1 is indicated by the recent identification of TIN2 mutations in patients suffering from the short telomere disease dyskeratosis congenita (39). Our findings suggest that inefficient telomerase recruitment might contribute to the pathogenesis of dyskeratosis congenita in these patients. It is not yet known whether additional factors are involved in TIN2-TPP1-mediated recruitment of telomerase to telomeres. It was previously demonstrated that TPP1 associates with telomerase in cell extracts (52); however, it is not clear whether TPP1 interacts directly with telomerase.

The results presented here support the emerging view that certain shelterin components act as both negative and positive regulators of telomerase function (14, 40, 48, 52). While collectively the shelterin proteins inhibit telomerase-telomere interactions, evidence indicates that particular telomere-associated proteins can also interact with and recruit telomerase. These proteins include Cdc13 (Saccharomyces cerevisiae), TEBP-beta (ciliates), and TPP1 (humans and other vertebrates [this study]) (9, 31, 35-37, 48, 52). Recent studies indicate that dynamic phosphorylation of these proteins switches them between negative and positive regulation of telomerase recruitment by modulating the ability of the protein to interact with specific partner proteins. For example, in yeast,

phosphorylation of Cdc13 by CDK1 favors an interaction with the Est1 subunit of telomerase (telomerase recruitment) over interaction with Stn1/Ten1 proteins (end protection) (30, 45). Likewise, in ciliates, phosphorylation of TEBP-beta stimulates the function of the protein in telomerase recruitment and interferes with formation of a heterodimer with TEBP-alpha, which functions in telomere protection (36). TPP1 is a structural homolog of the ciliate TEBP-beta protein (48, 52) and contains a conserved serine-rich domain with several predicted Cdk2 phosphorylation sites (48, 52). Our findings establish TPP1 as a central factor in telomerase recruitment in humans. While depletion of TPP1 reduces telomerase recruitment (this study), it can also lead to telomerase-mediated telomere extension (33, 54), suggesting that TPP1 is also poised to function as part of telomerase repressing and activating complexes in humans. To fully understand the mechanisms that underlie telomerase recruitment and understand the positive and negative roles of TPP1, POT1, and other shelterin components in telomerase regulation, it will be important to delineate the various telomeric states, identify the components of telomerase that associate with TPP1 during recruitment, and investigate whether phosphorylation of TPP1 plays a role in the regulated recruitment of telomerase to telomeres.

Acknowledgments

We thank Susan Smith for kindly providing us with the TIN2 antibodies and Peter Baumann and Titia de Lange for POT1 antibodies. We are grateful to David Hall (University of Georgia) for overseeing statistical analysis.

This work was supported by a grant from the National Cancer Institute (RO1 CA104676) to M.P.T. and R.M.T. and by a Swiss National Science Foundation grant, the European Community's Seventh Framework Programme FP7/2007-2011 (grant agreement number 200950), and a European Research Council advanced investigator grant (grant agreement number 232812) to J.L. Eladio Abreu was supported by an NIH (NRSA) Predoctoral Fellowship Award To Promote Diversity in Health-Related Research (F31GM087949).

References

1. Almeida, F., R. Saffrich, W. Ansorge, and M. Carmo-Fonseca. (1998). Microinjection of anti-coilin antibodies affects the structure of coiled bodies. J. Cell Biol. 142:899-912.
2. Armanios, M. Y., J. J. Chen, J. D. Cogan, J. K. Alder, R. G. Ingersoll, C. Markin, W. E. Lawson, M. Xie, I. Vulto, J. A. Phillips III, P. M. Lansdorp, C. W. Greider, and J. E. Loyd. (2007). Telomerase mutations in families with idiopathic pulmonary fibrosis. N. Engl. J. Med. 356:1317-1326.
3. Autexier, C., R. Pruzan, W. D. Funk, and C. W. Greider. (1996). Reconstitution of human telomerase activity and identification of a minimal functional region of the human telomerase RNA. EMBO J. 15:5928-5935.
4. Azzalin, C. M., and J. Lingner. (2006). The human RNA surveillance factor UPF1 is required for S phase progression and genome stability. Curr. Biol. 16:433-439.
5. Azzalin, C. M., P. Reichenbach, L. Khoriauli, E. Giulotto, and J. Lingner. (2007). Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. Science 318:798-801.
6. Baumann, P., and T. Cech. (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. Science 292:1171-1175.
7. Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C.-P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteinter, and W. E. Wright. (1998). Extension of life-span by introduction of telomerase into normal human cells. Science 279:349-352.
8. Broccoli, D., A. Smogorzewska, L. Chong, and T. de Lange. (1997). Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. Nat. Genet. 17:231-235.
9. Chandra, A., T. R. Hughes, C. I. Nugent, and V. Lundblad. (2001). Cdc13 both positively and negatively regulates telomere replication. Genes Dev. 15:404-414.
10. Cong, Y. S., W. E. Wright, and J. W. Shay. 2002. Human telomerase and its regulation. Microbiol. Mol. Biol. Rev. 66:407-425.

11. Crabbe, L., R. E. Verdun, C. I. Haggblom, and J. Karlseder. (2004). Defective telomere lagging strand synthesis in cells lacking WRN helicase activity. Science 306:1951-1953.
12. Cristofari, G., E. Adolf, P. Reichenbach, K. Sikora, R. M. Terns, M. P. Terns, and J. Lingner. (2007). Human telomerase RNA accumulation in Cajal bodies facilitates telomerase recruitment to telomeres and telomere elongation. Mol. Cell 27:882-889.
13. Cristofari, G., and J. Lingner. (2006). Telomere length homeostasis requires that telomerase levels are limiting. EMBO J. 25:565-574.
14. Cristofari, G., K. Sikora, and J. Lingner. (2007). Telomerase unplugged. ACS Chem. Biol. 2:155-158.
15. d'Adda di Fagagna, F., P. M. Reaper, L. Clay-Farrace, H. Fiegler, P. Carr, T. Von Zglinicki, G. Saretzki, N. P. Carter, and S. P. Jackson. (2003). A DNA damage checkpoint response in telomere-initiated senescence. Nature 426:194-198.
16. de Lange, T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev. 19:2100-2110.
17. Denchi, E. L., and T. de Lange.(2007). Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. Nature 448:1068-1071.
18. Greider, C. W., and E. H. Blackburn. (1989). A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature 337:331-337.
19. Guo, X., Y. Deng, Y. Lin, W. Cosme-Blanco, S. Chan, H. He, G. Yuan, E. J. Brown, and S. Chang. (2007). Dysfunctional telomeres activate an ATM-ATR-dependent DNA damage response to suppress tumorigenesis. EMBO J. 26:4709-4719.
20. Hockemeyer, D., W. Palm, T. Else, J. P. Daniels, K. K. Takai, J. Z. Ye, C. E. Keegan, T. de Lange, and G. D. Hammer. (2007). Telomere protection by mammalian Pot1 requires interaction with Tpp1. Nat. Struct. Mol. Biol. 14:754-761.

21. Hockemeyer, D., A. J. Sfeir, J. W. Shay, W. E. Wright, and T. de Lange. (2005). POT1 protects telomeres from a transient DNA damage response and determines how human chromosomes end. EMBO J. 24:2667-2678.

22. Jady, B. E., E. Bertrand, and T. Kiss. (2004). Human telomerase RNA and box H/ACA scaRNAs share a common Cajal body-specific localization signal. J. Cell Biol. 164:647-652.

23. Jady, B. E., P. Richard, E. Bertrand, and T. Kiss. (2006). Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. Mol. Biol. Cell 17:944-954.

24. Kibe, T., G. A. Osawa, C. E. Keegan, and T. de Lange.(2010). Telomere protection by TPP1 is mediated by POT1a and POT1b. Mol. Cell. Biol. 30:1059-1066.

25. Kim, S. H., C. Beausejour, A. R. Davalos, P. Kaminker, S. J. Heo, and J. Campisi. (2004). TIN2 mediates functions of TRF2 at human telomeres. J. Biol. Chem. 279:43799-43804

26. Kondo, T., N. Oue, K. Yoshida, Y. Mitani, K. Naka, H. Nakayama, and W. Yasui. (2004). Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. Cancer Res. 64:523-529.

27. Lansdorp, P. M. 2009. Telomeres and disease. EMBO J. 28:2532-2540.

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

29. Lei, M., E. R. Podell, and T. R. Cech. (2004). Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. Nat. Struct. Mol. Biol. 11:1223-1229.

30. Li, S., S. Makovets, T. Matsuguchi, J. D. Blethrow, K. M. Shokat, and E. H. Blackburn. (2009). Cdk1-dependent phosphorylation of Cdc13 coordinates telomere elongation during cell-cycle progression. Cell 136:50-61.

31. Lin, J. J., and V. A. Zakian. (1996). The *Saccharomyces CDC13* protein is a single-strand TG_{1-3} telomeric DNA-binding protein *in vitro* that affects telomere behavior *in vivo*. Proc. Natl. Acad. Sci. U. S. A. 93:13760-13765.

32. Lingner, J., T. R. Hughes, A. Sherchenko, M. Mann, V. Lundblad, and T. R. Cech. (1997). Reverse transcriptase motifs in the catalytic subunit of telomerase. Science 276:561-567.

33. Liu, D., A. Safari, M. S. O'Connor, D. W. Chan, A. Laegeler, J. Qin, and Z. Songyang. (2004). PTOP interacts with POT1 and regulates its localization to telomeres. Nat. Cell Biol. 6:673-680.

34. Loayza, D., and T. De Lange. (2003). POT1 as a terminal transducer of TRF1 telomere length control. Nature 424:1013-1018.

35. Nugent, C. I., T. R. Hughes, N. F. Lue, and V. Lundblad. (1996). Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. Science 274:249-252.

36. Paeschke, K., S. Juranek, T. Simonsson, A. Hempel, D. Rhodes, and H. J. Lipps. (2008). Telomerase recruitment by the telomere end binding protein-beta facilitates G-quadruplex DNA unfolding in ciliates. Nat. Struct. Mol. Biol. 15:598-604.

37. Paeschke, K., T. Simonsson, J. Postberg, D. Rhodes, and H. J. Lipps. (2005). Telomere end-binding proteins control the formation of G-quadruplex DNA structures in vivo. Nat. Struct. Mol. Biol. 12:847-854.

38. Palm, W., and T. de Lange. (2008). How shelterin protects mammalian telomeres. Annu. Rev. Genet. 42:301-334.

39. Savage, S. A., N. Giri, G. M. Baerlocher, N. Orr, P. M. Lansdorp, and B. P. Alter. (2008). TINF2, a component of the shelterin telomere protection complex, is mutated in dyskeratosis congenita. Am. J. Hum. Genet. 82:501-509

40. Smogorzewska, A., and T. De Lange. (2004). Regulation of telomerase by telomeric proteins. Annu. Rev. Biochem. 73:177-208.

41 Smogorzewska, A., B. van Steensel, A. Bianchi, S. Oelmann, M. R. Schaefer, G. Schnapp, and T. de Lange. (2000). Control of human telomere length by TRF1 and TRF2. Mol. Cell. Biol. 20:1659-1668.

42. Takai, H., A. Smogorzewska, and T. de Lange. (2003). DNA damage foci at dysfunctional telomeres. Curr. Biol. 13:1549-1556.

43. Tomlinson, R. L., E. B. Abreu, T. Ziegler, H. Ly, C. M. Counter, R. M. Terns, and M. P. Terns. (2008). Telomerase reverse transcriptase is required for the localization of telomerase RNA to cajal bodies and telomeres in human cancer cells. Mol. Biol. Cell 19:3793-3800.

44. Tomlinson, R. L., T. D. Ziegler, T. Supakorndej, R. M. Terns, and M. P. Terns. (2006). Cell cycle-regulated trafficking of human telomerase to telomeres. Mol. Biol. Cell 17:955-965.

45. Tseng, S. F., Z. J. Shen, H. J. Tsai, Y. H. Lin, and S. C. Teng. (2009). Rapid Cdc13 turnover and telomere length homeostasis are controlled by Cdk1-mediated phosphorylation of Cdc13. Nucleic Acids Res. 37:3602-3611.

46. Venteicher, A. S., E. B. Abreu, Z. Meng, K. E. McCann, R. M. Terns, T. D. Veenstra, M. P. Terns, and S. E. Artandi. (2009). A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. Science 323:644-648.

47. Vulliamy, T. J., and I. Dokal. (2008). Dyskeratosis congenita: the diverse clinical presentation of mutations in the telomerase complex. Biochimie 90:122-130.

48. Wang, F., E. R. Podell, A. J. Zaug, Y. Yang, P. Baciu, T. R. Cech, and M. Lei. (2007). The POT1-TPP1 telomere complex is a telomerase processivity factor. Nature 445:506-510.

49. Weinrich, S. L., R. Pruzan, L. Ma, M. Ouellette, V. M. Tesmer, S. E. Holt, A. G. Bodnar, S. Lichtsteiner, N. W. Kim, J. B. Trager, R. D. Taylor, R. Carlos, W. H. Andrews, W. E. Wright, J. W. Shay, C. B. Harley, and G. B. Morin. (1997). Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. Nat. Genet. 17:498-502.

50. Wenz, C., B. Enenkel, M. Amacker, C. Kelleher, K. Damm, and J. Lingner. (2001). Human telomerase contains two cooperating telomerase RNA molecules. EMBO J. 20:3526-3534.

51. Xin, H., D. Liu, and Z. Songyang. (2008). The telosome/shelterin complex and its functions. Genome Biol. 9:232.

52. Xin, H., D. Liu, M. Wan, A. Safari, H. Kim, W. Sun, M. S. O'Connor, and Z. Songyang. (2007). TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. Nature 445:559-562.

53. Ye, J. Z., and T. de Lange.(2004). TIN2 is a tankyrase 1 PARP modulator in the TRF1 telomere length control complex. Nat. Genet. 36:618-623.

54 Ye, J. Z., D. Hockemeyer, A. N. Krutchinsky, D. Loayza, S. M. Hooper, B. T. Chait, and T. de Lange. (2004). POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. Genes Dev. 18:1649-1654.

55. Yehezkel, S., Y. Segev, E. Viegas-Pequignot, K. Skorecki, and S. Selig. (2008). Hypomethylation of subtelomeric regions in ICF syndrome is associated with abnormally short telomeres and enhanced transcription from telomeric regions. Hum. Mol. Genet. 17:2776-2789.

56. Zhu, Y., R. L. Tomlinson, A. A. Lukowiak, R. M. Terns, and M. P. Terns. (2004). Telomerase RNA accumulates in Cajal bodies in human cancer cells. Mol. Biol. Cell 15:81-90.

Chapter 3:

The Role of Human Telomerase Reverse Transcriptase in Telomerase Trafficking

---

Eladio Abreu [1], Brad Culp [1], Clause Azzalin [2], Steven Artandi [4], Christopher Counter [3], Rebecca M. Terns [1], Joachim Lingner [2] Michael P. Terns [1]

To be submitted to The Journal of Cell Science

Abstract

DNA/protein complexes called telomeres protect eukaryotic chromosomes. Telomerase is the RNP responsible for synthesizing the repetitive DNA portion of the telomere. Although inactive in most adult somatic tissues, telomerase is reactivated in a majority of cancers. The role of the enzyme in tumorigenesis and maintenance make it the subject of anti-cancer therapeutic research. Telomerase has two primary components: hTR and hTERT. These components follow a well-defined pattern of nuclear trafficking. This trafficking pathway culminates with both of these components being recruited to the telomere end. It is hypothesized that the regulated trafficking of primary telomerase constituents contributes to the overall regulation of telomerase activity. Understanding this form of regulation may elucidate new strategies for cancer treatment. Significant progress has been made in identifying the roles of hTR and other factors in facilitating telomerase trafficking. However, little is known regarding the role of hTERT in the recruitment process. In this work, we dissect the role of hTERT in this process. Using site directed mutants and stable sub-fragments of hTERT, we have determined that the hTERT N-DAT domain is required for telomerase recruitment to telomeres. Furthermore, we have determined that the role of the N-DAT domain in recruitment is linked to the induction of novel telomerase-specific coilin positive bodies.

Introduction

Human telomerase is the ribonucleoprotein (RNP) enzyme responsible for synthesizing and maintaining telomeres at the 3' ends of eukaryotic chromosomes (21). The enzyme consists of telomerase RNA (hTR), which provides the template for the addition of telomere repeats, and several protein subunits including telomerase reverse transcriptase (hTERT), the primary catalytic component of the active enzyme (10, 17, 23). Other known enzyme subunits include dyskerin, Nhp2, Nop10, and GAR1 (16, 36, 41). These enzyme components have been shown to be required for hTR stabilization and accumulation in vivo (18). The most recently discovered telomerase holoenzyme component, telomerase Cajal body protein 1 (TCAB1), also interacts with hTR and required for telomerase traficking and telomere elongation (48). In an overwhelming majority of cancers, telomerase is reactivated (29). This re-activation is critical to

prolonged tumor maintenance and proliferation (20, 22). A better understanding of how this RNP and its primary components are recruited from their respective sites of biogenesis to their site of action at the telomere end may prove critical in revealing how telomerase enzyme activity is regulated in cancers.

Telomerase components follow a well-defined, cell cycle dependent recruitment pathway on the way to the site of action at the telomere. Throughout most of the cell cycle (G1 and G2), telomerase is found in Cajal bodies, the theorized sites of telomerase assembly and/or storage (28, 47, 56). During mid S-phase, both telomerase components mobilize to a specific subset of telomeres (28, 47). Limiting telomerase recruitment to this specific point in the cell cycle provides an opportunity to regulate the enzyme, ensuring that it only acts on the correct substrate (telomeres) at the proper time (S-phase). However, the manner in which telomerase function is restricted to this stage of the cell cycle is not known. We hypothesize that telomerase recruitment is facilitated by dynamic interactions between telomerase components and key factors at Cajal bodies and telomeres.

TPP1 is one of six core telomere-binding proteins that make up the shelterin complex (15). Although originally considered as a negative regulator of telomerase activity, new evidence suggests that TPP1functions as a recruitment factor and positive regulator of telomerase activity (45, 50, 53, 55). The TPP1/POT1 heterodimer contributes to *in vitro* telomerase processivity (50, 55). Additionally, telomere bound TPP1 is required for telomerase mediated telomere maintenance (45, 53). Data from one of these studies, suggests the TPP1 OB fold interacts with hTERT (53). It was later revealed that TPP1 does indeed function as a recruitment factor, and its presence is required to facilitate recruitment of both telomerase components to telomeres (1, 45, 55).

The role of hTR in the recruitment process has already been investigated and characterized. A specific domain within the RNA (the CAB box) is important for telomerase association with Cajal bodies (13, 26, 35). Furthermore, CAB box dependent telomerase recruitment to the Cajal body is required for telomerase trafficking to the telomere and subsequent telomere maintenance (13). As mentioned earlier, the newly discovered telomerase component and CAB box interacting protein, TCAB1, is required for telomerase trafficking to both Cajal bodies and telomeres (48).

These findings support the notion that telomerase is systematically recruited to telomeres through interactions between its own components and factors at telomeres. hTR requires the expression of hTERT to facilitate recruitment to Cajal bodies and telomeres (46). This suggests that hTR and hTERT may have to assemble into an active enzyme to take part in the recruitment process. Until now, little was known regarding the role of hTERT in this process.

Structure/function analysis of hTERT have revealed specific functional domains. The N-terminal half of hTERT functions in the regulation and assembly. The N-terminal end of hTERT contains the telomerase essential N-terminal (TEN) and telomerase RNA binding (TRBD) domains. The TEN domain encompasses critical domains required for interactions with telomeric DNA (25, 43). The TEN domain contains critical sequence motifs called anchor sites, which are thought to play a critical role in binding and translocation along the telomere end (34, 54). Evidence has emerged of two putative recruitment domains within the N and C terminal ends of the hTERT protein. The TEN domain contains one of these domains, the N-terminal disassociation of activity of telomerase (N-DAT) domain. The existence of these domains was revealed through mutational analysis. A series of deletion and substitution mutants were generated throughout the full-length hTERT protein to determine the necessity of each domain in both *in vivo* and *in vitro* activity (5, 6, 9). Each mutant was assessed for the ability to be expressed, immortalize cells, elongate telomeres and display *in vitro* activity. These experiments, along with subsequent studies, lead to the emergence of the N-DAT and its C-terminal counterpart, the C-DAT domain (5, 6, 9). Others obtained evidence that the DAT mutants used in the study have catalytic defects (32). However, roles for the DAT domains in both recruitment and catalysis are not mutually exclusive. Catalytic defects would later prove to be irrelevant in our understanding of telomerase recruitment if these mutants are never recruited the telomere.

In this work, we set out to determine the role of hTERT in telomerase recruitment and trafficking. We assessed a panel of stable hTERT mutants and fragments to determine which structural domains are needed to facilitate the recruitment of telomerase to Cajal bodies and telomeres. We used fluorescence microscopy techniques to monitor *in vivo* localization of the hTERT variants in the absence or presence of hTR expression.

We found that hTERT requires the expression of hTR to be recruited to both Cajal bodies and telomeres, indicating that both components need to assemble to facilitate recruitment. We also demonstrate that the N-DAT domain is required for telomerase localization to telomeres, but not Cajal bodies. Although an intact N-DAT domain is required, this portion of the protein is not sufficient to facilitate telomerase recruitment. Our data shows that the full-length protein is needed to accomplish this feat. Our data also suggests that higher order hTERT structure is likely needed at a key step in telomerase trafficking. Last, we found that over expression of both telomerase components leads to the induction of novel coilin-positive bodies, which specifically contain telomerase and occupy telomeres. Our observations suggest that these telomerase induced bodies may be important in telomerase delivery to telomeres.

Methods and Materials

The following wild-type hTERT and NAAIRS substitution constructs were used in pBabepuro vectors: FLAG -hTERT-pBabepuro (5), FLAG -hTERT-128NAA-pBabepuro (5), FLAG -hTERT-134NAA-pBabepuro (5), and FLAG -hTERT-1127NAA-pBabepuro (9). The following wild-type hTR and hTERT constructs were used in Bluscript vectors: pBS-U1-hTR (contains U1 promoter) (14), and pBS-FLAG -hTERT. The following full length and fragment hTERT constructs were used in PCDNA 3.1 vectors: FLAG -TERT-full (AA 1-1132) (49), FLAG -TERT-A (AA 1-589)(49) (49), FLAG -TERT-B (AA 1-792) (49), FLAG -TERT-C (AA 1-940) (49), FLAG -TERT-D (AA 545-940) (49) and FLAG -TERT-J (AA 546-1132)(49).

Cell culture and transfection

HeLa cells were grown on coverslips in DMEM media (Fisher scientific, Pittsburg, PA) supplemented with 10% fetal bovine serum (FBS) (Fisher scientific). Standard VA13 and VA13 cells stably expressing hTR and hTERT or hTR and mutant (D868A, catalytically dead) hTERT were provided by Hinh Ly (Emory University) and grown under the same conditions. All cells were cultured at 37°C with 5% CO_2. Transfections were carried out using Lipofectamine 2000 transfection reagent, according

to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Simultaneous over expression of both telomerase components was achieved with 5:1 (hTR/hTERT) plasmid ratio.

Fluorescence in situ hybridization (FISH) and Immunofluorescence (IF)

Three previously described DNA Probes (probes 1, 2, and 3), complementary to different regions of telomerase RNA, were used in hTR FISH (2). A fourth DNA probe complementary to the G-rich strand of the telomere was synthesized by Qiagen (Valencia, CA) as follows: CT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*A (T* indicates aminoallyl-modified thymidines) and used to detect telomeres. Probes were conjugated with Cy3 or Oregon green mono-functional reactive dye according to the manufacturer's protocol (GE Healthcare, Little Chalfront, Buckinghamshire, United Kingdom, and Invitrogen). 25 ng of each Cy3 labeled hTR FISH probe and 20 ng of telomere FISH probe (when FISH was used to detect telomeres) were used per coverslip. FISH was performed essentially as described previously (3, 47). However, when hTR FISH was performed in combination with telomere FISH, the cells were subjected to a 10 minute denaturation at 85°C in 70% formamide, 2X SSC prior to FISH.

Following FISH, cells were subjected to IF as described previously (47). Cells were washed three times with 1X PBS and blocked for 1hr in 0.05% Tween-20 in PBS (PBST) or 3% BSA in PBS. Next, cells were incubated with either one of several combinations of the following primary antibodies at the indicated dilution for 1 hr at room temperature: Π mouse anti-p80 coilin (1:5000, (4)), mouse anti-TRF2 (1:1000, Imgenex corp, San Diego, CA), rabbit anti-hTERT (1:400, Rockland, Gilbertsville, PA), mouse anti-FLAG (1:500, Sigma-Aldrich, St. Louis, MO), rabbit anti-dyskerin polyclonal sera (provided by Philip Mason (37)), mouse anti-SMN 7B10 (provided by Utz Fisher), mouse anti-Sm Y12 (33), anti-fibrillarin 17C12 (24), and rabbit anti-TCAB1 (48). Please note that the last six antibodies listed were used in Cajal body compositional analysis. In the case of such analysis, IF was performed prior to FISH due to antibody sensitivity to formamide. Performing IF prior to FISH requires a second fixation (fix as described previously) to preserve IF signal before proceeding with FISH procedure. Cells were washed three times in 1X PBS and then incubated with secondary antibody (1:100

Cy2 conjugated goat anti-rabbit IgG (H+L), 1:100 Cy2 conjugated goat anti-mouse IgG (H+L), 1:100 Cy5 conjugated goat anti-mouse IgGγ, 1:100 Cy5 conjugated goat anti-rabbit IgG (H+L), 1:100 AMCA conjugated goat anti-mouse IgG (H+L), or 1:100 AMCA conjugated goat anti-rabbit IgG (H+L); (all secondary antibodies obtained from Jackson ImmunoResearch laboratories, West Grove, PA) for 1 hr at room temperature. Primary antibodies were diluted in phosphate buffered saline supplemented with .05% tween (PBST) or 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS), while secondary antibodies were diluted in PBST only. Cells were then subjected to three final 1X PBS washes and mounted in Prolong Gold (Invitrogen).

Microscopy

Slides were analyzed using a Zeiss Axioskop 2 Mot Plus fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were acquired at 63x (Plan Apochromat objectives, numerical aperture 1.4) using a cooled charge-coupled device ORCA-ER digital camera (Hamamatsu photonics, Bridgewater, NJ) and IPLab Spectrum software (BioVision Technologies, Inc., Exton, PA

Results

hTERT requires the expression of hTR to facilitate recruitment to telomeres and coilin positive bodies.

Previous studies revealed that the reactivation of hTERT expression is sufficient to induce hTR localization to Cajal bodies and telomeres (46). However, it was not determined if hTR is required to facilitate hTERT localization. To address this, we observed the *in vivo* localization of ectopically expressed hTERT in the presence and absence of hTR co-expression (Fig. 13). HeLa cells were transiently transfected with plasmids encoding either hTR, hTERT, or both components. Transfected and untreated cells were subjected to IF to detect hTERT, telomeres, and Cajal bodies. When hTERT is transiently over expressed in HeLa cells, the protein is readily detected via IF within the nucleus of the cell (Fig. 13, panel A). Over expression of hTERT is necessary for detection presumably due to the low amount of endogenous hTERT molecules. The diffuse nuclear staining pattern observed upon hTERT expression alone exhibits no

significant colocalization to telomeres (labeled via anti-TRF2 IF) (Fig. 13, panel A). However, upon simultaneous expression with hTR, hTERT forms discrete foci. These induced hTERT foci correspond to telomeres (Fig. 13, panel A). Western blot analysis confirmed that the overexpression of hTR does not affect hTERT levels (Fig. 13, panel B). This suggests that the changes in hTERT localization observed are not a product of changes in hTERT levels due to hTR overexpression.

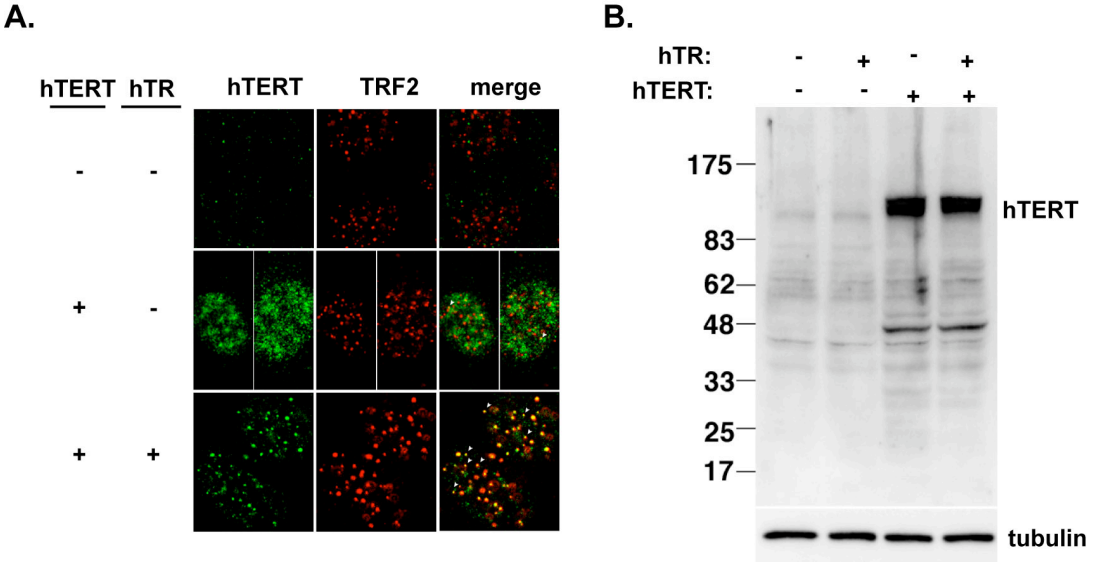


FIGURE 13. hTR overexpression is required to facilitate the recruitment of ectopically expressed hTERT to telomeres. (A) Images of HeLa cells ectopically overexpressing hTERT, or both hTR and hTERT simultaneously. Untransfected cells used as control. Fluorescence microscopy was employed to label hTERT (green) relative to TRF2 (telomere marker, red). Merge represents superimposition of hTERT and TRF2 channels (yellow indicates colocalization). (B) Western blot analysis of hTERT levels in HeLa cells ectopically overexpressing hTR, hTERT, or both simultaneously. Untransfected cells used as control. Anti-tubulin blot used as loading control.

Simultaneous overexpression of both telomerase components also appears to result in readily detectable colocalizations between hTERT and coilin foci (Fig. 14, panel A). We assumed this represented hTERT localization to Cajal bodies, as coilin is a traditional marker for Cajal bodies. Incidentally, we also observed a dramatic increase in what appeared to be Cajal bodies (Fig. 14, panel B). The average number of coilin

positive foci increased from 1.1 SEM +/-.13 (in untreated cells) to 16.3.SEM +/- 1.29 (for cells where both hTR and hTERT were expressed). Further investigation suggests that these coilin positive foci may actually represent a novel telomerase-specific body as opposed to traditional Cajal bodies.

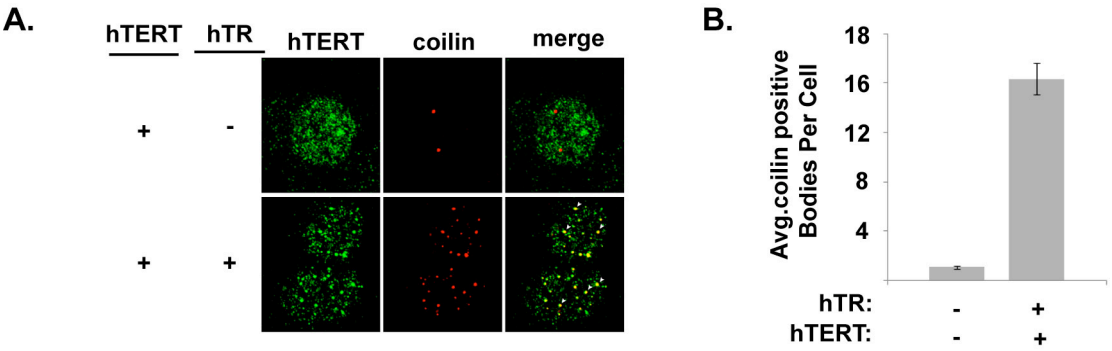


FIGURE 14. hTR overexpression is required to facilitate the recruitment of ectopically expressed hTERT to coilin-positive bodies. (A) Images of HeLa cells ectopically overexpressing hTERT, or both hTR and hTERT simultaneously. Untransfected cells used as control. Fluorescence microscopy was employed to label hTERT (green) relative to coilin (Cajal body marker, red). Merge represents superimposition of hTERT and coilin channels (yellow indicates colocalization). (B) Quantitation of average number of Coilin-positive bodies per cell before and after telomerase overexpression.

Simultaneous over expression of hTR and hTERT lead to an induction of large numbers of telomerase-containing coilin-positive bodies at telomeres.

Having observed that hTR expression induces expressed hTERT to colocalize with coilin–positive bodies and telomeres, we set out to determine if hTR was present at these foci as well. Additionally, we wanted to determine if the hTERT foci we observed were occupying coilin-positive bodies and telomeres simultaneously. Making these observations required simultaneous detection of both telomerase components with respect to coilin foci and telomeres. We employed FISH and IF in cells transiently overexpressing both primary telomerase components to detect hTR, hTERT, coilin, and

telomeres. While observing the over expressed cells under these three labeling conditions, we determined that all visible hTERT foci colocalized with hTR, telomeres, and coilin foci simultaneously (Fig. 15, panels A and B). Simultaneous labeling of telomeres and coilin foci confirmed that the numerous coilin-positive bodies we had previously observed (Fig. 14, panel A) all correspond to individual telomeres (Fig. 15, panel C). These observations link the presence of telomerase at telomeres to the appearance of numerous coilin-positive bodies at the same nuclear positions.

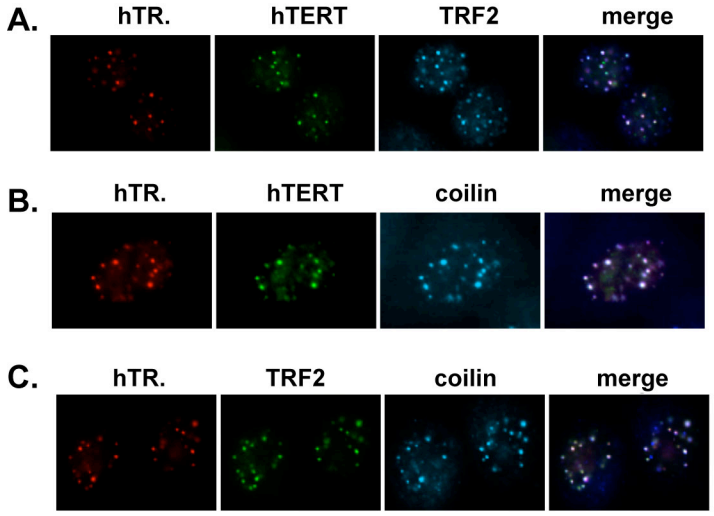


FIGURE 15: Simultaneous ectopic expression of both telomerase components results in four-way colocalization between hTR, hTERT, telomeres, and Coilin-positive bodies. Images of HeLa cells simultaneously overexpressing ectopic hTR and hTERT. Untransfected cells used as control. Fluorescence microscopy was employed to label hTR (red) and hTERT (green (Panels A and B)), relative to coilin (Cajal body marker, blue (Panels B and C)) and TRF2 (telomere marker, blue (Panel A) or green (Panel C)). Merge represents superimposition of red, green, and blue channels (white indicates 4 way colocalization).

Telomerase induced coilin-positive bodies are novel structures containing telomerase specific components

The Cajal body-like structures induced in the presence of hTR/hTERT expression initially exhibited some key differences from Cajal bodies. The observed coilin-positive bodies are considerably smaller and exist in much higher numbers then Cajal bodies (Fig.

14, panels A and B). Furthermore, these induced Cajal bodies associate with telomeres at very high frequency. Cajal bodies rarely associate with telomeres under normal conditions (28, 47). We set out to determine if the observed telomerase induced bodies were in fact traditional Cajal bodies or some novel coilin positive bodies, specific to telomerase. To test this, we performed a compositional analysis of the bodies by labeling for multiple components, previously reported to exhibit Cajal body association, in cells transiently overexpressing both telomerase components.

We used IF to label for traditional Cajal body markers. These markers include: coilin (primary structural constituent of Cajal bodies (39)), TCAB1 (telomerase holoenzyme component (48)), fibrillarin **(** rRNA 2'-O-methyltransferase**,** Box C/D snoRNP component (38, 39)), SMN (assists in assembly and trafficking of snRNPs and possibly snoRNPs and telomerase (8, 38, 39, 52), dyskerin (pseudouridine synthase, H/ACA snoRNP component, binds and contributes to the stabilization hTR (36)), and Sm (snRNP component (38, 39)). We also employed FISH to label for the small Cajal body RNA U85 (scaRNA, 2'-O-ribose methylation of snRNAs (27, 30)). Some of these components localize primarily at Cajal bodies (coilin, U85, and SMN). Other components do associate with Cajal bodies but display the majority of their signal at nucleoli (dyskerin and fibrillarin) or nuclear speckles (Sm).

In untransfected cells, each marker corresponded to the endogenous Cajal bodies as expected (Fig. 16, panels A, B, C, D, E, F). When both hTR and hTERT are over expressed, only telomerase specific factors associated with the newly formed coilin-positive bodies. In addition to hTR and hTERT (Fig. 15, panel B), we found that the newly induced coilin-positive bodies were positive for TCAB1 and dyskerin (Fig. 16, panels A and D).

The telomerase induced coilin positive bodies were nearly completely devoid of all other Cajal body associated factors (Fig. 16, panels B, C, E, F). In most cases, Cajal bodies containing any non-telomerase specific markers disappeared following telomerase overexpression. This lead to elimination of individual fibrillarin and U85 foci (Fig. 16, panels C, and E). In the case of fibrillarin, all nucleoplasmic foci disappeared, leaving only nucleolar staining (Fig. 16, panel E). In the cases of SMN and Sm, individual foci remained following telomerase overexpression (Fig. 16, panels B and F). However, the

residual foci corresponded to coilin foci only on rare occasion (observed in <5% of treated cells).

These observations suggest that these telomerase induced coilin-positive bodies contain only telomerase specific components (hTR, hTERT, TCAB1, and dyskerin). Considering that these bodies are only found associated with telomeres, indicates that these are a new class of telomerase specific Cajal body or some novel coilin-positive telomerase delivery body. From this point on we will refer to these bodies as telomerase induced bodies (TIBs).

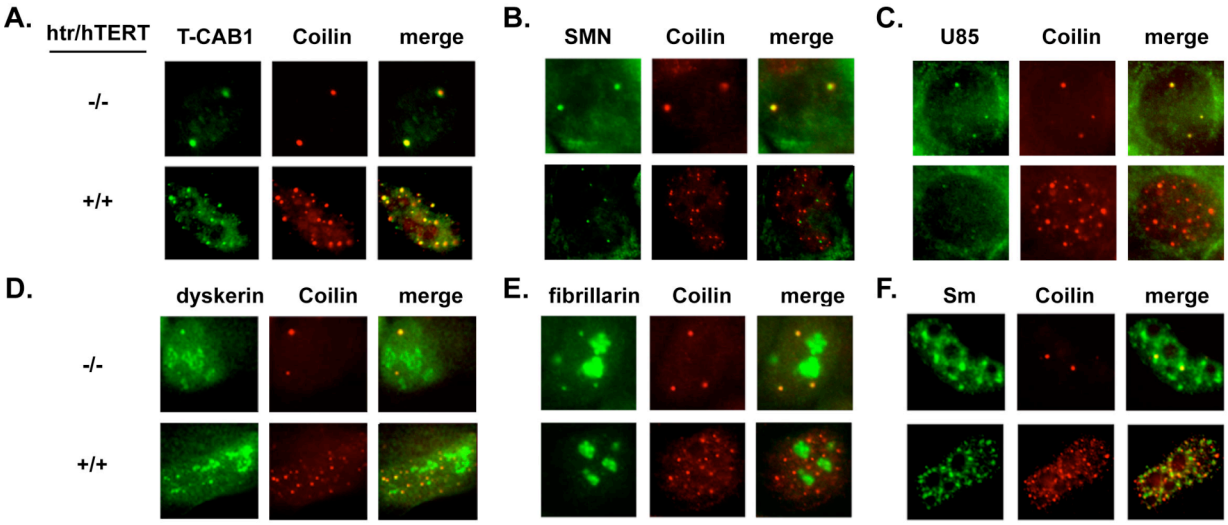


FIGURE 16. The observed telomerase induced bodies represent a novel class of coilin positive body, containing only telomerase specific components. (A, B, C, D, E, F) Images of HeLa cells simultaneously overexpressing ectopic hTR and hTERT. Untransfected cells used as control. Fluorescence microscopy was employed to label for several Cajal body markers (TCAB1, fibrillarin, SMN, dyskerin, U85, and SM, green) relative to coilin (Cajal body marker, red). Merge represents superimposition of the indicated marker and coilin channels (yellow indicates colocalization).

Various stable hTERT sub-fragments are insufficient to support telomerase trafficking or recruitment

Our next goal was to identify which portions of hTERT were sufficient to facilitate telomerase recruitment processes. We exploited a series of FLAG –tagged N and C terminal stable sub-fragments to achieve this goal. The fragments used in this assay consisted of the N-terminal or C-terminal halves of the protein with various segments of the RT domain attached (Fig. 17, Panel A). Each of these fragments has been shown to be stable *in vivo*, traffic into the nucleus of cells properly, and in some cases, exhibit protein binding activity (49). A Schematic of full-length hTERT is provided along with diagrams of the relative size and composition of each fragment (Fig. 17, Panel A). We tested the fragments for the ability to contribute to telomerase trafficking, evidenced by facilitating the telomerase delivery observed with wildtype telomerase overexpression. Each fragment was ectopically expressed with hTR in HeLa cells. Full-length hTERT was used as a control. Following transfection, anti FLAG (to detect hTERT) and TRF2 (to detect telomeres) IF was used to determine the trafficking pattern of each fragment. We found that none of the fragments tested formed any nuclear foci. FLAG staining showed that each fragment formed a nucleoplasmic staining pattern, which did not correspond to any nuclear structures such as telomeres (Fig. 17, Panel A).

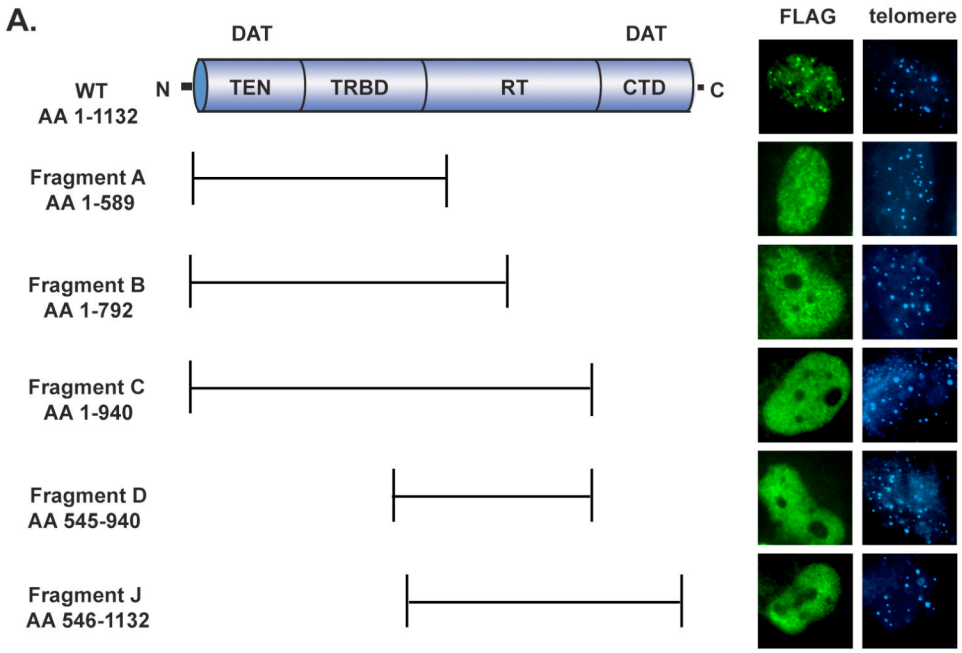


FIGURE 17: No stable sub-fragment of hTERT is sufficient to recruit telomerase to any nuclear foci. (A) Structural schematic of hTERT, stable sub-fragments, and images of corresponding trafficking patterns. Line segments indicate which portion

of full-length hTERT is contained in each fragment. Images of HeLa cells simultaneously overexpressing ectopic hTR along with hTERT or one of the indicated stable sub-fragments are provided. Fluorescence microscopy was employed to label for FLAG (marker for fragments, green) relative to telomeres (blue).

Catalytic activity is dispensable for telomerase recruitment to telomeres

Since no single portion of hTERT was sufficient to facilitate trafficking, we decided to change our approach and attempt to identify specific recruitment domains using site directed mutagenesis. We first wanted to determine if catalytic activity was needed for telomerase trafficking. We suspected that the DAT domains functioned as the recruitment domains within the hTERT protein. We predicted that if these domains operate as independent recruitment factors, the catalytic activity of the RT domain should not be required for telomerase recruitment. To address this, we studied VA13 cells stably expressing hTR along with WT hTERT, or a catalytically dead form of hTERT. The mutant used in this experiment included a mutation in the reverse transcriptase (RT) domain (D868A). This highly conserved domain is considered the catalytic portion of the protein (7). VA13 is an immortalized ALT cell line, which uses a telomerase-independent recombination mechanism to maintain its telomeres (51). VA13 cells are devoid of both telomerase components (51). The VA13 model allows us to supplement recombinant telomerase components without endogenous telomerase affecting our results. We used hTR FISH and anti-TRF2 IF to monitor telomerase trafficking relative to telomeres. We found that the catalytically dead construct readily induces telomerase localization to telomeres as compared to the wild type protein (Fig. 18, panel A). The cells expressing the catalytically dead mutant displayed telomerase foci at the majority of visible telomeres.

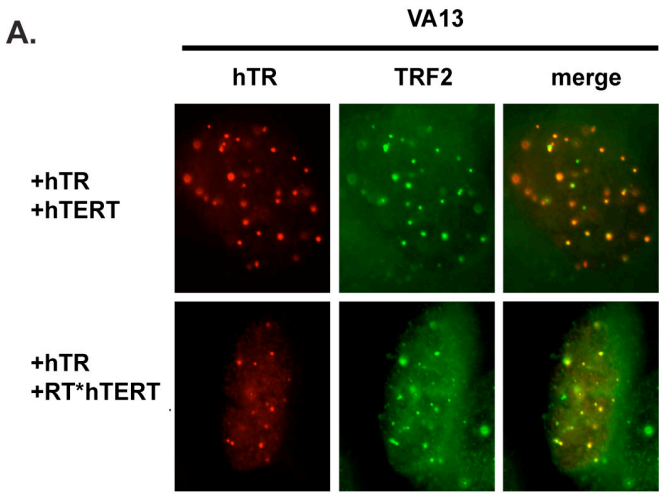


FIGURE 18. Catalytic activity is dispensable for hTERT to facilitate telomerase recruitment to telomeres. (A) Images of VA13 cells simultaneously overexpressing ectopic hTR along with hTERT or mutant hTERT (encompassing a mutation in the catalytic RT domain). Fluorescence microscopy was employed to label for hTR (red) relative to TRF2 (telomere marker, green). Merge represents superimposition of hTR and TRF2 channels (yellow indicates colocalization).

The N-DAT domain is required for hTERT recruitment to telomeres.

After determining that catalytic activity is dispensable for hTERT to function in telomerase recruitment, we set out to independently test the N-DAT and C-DAT domains for roles in telomerase trafficking. We exploited the telomerase overexpression phenotype to test stable substitution mutants of hTERT (5, 9). We assayed each mutant for the ability to contribute to telomerase localization to telomeres and Cajal bodies when transiently expressed with wild type hTR. The mutants used in these experiments contained previously described NAAIRS amino acid substitutions in the N-DAT (5), and C-DAT domains (9). Each mutant was ectopically expressed in HeLa simultaneously with hTR. Wild type hTERT was used as a control. Following transfection, cells were subjected to FISH and IF to detect hTR, hTERT, telomeres, and Cajal bodies (Fig. 19, Panels A and B). We found that the N-DAT mutant was capable of associating with hTR at Cajal bodies but insufficient for recruitment to telomeres (Fig. 19, panel A). We

observed that the C-DAT mutant behaved like the wild type protein, as it was able to associate with hTR and support telomerase localization to both TIBs and telomeres. Each mutant behaved in the same manner in VA13 cells (data not shown). In HeLa cells the N-DAT mutant displays the ability to localize to pre-existing Cajal bodies, but is apparently incapable of inducing the previously observed TIBs.

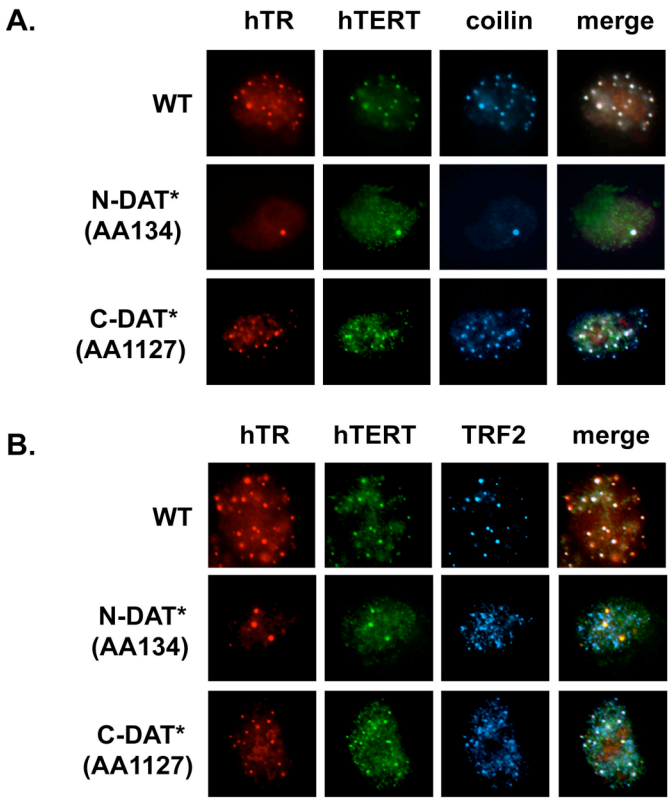


FIGURE 19. The N-DAT domain of hTERT is required for induction of TIBs and subsequent telomerase delivery to telomeres. (A, B) Images of HeLa cells simultaneously overexpressing ectopic hTR along with hTERT, or an hTERT mutant with an NAAIRS substitution in the N-DAT or C-DAT domain. Fluorescence microscopy was employed to label for hTR (red) and hTERT (green) relative to telomeres (blue, panel A) or coilin (Cajal body marker, blue Panel B).

Discussion

In summary, our study shows that hTERT functions in telomerase recruitment through the N-DAT recruitment domain. The N-DAT domain contributes directly to the telomere recruitment step in the telomerase trafficking pathway. This function of this domain is related to the induction of multiple telomere associated coilin-positive bodies we call TIBs, which correspond to telomerase delivery. Despite these contributions, hTERT remains dependent on hTR for recruitment to both Cajal bodies and telomeres.

hTR and hTERT function interdependently to facilitate telomerase recruitment to Cajal bodies and telomeres.

This work and previous studies illustrate that both primary telomerase components, hTR and hTERT, exhibit interdependency in recruitment/localization activity (56). This supports telomerase assembly as being critical in facilitating telomerase trafficking. It is well documented that hTR encompasses a specific motif which facilitates Cajal body localization (13, 26). Our work indicates that the hTERT protein must associate with hTR to facilitate telomerase localization (Figs. 13, 14). Perhaps this is in order to take advantage of the hTR CAB box as a Cajal body localization signal. However, why hTR requires the presence of hTERT to facilitate its own recruitment to Cajal bodies is unknown. One explanation is that the Cajal body must recognize the assembled structure of the enzyme in its entirety to allow for Cajal body localization. This may explain why hTERT fragments are insufficient for telomerase trafficking. As mentioned, Cajal body localization is critical for subsequent recruitment to telomeres (13). Therefore, if the entire enzyme is not recognized at the Cajal body localization step (and if this is indeed required for Cajal body localization) no subsequent trafficking to telomeres would occur.

The hTERT N-DAT domain is required for telomerase recruitment to telomeres and induction of TIBs.

This work identifies the hTERT N-DAT domain as important in recruitment of telomerase to telomeres, but not Cajal bodies (Fig. 19). In contrast, we have found no

evidence to support the C-DAT domain as a recruitment factor (Fig. 19). Structural analysis does reveal that the C-terminal end of hTERT, which contains the C-DAT domain, likely functions as an extended thumb to the RT domain (19) This supports the notion that at least one of the DAT domains functions in regulating catalytic activity (32). While it is evident that the N-DAT domain is integral to the recruitment process, the exact role of the domain in recruitment remains in question. Our data reveals that the domain is important at some step between Cajal body localization and recruitment to the telomere. Our results indicate that the N-DAT domain is dispensable for localization with endogenous Cajal bodies (Fig. 19). On the other hand, it is required for TIB induction and recruitment to telomeres. Others have shown that the role of TPP1 in recruitment may be based in the ability to interact with hTERT through the OB fold (53, 55). In the most recent of these two studies, it was found that a mutation within the N-DAT domain (Gly100) disrupts the functional interaction between TPP1 and telomerase (55). The N-DAT may be the domain of hTERT needed to facilitate such interactions. Future interaction assays with other key recruitment domains in the telomerase trafficking pathway may reveal how the domain functions in this capacity.

Alternatively, the N-DAT domain of hTERT may function in triggering the release of telomerase specific TIBS from normal endogenous Cajal bodies. Cajal bodies have already been observed splitting in two on some occasions as well as completely disassembling in response to changes in the cell cycle (11, 40). Interactions between the N-DAT domain and TPP1 may be a part of the recruitment process. In our previous studies we have presented evidence that hTR trafficking to Cajal bodies is a critical step in telomerase recruitment and telomere maintenance (13, 48). In this work we have presented several new lines of evidence supporting a role for a novel coilin-positive body in telomerase trafficking. Due to their telomerase specific composition and induction to telomerase overexpression, we call these structures telomerase induced bodies (Figs. 15, 16). We observe these telomerase-containing bodies, at telomeres (Fig. 15). We postulate that this is indicative of telomerase delivery to telomeres via TIBs. This role in recruitment is supported by the observation that hTERT mutants deficient in the ability to induce TIBs are subsequently deficient in the ability to be recruited to telomeres (Fig.

19). These new findings and previous studies support a critical role for coilin positive bodies in telomerase recruitment and trafficking (14, 47, 48, 56).

Although we cannot rule out the possibility that TIBs may be an artifact of telomerase overexpression, there are several lines of evidence that their induction is physiologically relevant. Under normal conditions, Cajal bodies are very responsive to the presence and levels of snRNP components and substrates (12, 40, 44). Cajal bodies are known to increase in number and size in response to increased transcription and RNA processing rates (31). Others have also regarded the Cajal body as a entropy driven assembly of specific macromolecules (42). When looking at Cajal bodies from this perspective, it makes the events we have seen appear as a natural response to increased levels of RNP components (in this case telomerase). This would be an otherwise invisible phenomenon, made detectable by overexpression.

Acknowledgments

This work was supported by grant from the National Cancer Institute (CA1D4676) to M.T. and R.T., and a Swiss National Science Foundation grant, the European Community's Seventh Framework Programme FP7/2007-2011 under grant agreement no. 200950 and an ERC advanced investigator grant to J.L. E.A was supported by an Ruth L. Kirschstein NRSA predoctoral fellowship from National Institutes of Health. We would also like to thank Hinh Ly, Phil Mason, and Utz Fisher for supplying cell lines and antibodies.

References

1. Abreu, E., E. Aritonovska, P. Reichenbach, G. Cristofari, B. Culp, R. M. Terns, J. Lingner, and M. P. Terns. TIN2-tethered TPP1 recruits human telomerase to telomeres in vivo. Mol Cell Biol 30:2971-2982.

2. Abreu, E., E. Aritonovska, P. Reichenbach, G. Cristofari, B. Culp, R. M. Terns, J. Lingner, and M. P. Terns. 2010. TIN2-tethered TPP1 recruits human telomerase to telomeres in vivo. Mol Cell Biol 30:2971-2982.
3. Abreu, E., R. M. Terns, and M. P. Terns. 2011. Visualization of Human Telomerase Localization by Fluorescence Microscopy Techniques. Methods Mol Biol 735:125-137.
4. Almeida, F., R. Saffrich, W. Ansorge, and M. Carmo-Fonseca. 1998. Microinjection of anti-coilin antibodies affects the structure of coiled bodies. J Cell Biol 142:899-912.
5. Armbruster, B. N., S. S. Banik, C. Guo, A. C. Smith, and C. M. Counter. 2001. N-terminal domains of the human telomerase catalytic subunit required for enzyme activity in vivo. Mol Cell Biol 21:7775-7786.
6. Armbruster, B. N., K. T. Etheridge, D. Broccoli, and C. M. Counter. 2003. Putative telomere-recruiting domain in the catalytic subunit of human telomerase. Mol Cell Biol 23:3237-3246.
7. Autexier, C., and N. F. Lue. 2006. The structure and function of telomerase reverse transcriptase. Annu Rev Biochem 75:493-517.
8. Bachand, F., F. M. Boisvert, J. Cote, S. Richard, and C. Autexier. 2002. The product of the survival of motor neuron (SMN) gene is a human telomerase-associated protein. Mol Biol Cell 13:3192-3202.
9. Banik, S. S., C. Guo, A. C. Smith, S. S. Margolis, D. A. Richardson, C. A. Tirado, and C. M. Counter. 2002. C-terminal regions of the human telomerase catalytic subunit essential for in vivo enzyme activity. Mol Cell Biol 22:6234-6246.
10. Beattie, T. L., W. Zhou, M. O. Robinson, and L. Harrington. 1998. Reconstitution of human telomerase activity in vitro. Curr Biol 8:177-180.
11. Carmo-Fonseca, M., J. Ferreira, and A. I. Lamond. 1993. Assembly of snRNP-containing coiled bodies is regulated in interphase and mitosis--evidence that the coiled body is a kinetic nuclear structure. J Cell Biol 120:841-852.
12. Cioce, M., and A. I. Lamond. 2005. Cajal bodies: a long history of discovery. Annu Rev Cell Dev Biol 21:105-131.

13. Cristofari, G., E. Adolf, P. Reichenbach, K. Sikora, R. M. Terns, M. P. Terns, and J. Lingner. 2007. Human telomerase RNA accumulation in Cajal bodies facilitates telomerase recruitment to telomeres and telomere elongation. Mol Cell 27:882-889.
14. Cristofari, G., and J. Lingner. 2006. Telomere length homeostasis requires that telomerase levels are limiting. EMBO J 25:565-574.
15. de Lange, T. 2005. Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev 19:2100-2110.
16. Dragon, F., V. Pogacic, and W. Filipowicz. 2000. In vitro assembly of human H/ACA small nucleolar RNPs reveals unique features of U17 and telomerase RNAs. Mol Cell Biol 20:3037-3048.
17. Feng, J., W. D. Funk, S. S. Wang, S. L. Weinrich, A. A. Avilion, C. P. Chiu, R. R. Adams, E. Chang, R. C. Allsopp, J. Yu, and et al. 1995. The RNA component of human telomerase. Science 269:1236-1241.
18. Fu, D., and K. Collins. 2003. Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. Mol Cell 11:1361-1372.
19. Gillis, A. J., A. P. Schuller, and E. Skordalakes. 2008. Structure of the Tribolium castaneum telomerase catalytic subunit TERT. Nature 455:633-637.
20. Greider, C. W. 1998. Telomerase activity, cell proliferation, and cancer. Proc Natl Acad Sci U S A 95:90-92.
21. Greider, C. W., and E. H. Blackburn. 1985. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 43:405-413.
22. Harley, C. B., and N. W. Kim. 1996. Telomerase and cancer. Important Adv Oncol:57-67.
23. Harrington, L., W. Zhou, T. McPhail, R. Oulton, D. S. Yeung, V. Mar, M. B. Bass, and M. O. Robinson. 1997. Human telomerase contains evolutionarily conserved catalytic and structural subunits. Genes Dev 11:3109-3115.
24. Hultman, P., S. Enestrom, S. J. Turley, and K. M. Pollard. 1994. Selective induction of anti-fibrillarin autoantibodies by silver nitrate in mice. Clin Exp Immunol 96:285-291.

25. Jacobs, S. A., E. R. Podell, and T. R. Cech. 2006. Crystal structure of the essential N-terminal domain of telomerase reverse transcriptase. Nat Struct Mol Biol 13:218-225.

26. Jady, B. E., E. Bertrand, and T. Kiss. 2004. Human telomerase RNA and box H/ACA scaRNAs share a common Cajal body-specific localization signal. J Cell Biol 164:647-652.

27. Jady, B. E., and T. Kiss. 2001. A small nucleolar guide RNA functions both in 2'-O-ribose methylation and pseudouridylation of the U5 spliceosomal RNA. EMBO J 20:541-551.

28. Jady, B. E., P. Richard, E. Bertrand, and T. Kiss. 2006. Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. Mol Biol Cell 17:944-954.

29. Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011-2015.

30. Kiss, A. M., B. E. Jady, X. Darzacq, C. Verheggen, E. Bertrand, and T. Kiss. 2002. A Cajal body-specific pseudouridylation guide RNA is composed of two box H/ACA snoRNA-like domains. Nucleic Acids Res 30:4643-4649.

31. Lafarga, M., M. T. Berciano, L. M. Garcia-Segura, M. A. Andres, and M. Carmo-Fonseca. 1998. Acute osmotic/stress stimuli induce a transient decrease of transcriptional activity in the neurosecretory neurons of supraoptic nuclei. J Neurocytol 27:205-217.

32. Lee, S. R., J. M. Wong, and K. Collins. 2003. Human telomerase reverse transcriptase motifs required for elongation of a telomeric substrate. J Biol Chem 278:52531-52536.

33. Lerner, E. A., M. R. Lerner, C. A. Janeway, Jr., and J. A. Steitz. 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. Proc Natl Acad Sci U S A 78:2737-2741.

34. Lue, N. F., and Z. Li. 2007. Modeling and structure function analysis of the putative anchor site of yeast telomerase. Nucleic Acids Res 35:5213-5222.
35. Lukowiak, A. A., A. Narayanan, Z. H. Li, R. M. Terns, and M. P. Terns. 2001. The snoRNA domain of vertebrate telomerase RNA functions to localize the RNA within the nucleus. RNA 7:1833-1844.
36. Mitchell, J. R., E. Wood, and K. Collins. 1999. A telomerase component is defective in the human disease dyskeratosis congenita. Nature 402:551-555.
37. Mochizuki, Y., J. He, S. Kulkarni, M. Bessler, and P. J. Mason. 2004. Mouse dyskerin mutations affect accumulation of telomerase RNA and small nucleolar RNA, telomerase activity, and ribosomal RNA processing. Proc Natl Acad Sci U S A 101:10756-10761.
38. Morris, G. E. 2008. The Cajal body. Biochim Biophys Acta 1783:2108-2115.
39. Nizami, Z., S. Deryusheva, and J. G. Gall. 2010. The Cajal body and histone locus body. Cold Spring Harb Perspect Biol 2:a000653.
40. Platani, M., I. Goldberg, A. I. Lamond, and J. R. Swedlow. 2002. Cajal body dynamics and association with chromatin are ATP-dependent. Nat Cell Biol 4:502-508.
41. Pogacic, V., F. Dragon, and W. Filipowicz. 2000. Human H/ACA small nucleolar RNPs and telomerase share evolutionarily conserved proteins NHP2 and NOP10. Mol Cell Biol 20:9028-9040.
42. Rippe, K. 2007. Dynamic organization of the cell nucleus. Curr Opin Genet Dev 17:373-380.
43. Rouda, S., and E. Skordalakes. 2007. Structure of the RNA-binding domain of telomerase: implications for RNA recognition and binding. Structure 15:1403-1412.
44. Sleeman, J. E., P. Ajuh, and A. I. Lamond. 2001. snRNP protein expression enhances the formation of Cajal bodies containing p80-coilin and SMN. J Cell Sci 114:4407-4419.
45. Tejera, A. M., M. Stagno d'Alcontres, M. Thanasoula, R. M. Marion, P. Martinez, C. Liao, J. M. Flores, M. Tarsounas, and M. A. Blasco. 2010. TPP1 is required for

TERT recruitment, telomere elongation during nuclear reprogramming, and normal skin development in mice. Dev Cell 18:775-789.

46. Tomlinson, R. L., E. B. Abreu, T. Ziegler, H. Ly, C. M. Counter, R. M. Terns, and M. P. Terns. 2008. Telomerase reverse transcriptase is required for the localization of telomerase RNA to cajal bodies and telomeres in human cancer cells. Mol Biol Cell 19:3793-3800.

47. Tomlinson, R. L., T. D. Ziegler, T. Supakorndej, R. M. Terns, and M. P. Terns. 2006. Cell cycle-regulated trafficking of human telomerase to telomeres. Mol Biol Cell 17:955-965.

48. Venteicher, A. S., E. B. Abreu, Z. Meng, K. E. McCann, R. M. Terns, T. D. Veenstra, M. P. Terns, and S. E. Artandi. 2009. A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. Science 323:644-648.

49. Venteicher, A. S., Z. Meng, P. J. Mason, T. D. Veenstra, and S. E. Artandi. 2008. Identification of ATPases pontin and reptin as telomerase components essential for holoenzyme assembly. Cell 132:945-957.

50. Wang, F., E. R. Podell, A. J. Zaug, Y. Yang, P. Baciu, T. R. Cech, and M. Lei. 2007. The POT1-TPP1 telomere complex is a telomerase processivity factor. Nature 445:506-510.

51. Wen, J., Y. S. Cong, and S. Bacchetti. 1998. Reconstitution of wild-type or mutant telomerase activity in telomerase-negative immortal human cells. Hum Mol Genet 7:1137-1141.

52. Whitehead, S. E., K. W. Jones, X. Zhang, X. Cheng, R. M. Terns, and M. P. Terns. 2002. Determinants of the interaction of the spinal muscular atrophy disease protein SMN with the dimethylarginine-modified box H/ACA small nucleolar ribonucleoprotein GAR1. J Biol Chem 277:48087-48093.

53. Xin, H., D. Liu, M. Wan, A. Safari, H. Kim, W. Sun, M. S. O'Connor, and Z. Songyang. 2007. TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. Nature 445:559-562.

54. Zaug, A. J., E. R. Podell, and T. R. Cech. 2008. Mutation in TERT separates processivity from anchor-site function. Nat Struct Mol Biol 15:870-872.

55. Zaug, A. J., E. R. Podell, J. Nandakumar, and T. R. Cech. 2010. Functional interaction between telomere protein TPP1 and telomerase. Genes Dev 24:613-622.

56. Zhu, Y., R. L. Tomlinson, A. A. Lukowiak, R. M. Terns, and M. P. Terns. 2004. Telomerase RNA accumulates in Cajal bodies in human cancer cells. Mol Biol Cell 15:81-90.

Chapter 4:
Discussion

Telomerase is recruited to telomeres via dynamic interactions with recruitment factors at Cajal bodies and telomeres.

The studies detailed here have provided tremendous insight into the molecular basis of telomerase trafficking in human cancer cells. As previously stated, past investigations have shown that telomerase components follow a well defined trafficking pathway (8). Our results indicate that the trafficking events are facilitated by interactions between factors intrinsic to telomerase components and at least one factor residing at telomeres. These key factors include the hTR CAB box, hTERT N-DAT domain, TCAB1, and the TPP1-OB fold, (1, 10). Due to the interdependent nature of primary telomerase components (hTR and hTERT) in facilitating recruitment, domains required for enzyme assembly would obviously be critical to recruitment as well (6). However, this has not been independently tested. For sometime, a strong correlation between Cajal bodies and regulation of telomerase recruitment has been apparent (2, 14). We have also reported that telomerase trafficking to Cajal bodies is a requirement for subsequent trafficking to telomeres (2).

The studies detailed in this dissertation have taken our inquires into the role of Cajal bodies in telomerase trafficking a bit further. We are the first to simultaneously visualize telomerase at telomeres along with Cajal body-like foci we call, TIBs. These new studies reveal that these bodies may be a new class of coilin-positive body, as they contain only telomerase specific components. Whether these bodies are derived from traditional Cajal bodies is yet to be determined. However, our previous work shows that telomerase trafficking to endogenous Cajal bodies is a required step in the recruitment pathway (2). Our recent discovery of telomerase component, TCAB1, offers another link between Cajal bodies and telomerase biogenesis/delivery (9, 10). TCAB1 interacts with the hTR CAB box facilitating Cajal body/telomere trafficking and subsequent *in vivo* telomerase activity.

Controlled recruitment of telomerase components to the telomere provides an excellent means of regulating telomerase action, restricting telomerase activity to the correct point in the cell cycle. This may insure that telomerase does not act on double

stranded breaks (DSBs), causing genomic instability. The recruitment pathway observed by our group is consistent, and readily observed in both telomerase positive cancers and immortalized cell lines (6, 8, 14). This recruitment process links telomerase delivery to the control mechanisms that govern the cell cycle, however, the exact molecular basis for this link remains unknown. While we have identified the key factors in this process, a better understanding of how these factors interact to facilitate recruitment may prove more valuable in the development of new therapeutic avenues for telomerase positive cancers.

Although interdependent, hTR and hTERT make unique contributions to telomere recruitment

We determined that hTERT expression was required to facilitate hTR localization shortly after our first observations of nuclear hTR foci (6). Although technical limitations prevented the detection of hTERT at these nuclear foci, the requirement of hTERT was the first clue that both primary telomerase components must assemble to facilitate recruitment. The discovery of the CAB box in hTR, and it's subsequent role in both Cajal body and telomere recruitment, would later illustrate that hTERT was not the sole contributor to telomerase recruitment (9, 10). However, the question remained as to what specific contributions were made by hTERT.

Our recent findings that the hTERT N-DAT domain is required for telomerase recruitment to telomeres show that each component displays a unique role in a multistep recruitment process (see chapter 3, Fig. 19). With no clear contribution to Cajal body localization, it is a fair assumption that hTERT depends on the hTR CAB box for recruitment to Cajal bodies. It can also be inferred that hTR depends upon the hTERT N-DAT domain for recruitment to telomeres, as it has no clear telomere recruitment signal. However, the question remains as to why hTR requires hTERT for recruitment to Cajal bodies. This requirement suggests that recognition of the higher order structure of assembled telomerase enzyme is required at the Cajal body localization step. The fact that no single telomerase fragment is sufficient for telomerase recruitment supports the notion

that higher order structure is required at some point in telomerase trafficking (see chapter 3, Fig. 17).

On another note, hTERT appears be critical in initiating the induction of TIBs (see chapter 3, Figs. 15, 16, 19). Our analysis of N-DAT mutants suggests that the domain is required for induction, and subsequent telomerase delivery to telomeres. This induction process may explain the necessity of hTERT at the Cajal body(6). In any case, the role of the Cajal body and TIBs in telomerase trafficking, recruitment and regulation is an interesting topic warranting further investigation.

TCAB1 must also be considered in this aspect of telomerase recruitment. This secondary telomerase component associates with all active telomerase and is required for in vivo telomerase activity, telomere maintenance, and cellular immortalization (9, 10). TCAB1 also happens to be required for sufficient telomerase trafficking to both Cajal bodies and telomeres (9, 10). We suspect that TCAB1 interacts with the CAB box to facilitate Cajal body localization. As part of the telomerase enzyme, hTERT may be required to stabilize interactions between TCAB1 and the rest of the telomerase enzyme. Biochemical interaction assays should eventually be employed to further characterize interactions between TCAB1 and hTR. Performing these assays in the presence and absence of hTERT would allow us to determine if hTERT is needed for such interactions.

Cajal bodies and telomerase induced bodies (TIBs) play a critical role in telomerase delivery to telomeres

Previous observations have suggested that Cajal bodies are involved telomerase assembly/delivery (3, 8, 14). Investigations carried out in this thesis continue to support this notion. The aforementioned discovery of T-CAB1 and the proposed role for the protein in recruitment activities is just one example. We have also uncovered novel coilin-positive bodies, which we have termed TIBs (see chapter 3, Figs. 15, 19). These TIBs are derived in response to high levels of primary telomerase components (see chapter 3, Figs. 13, 14). These bodies contain telomerase specific components (hTR, hTERT, TCAB1, and dyskerin) (see chapter 3, Fig. 16). Although TIBs are positive for

coilin, the primary constituent of Cajal bodies, they are devoid of all other traditional Cajal body constituents (scaRNAs, snRNP components, snoRNP components, etc.) (see chapter 3, Fig. 16). When TIBs are induced, traditional Cajal bodies all but completely disappear (see chapter 3, Fig. 16). In addition to this, the observation of these bodies at telomeres leads us to argue that TIBs break away from traditional Cajal bodies to facilitate telomerase delivery to telomeres (see chapter 3, Fig. 15). This may suggest that TIBs are derived from the preexisting pool of traditional Cajal bodies. Alternatively, these observations may be indicative of a physical interaction between telomerase and coilin, leading to a loss of coilin from CBs as telomerase is delivered to telomeres. In any case, our observations of the N-DAT mutant illustrate that the induction and localization to these novel bodies is a requirement for subsequent telomerase delivery to telomeres.

First, we must determine if the "Cajal bodies" we have observed at telomeres in the past are, in fact, Cajal bodies or TIBs. We have observed coilin-positive foci at telomeres in untreated HeLa cells, and VA13 stably expressing telomerase (1, 8). Now that we have some evidence that these may actually be TIBs it would make sense to go back and determine if we have been observing them for quite some time. The existence of TIBs in cells with stable levels of telomerase would confirm that TIBs are not an artifact of ectopic overexpression. If it is determined that TIBs are present at telomeres in these stable lines, cell synchronization coupled with further Cajal body compositional analysis could determine when telomerase associates with Cajal bodies versus TIBs. This knowledge would subsequently contribute to our understanding of the role of traditional Cajal bodies and TIBs in telomerase recruitment. In any case, our work has shown that traditional Cajal bodies are critical in the telomerase recruitment pathway. However, in this dissertation work, we have shown that the induction of a new class of coilin-positive body appears to be just as important.

Additionally, it is yet to be determined why Cajal bodies or TIBs are not observed in immortalized somatic cell lines (6). Despite the presence of both telomerase components, and telomere maintenance, neither TIBs nor Cajal bodies are observed in these cell lines (6). This may be a telomerase levels issue. It has already been shown that the size and number of Cajal bodies reflects the cellular levels of common Cajal body constituents (30-32. Perhaps the telomerase levels in these lines are not sufficient to

warrant Cajal bodies large enough for detection. One other explanation is that they simply are not there. We have shown that in mouse cells, where telomerase activity is common place, there seems to be no clear need for Cajal bodies (7). Perhaps, the need for Cajal bodies is specific to human cancers. Cajal bodies may have been pulled into the pathway as a compensatory measure to add regulation to illegitimately activated telomerase activity. Alternatively, Cajal bodies may be required to increase the efficiency of telomerase delivery/assembly in a cellular background otherwise unfavorable to the process.

Although, we do not have a definitive answer as to why coilin-positive bodies are required in the telomerase recruitment pathway specifically of human cancers, the correlation between these bodies and recruitment activities is too strong to be ignored. Additionally, we have found evidence to suggest that processing of telomerase in Cajal bodies is involved in determining whether the enzyme acts processively or distributively at telomere ends (see supplemental chapter III). Further investigations into the role of the Cajal body/TIBs in the recruitment process will one day contribute to our holistic understanding of telomerase regulation.

TIN2 tethered TPP1 recruits telomerase to the telomere end

At least two shelterin proteins have gained notoriety as positive regulators of telomerase activity (11, 12). While this flies in the face of conventional wisdom, from a functional perspective, it makes sense. Core telomere binding proteins must protect telomeres yet still provide flexibility to allow for telomerase accessibility during at least one point in the cell cycle. Thus, our identification of TIN2-tethered TPP1 as a recruitment factor was somewhat unsurprising (1) (see chapter 2). We have found that both TPP1 and TIN2 are required to facilitate telomerase recruitment to telomeres (1) (see chapter 2, Figs. 4, 6, 7). Our studies specifically implicate the TPP1 OB-fold as the recruitment domain at the telomere end (1) (see chapter 2, Fig. 9). We have argued that rather than functioning as a direct recruitment factor, TIN2 is required to stabilized TPP1 at the telomere end. However, there is some work that needs to be done to determine if

the TPP1-OB fold is the sole recruitment factor among shelterin components. We have collected some preliminary evidence that the loss of telomerase trafficking to telomeres associated with TIN2 knockdown can be rescued by tethering the TPP1-OB fold to the telomere end. There is also some evidence that that tethering TPP1–OB fold to the telomere can increase telomerase recruitment in HeLa cells. These kinds of experiments will conclusively identify TPP1 as the telomerase recruitment factor at the telomere end.

Although, TPP1 has been identified as a recruitment factor, there is still plenty of work to be done to further characterize the role of the protein. First, any interactions between TPP1 and hTERT need to be confirmed and evaluated. Although there has been some evidence presented that these proteins do interact, more conclusive evidence is needed (12, 13). *In vitro* binding assays between the TPP1-OB fold, hTERT, and stable hTERT sub-fragments, would go a long way in characterizing the proposed interactions.

Next, it is also important to know what changes occur within the shelterin complex to allow for telomerase recruitment rather then inhibition. Through out most of the cell cycle shelterin must restrict telomerase access to the telomere. However, immediately following replication, there must be some kind of dynamic switch that allows for telomerase recruitment. This switch may be indicative of a change in the conformation of the complex, or a change to a new subcomplex, exposing the TPP1-OB fold. Determining what facilitates this change provides a new avenue of investigation. Identifying what initiates this switch could provide the link between cell cycle control mechanisms and telomerase regulation.

We propose that post-translational modifications of core telomere binding proteins modulate their regulation of telomerase recruitment to the telomere. Cycles of phosphorylation and dephosphorylation among nuclear proteins play critical roles in cell cycle maintenance and control. Phosphorylation by any of a number of cell cycle and DNA damage specific kinases may provide an excellent means by which to couple the proposed recruitment activities of shelterin with the regulation of the cell cycle. Preliminary bioinformatics predictions from Ying Xu's group at the University of Georgia have already identified several consensus sequences in TPP1, corresponding to kinases involved in both DNA damage and cell cycle control. TEBP-β, the ciliate homolog of TPP1, has been found to undergo cycles of phosphorylation and

dephosphorylation correlating with the cell cycle (5). In the same studies cyclin-dependent kinase (CDK)2 was identified as the kinase involved in phosphorylating TEBP-β (5). CDK2 is just one of many CDKs which control the events of the cell cycle. CDK2 is specifically critical for entry into S-phase. The dynamic changes in TEBP-β phosphorylation observed in *O. nova* have been linked to telomerase recruitment to the telomere end *(5)*. Similarly, CDK1 phosphorylates CDC13 as a key step in the regulation of telomerase recruitment in budding yeast (4). Could similar phenomena occur with TPP1 in humans? The multiple similarities in function and structure between TEBP-β and TPP1 lead us to theorize that this may be true (11, 12).

Future investigations should include a series of studies to evaluate whether or not the components of the shelterin complex are phosphorylated as a product of the cell cycle. Additional inquiry into the consequences of such modifications on the recruitment of telomerase to telomeres would also be needed. Parallel studies based in autoradiography and mass spectrometry should provide us with both a quantitative and qualitative means of studying *in vivo* phosphorylation of shelterin targets.

Last, there needs to be some functional assays performed to assess the significance of the trafficking patterns observed by our group. It needs to be determined if the foci observed at telomeres are truly indicative of increased telomerase activity and subsequent telomere maintenance. For some time it has been reported that the loss of shelterin components leads to rapid telomere lengthening. However, in many cases we are actually seeing a loss of telomerase from telomere ends. We need to reconcile these seemingly opposing observations. We will not be able make clear conclusions on the consequences of the various observed trafficking patterns until we can combine our imaging techniques with telomere length, and long term cellular proliferation assays. Unfortunately, we are limited in this capacity due to the long-term secondary effects on telomere stability and/or cell viability following loss of shelterin components and mutant hTERT expression.

In conclusion, our investigations have contributed greatly to the field's understanding of telomerase trafficking in cancer cells. Our findings have yielded a clear model for recruitment through interactions between telomerase and recruitment factors residing at telomeres. Now that the key factors in this process have been identified,

further functional analysis can be employed to reveal how these factors interact, and/or modified to facilitate telomerase regulation and recruitment.

References

1. Abreu, E., E. Aritonovska, P. Reichenbach, G. Cristofari, B. Culp, R. M. Terns, J. Lingner, and M. P. Terns. 2010. TIN2-tethered TPP1 recruits human telomerase to telomeres in vivo. Mol Cell Biol 30:2971-2982.
2. Cristofari, G., E. Adolf, P. Reichenbach, K. Sikora, R. M. Terns, M. P. Terns, and J. Lingner. 2007. Human telomerase RNA accumulation in Cajal bodies facilitates telomerase recruitment to telomeres and telomere elongation. Mol Cell 27:882-889.
3. Jady, B. E., P. Richard, E. Bertrand, and T. Kiss. 2006. Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. Mol Biol Cell 17:944-954.
4. Li, S., S. Makovets, T. Matsuguchi, J. D. Blethrow, K. M. Shokat, and E. H. Blackburn. 2009. Cdk1-dependent phosphorylation of Cdc13 coordinates telomere elongation during cell-cycle progression. Cell 136:50-61.
5. Paeschke, K., T. Simonsson, J. Postberg, D. Rhodes, and H. J. Lipps. 2005. Telomere end-binding proteins control the formation of G-quadruplex DNA structures in vivo. Nat Struct Mol Biol 12:847-854.
6. Tomlinson, R. L., E. B. Abreu, T. Ziegler, H. Ly, C. M. Counter, R. M. Terns, and M. P. Terns. 2008. Telomerase reverse transcriptase is required for the localization of telomerase RNA to cajal bodies and telomeres in human cancer cells. Mol Biol Cell 19:3793-3800.
7. Tomlinson, R. L., J. Li, B. R. Culp, R. M. Terns, and M. P. Terns. 2010. A Cajal body-independent pathway for telomerase trafficking in mice. Exp Cell Res 316:2797-2809.
8. Tomlinson, R. L., T. D. Ziegler, T. Supakorndej, R. M. Terns, and M. P. Terns. 2006. Cell cycle-regulated trafficking of human telomerase to telomeres. Mol Biol Cell 17:955-965.

9. Tycowski, K. T., M. D. Shu, A. Kukoyi, and J. A. Steitz. 2009. A conserved WD40 protein binds the Cajal body localization signal of scaRNP particles. Mol Cell 34:47-57.
10. Venteicher, A. S., E. B. Abreu, Z. Meng, K. E. McCann, R. M. Terns, T. D. Veenstra, M. P. Terns, and S. E. Artandi. 2009. A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. Science 323:644-648.
11. Wang, F., E. R. Podell, A. J. Zaug, Y. Yang, P. Baciu, T. R. Cech, and M. Lei. 2007. The POT1-TPP1 telomere complex is a telomerase processivity factor. Nature 445:506-510.
12. Xin, H., D. Liu, M. Wan, A. Safari, H. Kim, W. Sun, M. S. O'Connor, and Z. Songyang. 2007. TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. Nature 445:559-562.
13. Zaug, A. J., E. R. Podell, J. Nandakumar, and T. R. Cech. 2010. Functional interaction between telomere protein TPP1 and telomerase. Genes Dev 24:613-622.
14. Zhu, Y., R. L. Tomlinson, A. A. Lukowiak, R. M. Terns, and M. P. Terns. 2004. Telomerase RNA accumulates in Cajal bodies in human cancer cells. Mol Biol Cell 15:81-90.

Supplemental Chapter I:

Telomerase Reverse Transcriptase Is Required for the Localization of Telomerase RNA to Cajal Bodies and Telomeres in Human Cancer Cells

---

Rebecca L. Tomlinson[*], Eladio B. Abreu[*], Tania Ziegler[*], Hinh Ly[†], Christopher M. Counter[‡], Rebecca M. Terns[*], and Michael P. Terns[*]

Published in Molecular Biology of the Cell 19, 3793–3800, September 2008.

Abstract

Telomere maintenance by telomerase is critical for the unlimited division potential of most human cancer cells. The two essential components of human telomerase, telomerase RNA (hTR) and telomerase reverse transcriptase (hTERT), are recruited from distinct subnuclear sites to telomeres during S phase. Throughout the remainder of the cell cycle hTR is found primarily in Cajal bodies. The localization of hTR to Cajal bodies and telomeres is specific to cancer cells where telomerase is active and is not observed in primary cells. Here we show that the trafficking of hTR to both telomeres and Cajal bodies depends on hTERT. RNA interference–mediated depletion of hTERT in cancer cells leads to loss of hTR from both Cajal bodies and telomeres without affecting hTR levels. In addition, expression of hTERT in telomerase-negative cells (including primary and ALT cancer cell lines) induces hTR to localize to both sites. Factors that did not stimulate hTR localization in our experiments include increased hTR RNA levels and Cajal body numbers, and expression of SV40 large T antigen and oncogenic Ras. Our findings suggest that the trafficking of telomerase to Cajal bodies and telomeres in cancer cells correlates with and depends on the assembly of the enzyme.

Introduction

Telomerase is a ribonucleoprotein (RNP) reverse transcriptase that utilizes a short motif within its integral RNA subunit (telomerase RNA or TR) to synthesize the DNA repeats that form the core of the telomere (1). In humans, the enzyme's activity is restricted to early prenatal development and the resulting telomeric repeats last throughout the normal lifespan of the organism (2, 3). Most adult somatic tissues contain low or undetectable levels of the catalytic telomerase reverse transcriptase (hTERT) and telomerase activity, resulting in shortening of the telomeres and a limited replicative capacity (4, 5, 6, 3, 7, 8). Although relatively inactive in the majority of adult somatic tissues, telomerase is reactivated in over 90% of human cancers, and telomere maintenance by telomerase is critical for the continued proliferation of these cells (6, 9, 10).

Previously, our lab and that of Tamas Kiss reported cell cycle-regulated trafficking of hTR (and hTERT) in cancer cells that appears to reflect a cellular mechanism by which human telomerase activity is controlled (11, 12). The data indicate that access of hTR and hTERT to telomeres is restricted to S phase (11, 12) when telomere synthesis occurs (13, 14). hTR is found in Cajal bodies in cancer cells during most of the cell cycle (11, 12). hTERT is similarly mobilized from separate nuclear foci to telomeres at S phase in human cancer cells (11, 12). In addition, during S phase near the time of telomere localization, both hTR and hTERT can be observed in foci directly associated with Cajal bodies (11, 12). Cajal bodies have been postulated to serve as sites of telomerase maturation and assembly and to deliver active telomerase to the telomere (15, 11, 16, 17, 12, 18). Although hTR is expressed in primary (normal) human cells, it does not accumulate at Cajal bodies, telomeres, or any other intranuclear structures and is instead found diffusely distributed throughout the nucleoplasm (16).

Here, we have taken advantage of the differences between normal and cancer cells to better understand the trafficking of hTR. We examined a variety of factors that differ between normal and cancer cells in order to identify factors that impact hTR localization. Our results indicate that hTERT is a key determinant in hTR trafficking and is essential for the localization of hTR to both Cajal bodies and telomeres.

Materials and Methods

Cell Culture and Transfection

HeLa, VA13, VA13+hTR, VA13+hTR+hTERT, GM847, and GM847+hTERT cells were grown on coverslips in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (FCS; Mediatech, Manassas, VA). IIICF-T/C3 cells (19) were grown in RPMI (American Type Culture Collection [ATCC], Manassas, VA) supplemented with 10% FCS. IMR90 and IMR90+hTERT primary fibroblasts were grown in minimum essential Eagle's medium (ATCC) with 10% FCS. MCF7 cells were cultured in minimum essential Eagle's media supplemented with 0.01 mg/ml bovine insulin (Sigma-Aldrich, St. Louis, MO) and 10% FCS. All human mammary epithelial (HME) cells (20) were grown in

MEGM media supplemented with bovine pituitary extract (BPE; Cambrex, Walkersville, MD). All cells were cultured at 37°C with 5% CO_2. To increase Cajal body numbers in primary cells, IMR90 and HME cells were cultured at 32°C for 24 h before fixation as described (21). Transfections were carried out using Fugene transfection reagent, according to the manufacturers protocol (Roche, Indianapolis, IN). For hTR overexpression, cells were transfected with construct encoding hTR under control of the U1 promoter (22). For hTERT RNAi, cells were transfected with an shRNA against hTERT or empty vector control plasmid (7). Twenty-four hours after transfection, cells were selected with 1 μg/ml puromycin for 48 h before analysis.

hTR Fluorescence In Situ Hybridization

hTR fluorescence in situ hybridization (FISH) was performed using a combination of three cy3-conjugated DNA probes complementary to different regions of telomerase RNA (nts. 128–183, 331–383, and 393–449) essentially as described (16, 12). Cells were grown on coverslips overnight and then washed once with 1x phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, and 1.4 mM KH_2PO_4, pH7.4) and fixed with 4% formaldehyde (Electron Microscope Sciences, Fort Washington, PA), 10% acetic acid, and 1x PBS for 10 min at room temperature. After two PBS washes, cells were permeablized in 70% ethanol overnight at 4°C. When hTR FISH was combined with visualization of green fluorescent protein (GFP), cells were fixed in 4% formaldehyde in 1x PBS for 10 min at room temperature. After two PBS washes, cells were permeablized in 0.2% Triton X-100 (Sigma-Aldrich) in 1x PBS for 5 min at 4°C. Cells were rinsed twice in 1x PBS and once in 50% formamide (Sigma-Aldrich), 2x SSC before FISH.

Immunofluorescence

After hTR FISH, cells were washed three times with 1x PBS and were incubated with either one or both of the following primary antibodies at the indicated dilution for 1 h at room temperature: mouse anti-p80 coilin to mark Cajal bodies (1:10,000; gift from G. Matera, Case Western Reserve University, Cleveland, OH), mouse anti-TRF2 (1:1000,

Upstate/Millipore, Billerica, MA) or rabbit anti-TRF1 (1:100, gift from Susan Smith, Skirball Institute, New York, NY) to mark telomeres. Cells were washed three times in 1x PBS and then incubated with secondary antibody (1:100 of Cy2 conjugated goat anti-rabbit IgG (H+L), 1:100 cy2 conjugated goat anti-mouse IgG (H+L), and/or 1:100 Cy5 conjugated goat anti-mouse IgG) for 1 h at room temperature. hTERT immunofluorescence (IF) was performed essentially as described (7, 12) using a 1:5000 dilution of 2C4 monoclonal hTERT antibody (Abcam, Cambridge, MA) and a 1:100 dilution of cy2-conjugated goat anti-mouse IgM secondary antibody. All antibodies were diluted in 0.05% Tween-20 in PBS (PBST) and all secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Cells were then subjected to three final 1x PBS washes and mounted in either 90% glycerol, 1 mg/ml p-phenylenediamine, 1x PBS, and 0.1 µg/ml 4'6-diamidino-2-phenylindole (DAPI) or Prolong Gold (Molecular Probes/Invitrogen, Carlsbad, CA).

5-Bromodeoxyuridine Labeling

Before fixation, cells were incubated with 100 µM bromodeoxyuridine (BrdU; Sigma-Aldrich) for 30 min at 37°C. The cells were fixed as described above and denatured in 70% formamide (Sigma-Aldrich), and 2x SSC for 5 min at 80°C. After three PBS washes, BrdU was detected using a monoclonal BrdU antibody (1:1000, G3G4; Developmental Studies Hybridoma Bank, Iowa City, IA) and aminomethylcoumarin acetate (AMCA)-conjugated secondary antibody (1:100; Jackson ImmunoResearch) for 2 h at room temperature. Both antibodies were diluted in PBST. After three PBS washes, coverslips were mounted as described above. In cases where hTR FISH was to be performed, cells were fixed again in 4% formaldehyde in 1x PBS for 10 min at room temperature and washed twice in PBS.

S phase Synchronization

Synchronous populations of IMR90 and IMR90+TERT cells were obtained by double thymidine block. Cells were treated with 2 mM thymidine (Sigma-Aldrich) for 36 h. Cells

were released by rinsing twice with 1x PBS and incubated in normal growth media for 12 h. Cells were retreated with 2 mM thymidine for another 36 h. At various time points after release, cells were fixed and analyzed by BrdU labeling and FISH.

Microscopy

Images were obtained with the Zeiss Axioskop 2 Mot Plus fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY) at 63x (Plan Apochromat objectives, NA 1.4) using a cooled charge-coupled device Retiga Exi Fast 1394 camera (Qimaging, Burnaby, BC, Canada) and IPLab Spectrum software (Scanalytics, Billerica, MA). Unless otherwise noted, the results reported are from analysis of at least 100 cells and at least two separate experiments.

RNase Protection Analysis

Total RNA samples were prepared from cells using Trizol according to the manufacturer's protocol (Invitrogen). RNase A/T1 protection was carried out as described (23). ^{32}P (UTP)-labeled, antisense U3 and U1 probes were synthesized from EcoRI linearized plasmids (24, 25) using SP6 polymerase. The template for the hTR probe was generated by PCR amplification of an hTR plasmid (26) using the following oligos: 5'-AGCCGCGAGAGTCAGCTTGG-3' and 5'-ATTTAGGTGACACTATAGAGGTGACGGATGCGCACGATC-3'. The antisense hTR probe was generated by in vitro transcription of this PCR product using SP6 polymerase.

RESULTS

Identification of hTERT as a Factor in Nuclear Trafficking of hTR

To better understand the mechanisms governing trafficking of hTR to Cajal bodies and telomeres, we tested a series of factors that distinguish cancer cells (tumor-derived

cancer lines) from normal cells (isolated primary strains), beginning with hTR expression levels.

Although hTR is expressed in both normal and cancer cells, on average cancer cells contain twofold higher levels of hTR than normal cells (27, 28). To determine whether expression of higher levels of hTR could account for hTR accumulation at intranuclear foci (like Cajal bodies and telomeres), we transiently expressed a construct encoding hTR (22) in IMR90 primary lung fibroblasts. On this construct, hTR is expressed from a strong U1 snRNA promoter, which produces elevated levels of functional hTR (22). The construct also encodes a GFP reporter gene, allowing us to determine which cells were transfected. After transfection, no hTR foci were observed in the GFP-positive cells; in fact, hTR exhibited the same diffuse localization pattern in transfected and untransfected cells (Figure 1A). Furthermore, expression of hTR from this same construct stably maintained in VA13 cells (an unusual cell line that lacks endogenous hTR transcripts; 19) produced higher hTR levels than are found in HeLa (cancer) cells (Figure 1B); however, no hTR foci were observed in the stable VA13+hTR lines (Figure 1A). The results indicate that increased hTR levels are not sufficient for localization of hTR into subnuclear structures.

In addition to lower hTR levels, primary cell lines generally contain fewer well-formed Cajal bodies. Less than 10% of primary cells in a given population organize typical Cajal body constituents such as coilin into distinct Cajal bodies (29). In contrast, over 90% of cancer cell lines have at least one Cajal body per nucleus (29). Therefore, we increased Cajal body numbers in HME cells and IMR90 cells by the previously established method of culturing the cells at 32°C (instead of 37°C) for 24 h (21). Data for the HME cells is shown in Figure 1C. We confirmed that culturing at reduced temperature did not disrupt hTR localization in a cancer cell line (MCF7 breast cancer cells, Figure 1C). As expected, growing primary cells at 32°C produced detectable Cajal bodies (monitored by coilin IF) in over 40% of cells examined. However, no change in hTR localization was detectable in the HME and IMR90 cells grown at 32°C (Figure 1C and data not shown), indicating that the presence of well-formed Cajal bodies is also not sufficient to bring about hTR localization to Cajal bodies (or other subnuclear structures).

Finally, we addressed these two factors in combination in the ALT (alternative lengthening of telomeres) cell lines IIICF-T/C3 and GM847. ALT cells are immortalized cell lines that do not have active telomerase and instead maintain telomeres by a telomerase-independent mechanism (30). Importantly, these two cell lines express levels of hTR comparable to the commonly studied cancer line HeLa, and approximately one-third of these cells contain well-formed Cajal bodies (Figure 1B and 19). As shown in Figure 1D, hTR does not accumulate within Cajal bodies in either IIICF-T/C3 or GM847 cells. These results confirm that hTR levels and the presence of well-formed Cajal bodies do not account for the accumulation of hTR at Cajal bodies and telomeres in cancer cells.

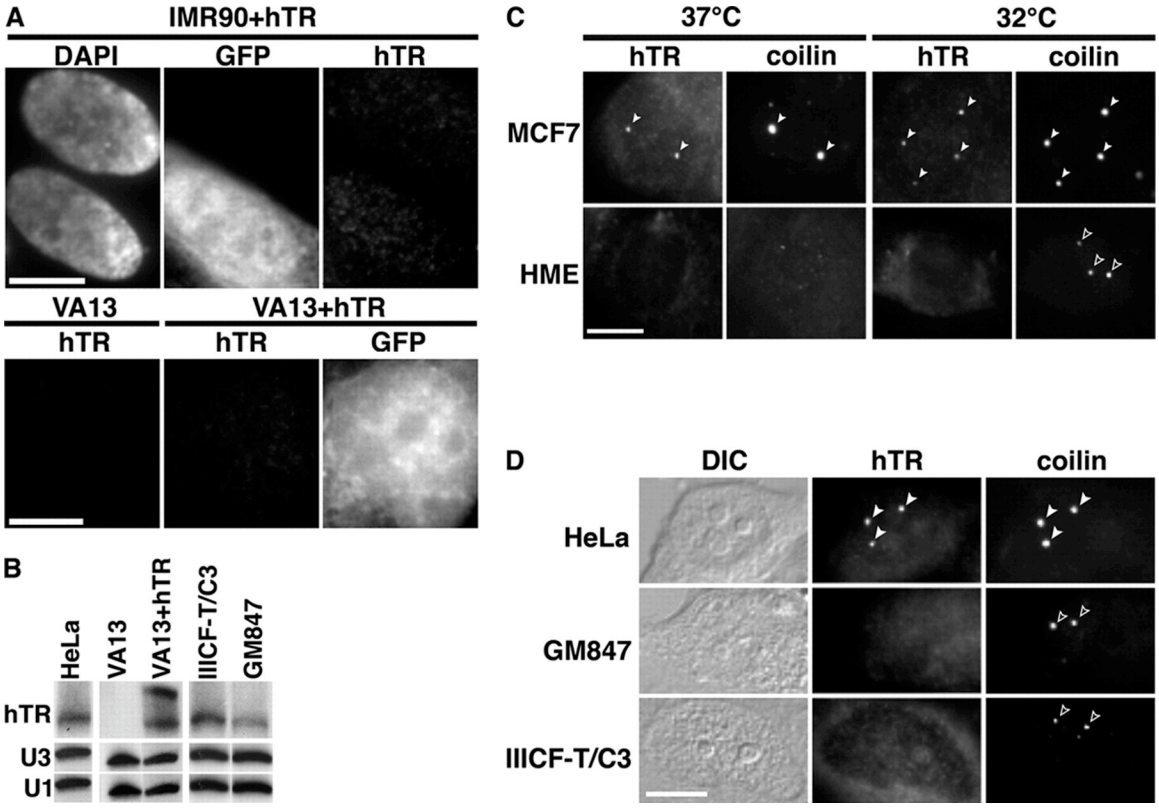


FIGURE 1. Localization of hTR to intranuclear foci is not stimulated by increased levels of hTR or Cajal bodies. (A) Ectopic overexpression of hTR does not lead to localization of hTR to nuclear foci. IMR90 primary fibroblasts transiently transfected with an hTR construct (22), VA13, and VA13 cells stably expressing the same construct were analyzed by hTR FISH. GFP marks cells transfected with the

hTR construct. Nuclei are stained with DAPI. (B) Comparison of hTR levels. RNase protection assay on total RNA from HeLa, IIICF-T/C3, GM847, VA13, and VA13+hTR cells. Note that hTR sometimes migrates as a doublet. U3 snoRNA and U1 snRNA were used as loading controls. (C) Increasing Cajal body numbers does not promote hTR localization. MCF7 breast cancer cells and human mammary epithelial (HME) primary cells were grown at 37°C (normal growth temperature) or 32°C (to increase Cajal body formation). Cells were costained by hTR FISH (hTR) and coilin IF (marker protein for Cajal bodies, coilin). Filled arrowheads, Cajal bodies that contain hTR; open arrowheads, Cajal bodies without hTR. (D) hTR does not localize to Cajal bodies in the ALT cell lines GM847 and IIICF-T/C3. HeLa, GM847, and IIICF-T/C3 cells were coanalyzed for hTR (detected by FISH, hTR) and coilin localization (detected by IF, coilin). Filled arrowheads denote Cajal bodies that contain hTR; open arrowheads indicate Cajal bodies without hTR. DIC, differential interference contrast. Scale bars, 10 μm.

We then examined a series of three gene products that is known to transform primary cells to an oncogenic state: large T antigen of SV40, hTERT, and oncogenic Ras (31, 20). We examined these potential factors in a series of cell lines, each stably expressing one or more of the three proteins (20). The lines were derived from HME primary cells, which do not accumulate hTR in detectable intranuclear foci (Figure 2A, HME). Expression of all three of these gene products in HME cells results in the appearance of hTR in nucleoplasmic foci in approximately one-third of cells examined (Figure 2A, HME + hTERT + large T + onc. Ras). However, we found that coexpression of large T antigen and oncogenic Ras (in the absence of hTERT) did not induce hTR localization (Figure 2A). At the same time, expression of hTERT alone was sufficient for the emergence of hTR foci (Figure 2, HME + hTERT), also producing hTR foci in one-third of cells examined. Finally, a similar pattern was observed in cells expressing both large T antigen and hTERT, whereas expression of large T antigen alone did not induce hTR localization (data not shown). Figure 2B shows that expression of hTERT in the HME cells did not produce a detectable increase in hTR levels. These results suggest that

hTERT is important for the localization of hTR to specific nuclear foci and that this effect is not a consequence of increased hTR levels.

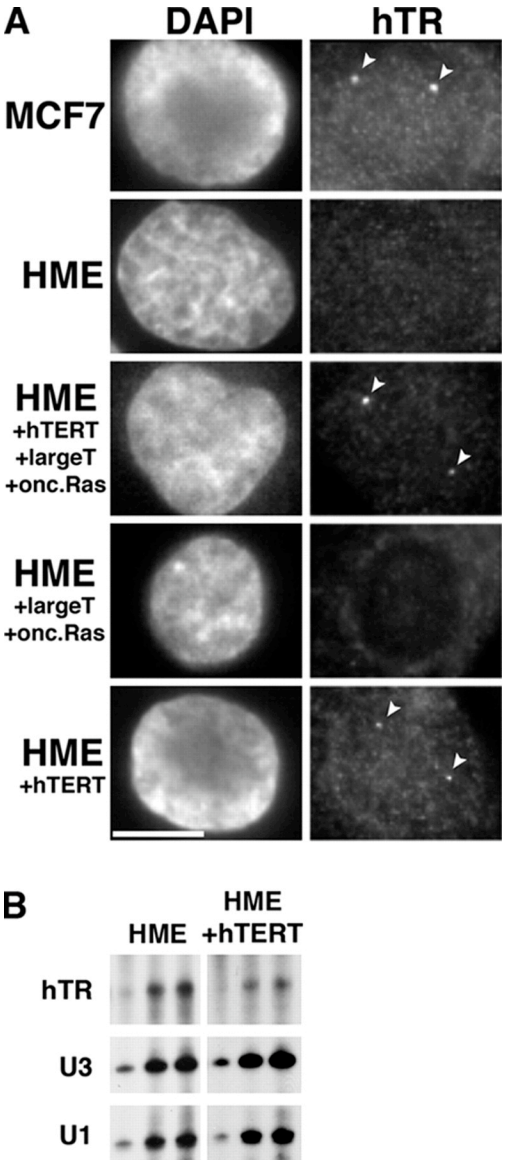


FIGURE 2. hTERT expression leads to accumulation of hTR within nuclear foci. (A) MCF7 breast cancer cells, human mammary epithelial (HME) normal cells, and HME cells expressing combinations of hTERT, large T antigen, and oncogenic (onc.) Ras were subjected to hTR FISH (hTR). Nuclei are stained with DAPI. Arrowheads denote hTR foci. Scale bar, 10 μm. (B) RNase protection assay on total

RNA from HME and HME+hTERT cells. U3 snoRNA and U1 snRNA were used as loading controls.

hTERT Expression Is Essential for Localization of hTR to Cajal Bodies

To determine whether hTERT may play a role in hTR localization specifically to Cajal bodies, we examined the effect of hTERT expression in HME cell lines cultured at 32°C (to increase the frequency of readily detectable Cajal bodies). Although no accumulation of hTR in Cajal bodies was observed in HME cells cultured at 32°C in the absence of ectopic hTERT expression (Figure 1C), we found that approximately half of the Cajal bodies accumulated hTR in the HME cells expressing hTERT (Figure 3). Similar results were observed with IMR90+hTERT cells (Figure 3).

Additionally, we tested the effect of hTERT expression on hTR localization in the ALT line GM847, where Cajal bodies are more prevalent but hTR is also not found in Cajal bodies (Figure 1D). Upon stable expression of hTERT in the GM847 cells, hTR was found in nearly every Cajal body observed (Figure 3). These results suggest that hTERT is important for the localization of hTR to Cajal bodies.

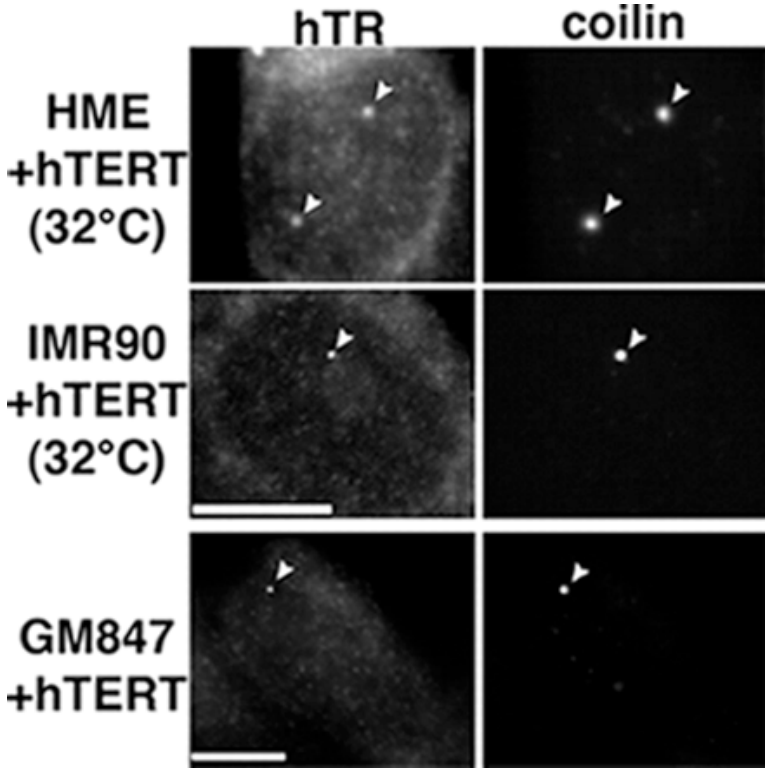


FIGURE 3. Expression of hTERT induces hTR localization to Cajal bodies. HME and IMR90 cells that ectopically express hTERT were cultured at 32°C to increase Cajal body numbers. These cells and GM847 (ALT) cells that stably express hTERT were analyzed by hTR FISH (hTR) and coilin IF (coilin). Arrowheads indicate hTR foci that colocalize with coilin (Cajal bodies). Scale bars, 10 μm.

As a reciprocal approach, we knocked down expression of hTERT in HeLa and MCF7 cancer cells (where hTR is found in Cajal bodies) using RNA interference. Figure 4 shows data from the HeLa cell lines. We expressed a short hairpin RNA (shRNA) against the hTERT message and monitored hTERT knockdown efficiency by IF with an antibody against hTERT (7). In the HeLa cells transfected with an empty vector (and in untransfected cells, data not shown), 5–20 small hTERT foci were observed in over 90% of nuclei (Figure 4A, empty vector). Expression of the hTERT shRNA eliminated detectable hTERT foci in over 70% of cells and reduced hTERT signals in the remainder, indicating effective hTERT knockdown (Figure 4A, hTERT shRNA).

We found that hTERT knockdown disrupted hTR localization to Cajal bodies in the cancer cell lines. We performed hTR FISH and coilin IF on cells expressing the hTERT shRNA and found no detectable hTR within Cajal bodies (or at any other nuclear sites) in over 70% of HeLa cells and reduced hTR signal intensity in other cells (Figure 4B, hTERT shRNA). By comparison, hTR was observed in Cajal bodies in over 80% of untransfected cells and cells transfected with empty vector (Figure 4B, empty vector; and data not shown). Importantly, RNase protection assays show that the effect on hTR localization to Cajal bodies is not attributable to a decline in hTR levels in the cells expressing the hTERT shRNA (Figure 4C). Similar results were seen with hTERT knockdown in MCF7 cancer cells where hTR was not observed in Cajal bodies in over 60% of cells (data not shown). Together, the findings presented here strongly indicate the importance of hTERT in the localization of hTR to Cajal bodies.

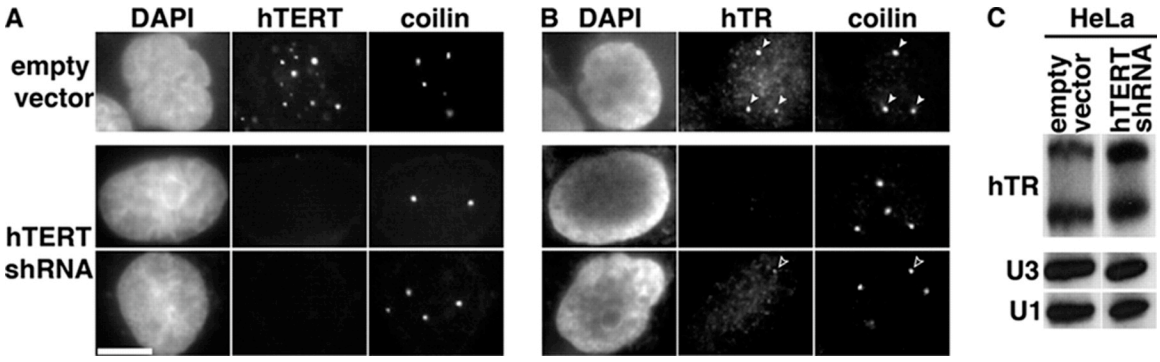


FIGURE 4. hTERT expression is necessary for localization of hTR to Cajal bodies. (A and B) RNA interference mediated depletion of hTERT leads to a loss of hTR within Cajal bodies. HeLa cells were transfected with hTERT shRNA (middle and bottom rows) or an empty vector control (top row). (A) Cells were coanalyzed for hTERT and coilin localization (detected by IF); (B) hTR (detected by FISH) and coilin localization (detected by IF). Data in each set of panels were normalized relative to the empty vector control to allow direct visual comparison. Filled arrowheads, hTR in Cajal bodies; open arrowhead, a Cajal body with reduced level of hTR. DAPI was used to stain the DNA. Scale bar, 10 μm. (C) RNA interference mediated depletion of hTERT does not affect hTR levels. RNase protection assay on total RNA from HeLa cells transfected with hTERT shRNA or empty vector. Note

that hTR sometimes migrates as a doublet. U3 snoRNA and U1 snRNA are used as loading controls.

hTERT Promotes S phase–specific Recruitment of hTR to Telomeres

As shown in Figure 2, we found that hTERT induced the appearance of hTR foci in approximately one-third of normal cells in the absence of Cajal body induction, a pattern suggestive of hTERT-induced hTR localization to telomeres during S phase. First, to determine whether localization of hTR to these foci occurred during S phase, we performed hTR FISH in combination with BrdU labeling on normal cells ectopically expressing hTERT (IMR90+hTERT and HME+hTERT). Figure 5 shows data from the IMR90 cell lines. BrdU is incorporated into the DNA of cells undergoing DNA replication (i.e., cells in S phase) and distinct BrdU staining patterns are found in cells in early, mid, or late S phase (32). Ectopic expression of hTERT in the normal cells resulted in the appearance of hTR foci as was previously observed (Figure 2), and we found that ~70% of the cells that contained the hTR foci were in S phase (Figure 5A, IMR90+hTERT; and data not shown). No hTR foci were found in IMR90 or HME cells lacking ectopic hTERT expression in S phase or any other stage of the cell cycle (Figure 5A, IMR90; and data not shown).

We quantified the relationship of the hTERT-induced hTR foci to the cell cycle in synchronized cells (Figure 5B). We synchronized IMR90 and IMR90+hTERT cells at the G1/S transition using a double thymidine block and analyzed cells at various time points after release by hTR FISH and BrdU labeling (to assess synchronization efficiency). The incidence of hTR foci in IMR90+hTERT cells peaks in mid-S phase (4 h after release from the block, assessed by BrdU staining, 79% of cells in S phase), where we found that 66% of cells contained hTR foci (Figure 5B). By 8 h after release (20% of cells still in S phase; majority of cells in G2), the frequency of cells with hTR foci had dropped to 18%. IMR90 cells do not display hTR foci in the absence of hTERT expression (Figure 5A) and the synchronization procedure did not induce the formation of hTR foci in IMR90

cells (data not shown). Together, the results indicate that hTR localization to the majority of the nuclear foci observed in hTERT-expressing normal cells occurs during S phase. To determine whether the hTERT-induced hTR foci observed during S phase corresponded to telomeres, we performed hTR FISH in combination with IF using antibodies against the telomere-binding proteins TRF1 or TRF2 in IMR90+hTERT and HME+hTERT cells (Figure 6A). We found that hTR is present at telomeres in over 65% of the cells that have hTR foci. Localization of hTR to the telomere was only observed in BrdU-positive cells (data not shown), indicating that it is an S phase–specific event. In addition, we synchronized IMR90+TERT cells and found that greater than 90% of the hTR foci in S phase cells colocalize with telomeres (data not shown).

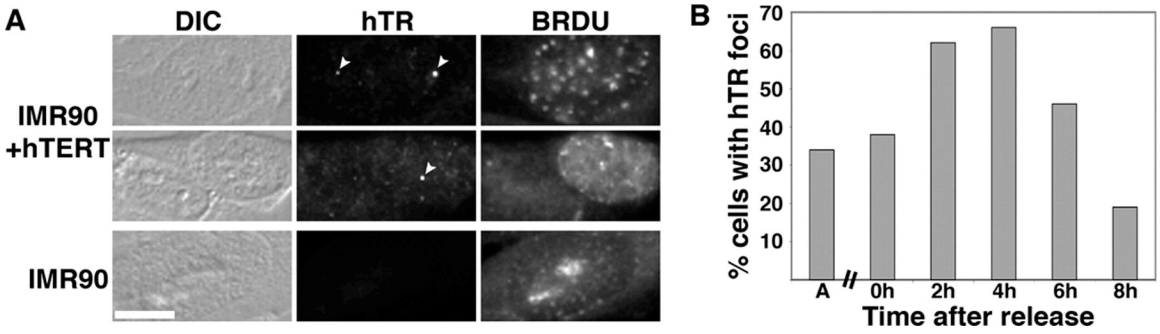


FIGURE 5. hTR foci in normal cells that express hTERT occur primarily in S phase. (A) hTR foci are predominantly found in S phase cells. IMR90 and IMR90+hTERT cells were subjected to hTR FISH (hTR) and BrdU labeling (BRDU). Arrowheads indicate hTR foci, which are present in BrdU-positive IMR90+TERT cells. Note that the BrdU-negative IMR90+hTERT cell in the middle row of panels does not contain hTR foci. DIC, differential interference contrast. Scale bar, 10 μm. (B) The percentage of cells with hTR foci increases in S phase the percentage of cells with hTR foci. The percentage of IMR90+hTERT cells with hTR foci is graphed relative to time after release from a double thymidine block. Data are compiled from over 100 cells and two independent experiments. A, asynchronous cells.

Finally, we examined hTR localization in VA13 ALT cells stably expressing both hTR and hTERT (VA13+hTR+hTERT). As described above, VA13 cell lines are normally devoid of both hTR and hTERT (19) and expression of hTR alone in VA13 cells resulted in no hTR foci (see VA13+hTR, Figure 1A). In striking contrast, stable ectopic expression of hTR and hTERT in these cells results in numerous hTR foci, nearly all of which coincide with a telomere (as indicated by TRF2 staining; Figure 6B).

As shown in Figure 4, hTERT knockdown disrupted the accumulation of hTR in any detectable nuclear foci in cancer cells, indicating that hTERT is required for hTR localization to telomeres (as well as Cajal bodies). Taken together, our data suggest that hTERT expression is necessary and sufficient to enable localization of hTR to the telomere during S phase.

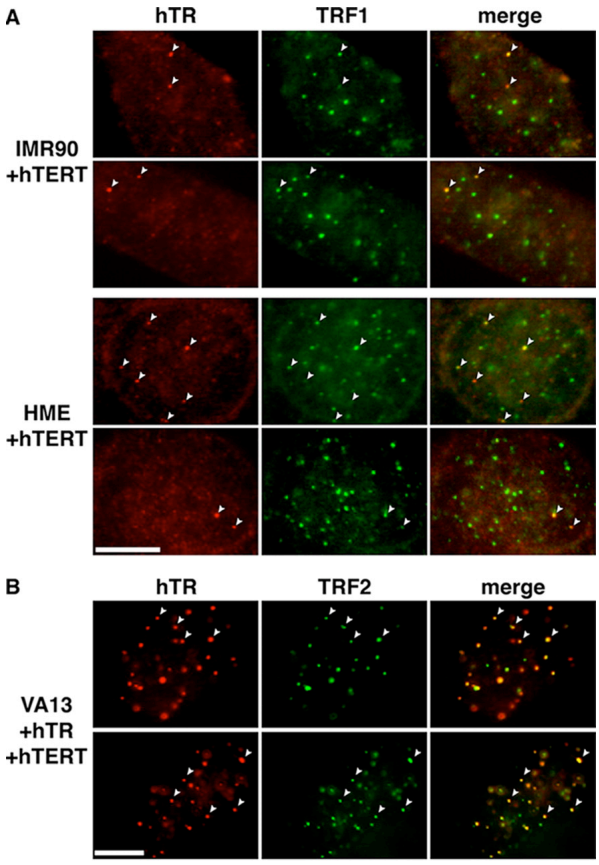


FIGURE 6. hTERT expression is sufficient to stimulate localization of hTR to telomeres. (A) hTR localizes to a subset of telomeres in normal cells that ectopically express hTERT. IMR90+ hTERT (top panels) and HME+hTERT cells were

analyzed by hTR FISH (hTR, red) and TRF1 IF (TRF1, green). Merge panels show superimposition of hTR and TRF1 panels; yellow indicates signal overlap. Some colocalizations of hTR and telomeres are denoted with arrowheads. (B) hTR localizes to most telomeres in VA13+hTR+hTERT cells. hTR FISH (hTR, red) and TRF2 IF (TRF2, green) were performed on VA13 cells that stably express both hTR and hTERT. Arrowheads point to representative examples of hTR and TRF2 colocalizations in each panel. Scale bars, 10 μm.

Discussion

Current evidence suggests that the restriction of human telomerase activity to S phase of the cell cycle is accomplished by regulated trafficking of the enzyme. Access of both hTR and hTERT to the telomere is restricted to S phase (11, 12). Outside of S phase, the two components appear to be held primarily in separate intranuclear compartments: hTR in Cajal bodies and hTERT in other nuclear foci ("TERT foci", 11, 12). Because trafficking appears to be a mechanism for cell cycle–based regulation of the activity of telomerase, we undertook studies to further understand the trafficking of hTR. We found that the localization of hTR to both Cajal bodies and telomeres depends on hTERT. These findings explain the presence of hTR at Cajal bodies and telomeres in the majority of cancer cells but not normal cells and identify hTERT as the molecular basis for this difference. Moreover, our results suggest that the intranuclear trafficking of telomerase is coupled to telomerase biogenesis or activity as described below.

Getting to Telomeres during S phase

hTR and hTERT function together as integral components of the telomerase enzyme. Previously, we and others found that both hTR and hTERT are specifically recruited to subsets of telomeres in cancer cells during S phase (11, 12), the time when telomeres are replicated and presumably extended by telomerase (13, 14, 33). The current studies revealed that the trafficking of hTR to telomeres during S phase depends on hTERT. The simplest model suggested by these findings is that hTR is only transported to

telomeres with hTERT as an assembled complex (Figure 7). Consistent with this concept, we have also found that the localization of hTERT to telomeres depends on expression of telomerase RNA (manuscript in preparation). Moreover, biochemical and genetic studies in *S. cerevisiae* suggest that the localization of TERT (called Est2p in yeast) to telomeres also requires the expression of TR (TLC1 in yeast, 34, 35). This model suggests that telomerase (i.e., hTR and hTERT) assembly occurs before transport to telomeres. However, it is also possible that our results reflect more complicated scenarios wherein hTERT levels indirectly influence the trafficking of hTR or a transient interaction of the two components contributes to hTR localization.

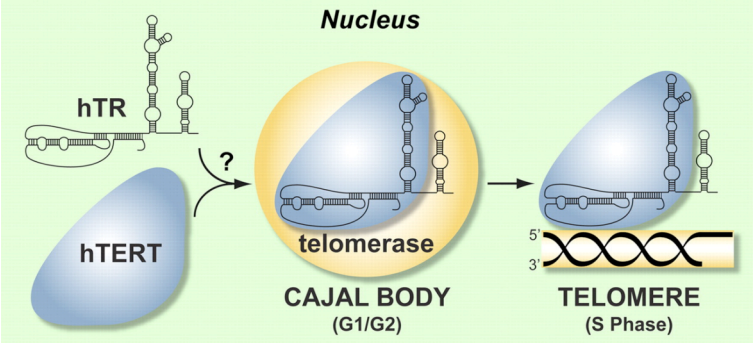


Figure 7.

Localization of human telomerase RNA (hTR) to Cajal bodies and telomeres is linked to assembly with telomerase reverse transcriptase (hTERT). The simplest model that arises from the current data are that assembly of hTR and hTERT is a prerequisite for the trafficking of telomerase to Cajal bodies and telomeres. It is not clear whether hTR and hTERT assemble before or after localization to Cajal bodies (indicated by question mark). See *Discussion* for details.

Cajal Bodies and Telomerase

hTR is observed in Cajal bodies throughout most of the cell cycle in cancer cells (15, 16, 11, 12) and in this study we found that this localization depends on hTERT. Because hTERT does not appear to be present at Cajal bodies outside of S phase (12), it does not seem likely that cotransport to Cajal bodies as an assembled complex accounts for the hTERT dependence of hTR accumulation in Cajal bodies (though, it is possible

that hTERT is present with hTR in Cajal bodies at concentrations below current detection limits). However, the observation that hTR accumulates in Cajal bodies only in the presence of hTERT points to a strong link between Cajal bodies and telomerase biogenesis. Although the mechanism by which hTERT promotes hTR trafficking is unclear, our results indicate that the localization of hTR to Cajal bodies is coordinated with ongoing or potential telomerase assembly (Figure 7).

Based initially on the observation that telomerase RNA accumulates in Cajal bodies, Cajal bodies have been implicated in various aspects of telomerase biogenesis (36; 15, 16, 12). Evidence indicates that these subnuclear structures are general sites of RNP assembly and RNA modification (37, 38, 39, 17). Some evidence suggests that the assembly of telomerase may be mediated by the SMN (survival of motor neurons) complex, an RNP chaperone associated with Cajal bodies (40). SMN has been reported to interact with functional telomerase, and in particular with the telomerase-associated proteins Gar1 and hTERT (41, 42). Our new findings—that hTR does not accumulate in Cajal bodies in cancer cells in the absence of hTERT and that hTERT expression can induce hTR targeting to Cajal bodies in normal cells—strongly support the idea that Cajal body localization is intimately connected to telomerase biogenesis.

The mechanisms involved in the targeting and retention of hTR at Cajal bodies are not fully understood. Mutational analysis has demonstrated the importance of a motif called the CAB (Cajal body) box (ugAG) for localization of hTR to Cajal bodies (15, 18, 43). The CAB box of hTR is located in a terminal loop in the CR7 domain of the molecule (15). Our finding that hTR fails to accumulate at Cajal bodies in cells that do not express hTERT indicates that the function of the CAB box of hTR depends (directly or indirectly) on the hTERT protein. Direct interaction of hTERT with hTR may activate the function of the CAB box. However, the CAB box is also present in a subset of functionally unrelated scaRNAs (small Cajal body RNAs), and it is unlikely that hTERT is required for the Cajal body association of this class of RNAs (44, 45).

Cajal bodies have also been proposed to function in the regulated delivery of telomerase to telomeres (11, 12, 18). Cajal bodies are mobile within the nucleus and have been shown to interact with several chromosomal loci, including telomeres during S phase (37, 39, 11). Recently, we found that mutations in the CAB box element of hTR

that reduce association of hTR with Cajal bodies (15) also reduce the frequency of association of hTR with telomeres and lead to shorter average telomere length (relative to cells expressing a wild-type copy of hTR; 18). Because the CAB box mutations tested did not significantly affect hTR stability or ability to assemble into active enzyme in vivo, the results indicate that accumulation of hTR in Cajal bodies is critical in the localization of telomerase to telomeres.

The results of this study support the emerging paradigm of strong linkages between the physical trafficking pathways and biogenesis pathways of noncoding RNPs (46).

Acknowledgements

We are grateful to the following people for providing cell lines, plasmids, and antibodies: William Hahn (Dana-Farber Cancer Institute, Harvard Medical School; all HME cell lines, GM847+hTERT; 47), Gregory Matera (Case Western Reserve University; HeLa cell line and anti-coilin antibody), Jerry Shay (UT Southwestern; IMR90+hTERT and GM847 cells); Roger Reddel (Children's Medical Research Institute, Sydney; IIICF-T/C3 cells), Michael Pierce (University of Georgia;MCF7 cells); Liz Blackburn (University of California, San Francisco; hTR plasmid for transfection), Kathy Collins (University of California, Berkley; hTR plasmid for RNase protection assay), Elsebet Lund (University of Wisconsin-Madison; antisense U1 plasmid), Tamas Kiss (Laboratoire de Biologie Moleculaire Eucaryote du CNRS; antisense U3 plasmid), and Susan Smith (Skirball Institute; anti-TRF1 antibodies). This work was supported by National Cancer Institute (NCI) Grant R01 CA104676 to M.P.T. and R.M.T, and R.L.T. was supported by a National Institutes of Health (NIH) training grant to the Department of Genetics at University of Georgia (GM07103) and a University of Georgia Dissertation Award. E.B.A. was supported by a University of Georgia GRO award and an NCI Diversity Supplement. H.L. was supported by the American Cancer Society (RSG-06-162-01-GMC). C.M.C. was supported by NIH Grant CA082481 and the Werner and Elaine Dannheisser Fund for Research in the Biology of Aging from the Lymphoma Foundation. C.M.C. is a Leukemia and Lymphoma Society Scholar.

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. Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W., and Shay, J. W. (1996). Telomerase activity in human germline and embryonic tissues and cells. Dev. Genet *18*, 173–179.
3. Cong, Y. S., Wright, W. E., and Shay, J. W. (2002). Human telomerase and its regulation. Microbiol. Mol. Biol. Rev *66*, 407–425. table of contents.
4. Harley, C. B., Futcher, A. B., and Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts. Nature *345*, 458–460.
5. Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W., and Harley, C. B. (1992). Telomere length predicts replicative capacity of human fibroblasts. Proc. Natl. Acad. Sci. USA *89*, 10114–10118.
6. Kim, N. W. *et al.* (1994). Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011–2015.
7. Masutomi, K. *et al.* (2003). Telomerase maintains telomere structure in normal human cells. Cell *114*, 241–253.
8. Shay, J. W., and Wright, W. E. (2005). Senescence and immortalization: role of t elomeres and telomerase. Carcinogenesis *26*, 867–874.
9. Shay, J. W., and Bacchetti, S. (1997). A survey of telomerase activity in human cancer. Eur. J. Cancer *33*, 787–791.
10. Shay, J. W., and Wright, W. E. (2002). Telomerase: a target for cancer therapeutics. Cancer Cell *2*, 257–265.
11. Jady, B. E., Richard, P., Bertrand, E., and Kiss, T. (2006). Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. Mol. Biol. Cell *17*, 944–954.
12. Tomlinson, R. L., Ziegler, T. D., Supakorndej, T., Terns, R. M., and Terns, M. P. (2006). Cell cycle-regulated trafficking of human telomerase to telomeres. Mol. Biol. Cell *17*, 955–965.

13. Ten Hagen, K. G., Gilbert, D. M., Willard, H. F., and Cohen, S. N. (1990). Replication timing of DNA sequences associated with human centromeres and telomeres. Mol. Cell Biol *10*, 6348–6355.
14. Wright, W. E., Tesmer, V. M., Liao, M. L., and Shay, J. W. (1999). Normal human telomeres are not late replicating. Exp. Cell Res *251*, 492–499.
15. Jady, B. E., Bertrand, E., and Kiss, T. (2004). Human telomerase RNA and box H/ACA scaRNAs share a common Cajal body-specific localization signal. J. Cell Biol *164*, 647–652.
16. Zhu, Y., Tomlinson, R. L., Lukowiak, A. A., Terns, R. M., and Terns, M. P. (2004). Telomerase RNA accumulates in Cajal bodies in human cancer cells. Mol. Biol. Cell *15*, 81–90.
17. Matera, A. G., and Shpargel, K. B. (2006). Pumping RNA: nuclear bodybuilding along the RNP pipeline. Curr. Opin. Cell Biol *18*, 317–324.
18. Cristofari, G., Adolf, E., Reichenbach, P., Sikora, K., Terns, R. M., Terns, M. P., and Lingner, J. (2007). Human telomerase RNA accumulation in Cajal bodies facilitates telomerase recruitment to telomeres and telomere elongation. Mol. Cell *27*, 882–889.
19. Bryan, T. M., Marusic, L., Bacchetti, S., Namba, M., and Reddel, R. R. (1997). The telomere lengthening mechanism in telomerase-negative immortal human cells does not involve the telomerase RNA subunit. Hum. Mol. Genet *6*, 921–926.
20. Elenbaas, B. *et al.* (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev *15*, 50–65.
21. Carmo-Fonseca, M., Ferreira, J., and Lamond, A. I. (1993). Assembly of snRNP-containing coiled bodies is regulated in interphase and mitosis-evidence that the coiled body is a kinetic nuclear structure. J. Cell Biol *120*, 841–852.
22. Li, S., Rosenberg, J. E., Donjacour, A. A., Botchkina, I. L., Hom, Y. K., Cunha, G. R., and Blackburn, E. H. (2004). Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA. Cancer Res *64*, 4833–4840.
23. Goodall, G. J., Wiebauer, K., and Filipowicz, W. (1990). Analysis of pre-mRNA processing in transfected plant protoplasts. Methods Enzymol *181*, 148–161.

24. Ganot, P., Caizergues-Ferrer, M., and Kiss, T. (1997). The family of box ACA small nucleolar RNAs is defined by an evolutionarily conserved secondary structure and ubiquitous sequence elements essential for RNA accumulation. Genes Dev *11*, 941–956.
25. Lund, E. (1988). Heterogeneity of human U1 snRNAs. Nucleic Acids Res *16*, 5813–5826.
26. Fu, D., and Collins, K. (2003). Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. Mol. Cell *11*, 1361–1372.
27. Yi, X., Tesmer, V. M., Savre-Train, I., Shay, J. W., and Wright, W. E. (1999). Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels. Mol. Cell Biol *19*, 3989–3997.
28. Yi, X., Shay, J. W., and Wright, W. E. (2001). Quantitation of telomerase components and hTERT mRNA splicing patterns in immortal human cells. Nucleic Acids Res *29*, 4818–4825.
29. Spector, D. L., Lark, G., and Huang, S. (1992). Differences in snRNP localization between transformed and nontransformed cells. Mol. Biol. Cell *3*, 555–569.
30. Henson, J. D., Neumann, A. A., Yeager, T. R., and Reddel, R. R. (2002). Alternative lengthening of telomeres in mammalian cells. Oncogene *21*, 598–610.
31. Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., and Weinberg, R. A. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464–468.
32. O'Keefe, R. T., Henderson, S. C., and Spector, D. L. (1992). Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally defined replication of chromosome-specific alpha-satellite DNA sequences. J. Cell Biol *116*, 1095–1110.
33. Marcand, S., Brevet, V., Mann, C., and Gilson, E. (2000). Cell cycle restriction of telomere elongation. Curr. Biol *10*, 487–490.
34. Taggart, A. K., Teng, S. C., and Zakian, V. A. (2002). Est1p as a cell cycle-regulated activator of telomere-bound telomerase. Science *297*, 1023–1026.
35. Fisher, T. S., Taggart, A. K., and Zakian, V. A. (2004). Cell cycle-dependent regulation of yeast telomerase by Ku. Nat. Struct. Mol. Biol *11*, 1198–1205.

36. Lukowiak, A. A., Narayanan, A., Li, Z. H., Terns, R. M., and Terns, M. P. (2001). The snoRNA domain of vertebrate telomerase RNA functions to localize the RNA within the nucleus. RNA *7*, 1833–1844.

37. Gall, J. G. (2000). Cajal bodies: the first 100 years. Annu. Rev. Cell Dev. Biol *16*, 273–300.

38. Gall, J. G. (2003). The centennial of the Cajal body. Nat. Rev. Mol. Cell Biol *4*, 975–980.

39. Cioce, M., and Lamond, A. I. (2005). Cajal bodies: a long history of discovery. Annu. Rev. Cell Dev. Biol *21*, 105–131.

40. Terns, M. P., and Terns, R. M. (2001). Macromolecular complexes: SMN-the master assembler. Curr. Biol *11*, R862–864.

41. Bachand, F., Boisvert, F. M., Cote, J., Richard, S., and Autexier, C. (2002). The product of the survival of motor neuron (SMN) gene is a human telomerase-associated protein. Mol. Biol. Cell *13*, 3192–3202.

42. Whitehead, S. E., Jones, K. W., Zhang, X., Cheng, X., Terns, R. M., and Terns, M. P. (2002). Determinants of the interaction of the spinal muscular atrophy disease protein SMN with the dimethylarginine-modified box H/ACA small nucleolar ribonucleoprotein GAR1. J. Biol. Chem *277*, 48087–48093.

43. Theimer, C. A., Jady, B. E., Chim, N., Richard, P., Breece, K. E., Kiss, T., and Feigon, J. (2007). Structural and functional characterization of human telomerase RNA processing and cajal body localization signals. Mol. Cell *27*, 869–881.

44. Kiss, T., Fayet, E., Jady, B. E., Richard, P., and Weber, M. (2006). Biogenesis and intranuclear trafficking of human box C/D and H/ACA RNPs. Cold Spring Harb. Symp. Quant. Biol *71*, 407–417.

45. Terns, M., and Terns, R. (2006). Noncoding RNAs of the H/ACA family. Cold Spring Harb. Symp. Quant. Biol *71*, 395–405.

46. Matera, A. G., Terns, R. M., and Terns, M. P. (2007). Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. Nat. Rev. Mol. Cell Biol *8*, 209–220.

47. Counter, C. M., Meyerson, M., Eaton, E. N., Ellisen, L. W., Caddle, S. D., Haber, D. A., and Weinberg, R. A. (1998). Telomerase activity is restored in human cells

by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase. Oncogene *16*, 1217–1222.

Supplemental Chapter II:

A Human Telomerase Holoenzyme Protein Required for Cajal Body Localization and Telomere Synthesis

---

Andrew S. Venteicher,[1,2] Eladio B. Abreu,[3*] Zhaojing Meng,[4*] Kelly E. McCann,[1,5] Rebecca M. Terns,[3] Timothy D. Veenstra,[4] Michael P. Terns,[3] and Steven E. Artandi[1,2,5,6]

Published in Science. 323(5914): 644–648. January 30, 2009. Reprinted here with permission of publisher

Abstract

Telomerase is a ribonucleoprotein (RNP) complex that synthesizes telomere repeats in tissue progenitor cells and cancer cells. Active human telomerase consists of at least three principal subunits, including the telomerase reverse transcriptase (TERT), the telomerase RNA (TERC), and dyskerin. Here, we identify a holoenzyme subunit, TCAB1 (**t** elomerase **Ca** jal **b** ody protein1), uniquely enriched in Cajal bodies, nuclear sites of RNP processing important for telomerase function. TCAB1 associates with active telomerase enzyme, with established telomerase components, and with small Cajal body RNAs involved in modifying splicing RNAs. Depletion of TCAB1 using RNA interference prevents TERC from associating with Cajal bodies, disrupts telomerase-telomere association and abrogates telomere synthesis by telomerase. Thus, TCAB1 controls telomerase trafficking and is required for telomere synthesis in human cancer cells.

Introduction

TERT and TERC comprise the minimal catalytic core of the telomerase enzyme (1), whereas dyskerin is an RNA binding protein that recognizes the H/ACA sequence motif shared by TERC and two groups of non-coding RNAs involved in RNA modification - small Cajal body (sca) RNAs and small nucleolar (sno) RNAs (2, 3). Dyskerin functions in part to support telomerase RNP biogenesis and TERC stability (4, 5). TERT, TERC and dyskerin are all components of active telomerase (6), and mutations in any of these genes can cause the human stem cell disorder dyskeratosis congenita (7). Other potential components of active telomerase include three evolutionarily conserved dyskerin-associated proteins, NOP10, NHP2 and GAR1 (8-10), and EST1A, a homologue of the yeast telomerase protein Est1p (11, 12). However, the size of active human telomerase, estimated in the 0.65 to 2 MDa range (6, 13, 14), suggests the existence of additional components. We reasoned that other dyskerin-associated proteins may be telomerase components, and we therefore sought to purify dyskerin complexes.

Materials and Methods

Dyskerin complex purification and mass spectrometry

Dyskerin with an N-terminal AH3 tag was expressed in and purified from suspension HeLa S3 cells for mass spectrometry as previously described (14). In brief, nuclear extracts dialyzed to 150mM KCl were bound to rabbit IgG resin (Sigma), washed, and eluted with TEV protease. TEV eluants were precleared with mouse IgG resin and then bound again to 12CA5 anti-HA resin. After washing, both mouse IgG (negative control) and anti-HA resins was eluted with denaturing $MgCl_2$ and precipitated for mass spectrometry. All steps were performed at 4°C.

Mass spectrometry

Purified dyskerin complexes were fractionated by SDS-PAGE and stained with Coomassie blue. Gel slices from the entire lane were trypsin-digested and extracted peptides were then analyzed by nanoflow reversed-phase liquid chromatography (RPLC)-tandem mass spectrometry (MS/MS) as described previously (14). In brief, the nanoflow RPLC column was coupled online to a linear ion-trap (LIT)-mass spectrometer (ThermoElectron, San Jose, CA) using the manufacturer's nanoelectrospray source with an applied electrospray potential of 1.5 kV and capillary temperature of 160°C. The LIT-mass spectrometer was operated in a data-dependent mode in which each full MS scan was followed by five MS/MS scans, where the five most abundant molecular ions were dynamically selected for MS/MS using a normalized collisional-induced dissociation (CID) energy of 35%. Dynamic exclusion was utilized to minimize redundant MS/MS acquisition. The CID spectra were analyzed using SEQUEST against a UNIPROT derived human proteome database downloaded from the European Bioinformatics Institute.

Small scale cell culture and retroviral transductions

Adherent HeLa cultures were grown in DMEM/5% newborn calf serum/1% penicillin-streptomycin (PS); 293T cultures were grown in DMEM/10% bovine growth serum/1% PS. Retroviruses were generated from 293T cells by cotransfecting plasmids encoding RSV(Gag+Pol), VSV-G, and the retroviral expression or shRNA plasmids (15) by calcium phosphate precipitation. To generate "replacement" cell lines, HeLa cells were first transduced with N-terminal Flag-tagged shRNA-resistant expression constructs in the pMGIB vector, selected with blasticidin S, transduced with the corresponding LMP shRNA vector, and finally selected with puromycin. For shRNA vectors, hairpin sequences were amplified, ligated, and sequenced as previously described (15). Template sequences were: NAF1 A – 5'-tgctgttgacagtgagcgctgggatggtttcaagtattattagtgaagccacagatgtaataatacttgaaaccatcccaatgcctactgcctcgga-3'; TCAB1 A – 5'-tgctgttgacagtgagcgaggttcctgcatcttgaccaattagtgaagccacagatgtaattggtcaagatgcaggaaccgtgcctactgcctcgga-3'; TCAB1 B – 5'-tgctgttgacagtgagcgcgccgggagaacccgattcatatagtgaagccacagatgtatatgaatcgggttctcccggcttgcctactgcctcgga-3'. Dyskerin and pontin shRNA sequences were previously described (14).

Immunoprecipitations, western blots, and northern blots

Cells were lysed in NP40 buffer (25mM HEPES-KOH, 150mM KCl, 1.5mM $MgCl_2$, 10% glycerol, 0.5% NP40, 5mM 2ME, pH 7.5 supplemented with protease inhibitors) for 10 min on ice. Extracts clarified by centrifugation for 16,000g for 10 min were quantified by Bradford assay. Flag tagged proteins were immunoprecipitated/immunodepleted with 10-15uL M2 anti-Flag resin (Sigma), and endogenous proteins were immunoprecipitated/depleted with 1ug affinity-purified antibody supplemented with 10uL protein A resin (Sigma) for 1-2 hr at 4°C. Where indicated, RNase A was included during the incubation at 0.1mg/mL. For immunoprecipitation-TRAP assays, resins were preblocked in 5% nonfat dry milk for 30 min prior to use. Resins were then washed 5 times for 10 min each with 1mL NP40 buffer, boiled in Laemmli sample buffer, and

fractionated by SDS-PAGE. Gels were transferred to nitrocellulose filters (Whatman S&S), blocked in 2.5% nonfat dry milk in TBST for 30 min, and incubated in primary antibody overnight at 4°C. Primary antibodies were used at the following: Flag M2 - 100ng/mL; TCAB1 - 20 ng/mL; NAF1 - 20 ng/mL; pontin - 20ng/mL; TERT - 300ng/mL; dyskerin (serum; (5)) - 1:30,000; a-tubulin (DM1A; Sigma) - 1:150,000. Anti-TERT and anti-pontin antibodies were validated previously (14). The next day, filters were washed 3 times for 10 min each in TBST, incubated for 1 hr in 1:10,000 peroxidase-conjugated secondary antibody (Jackson Immunoresearch), washed again 5 times for 5 min each in TBST and developed with ECL+ (Amersham). For northern blots, equal portions for each immunoprecipitation (into which a recovery control RNA was spiked to control for differential recovery in subsequent steps) was extracted with phenol:chloroform:isoamyl alcohol and precipitated with glycogen carrier. RNA pellets were boiled in formamide loading buffer, loaded onto 5% polyacrylamide-8M urea gels, transferred to Hybond N+ (Amersham), and hybridized with ^{32}P-a-dCTP labeled full-length hTR, U3, or U1 probe in Ultrahyb (Ambion) as previously described (14). For all other RNAs, ^{32}P-g-ATP end-labeled antisense DNA oligonucleotides were used as northern probes. For total RNA analysis, 2ug of Trizol (Invitrogen) extracted RNA was used.

Polyclonal antibody production

ORFs for full-length NAF1 and TCAB1 were cloned into pMAL-c2 and pGEX6P-3 vectors to make recombinant MBP and GST tagged versions, respectively. All fusions were expressed in BL21(DE3) bacteria, induced for 4 hr with 0.3mM IPTG at 32°C, and produced soluble proteins. MBP fusions, purified using standard amylose affinity chromatography (NEB) and competitive maltose elution, were injected into rabbits for polyclonal antibody production (Covance). All antibodies were affinity purified. For this, the corresponding GST fusions were expressed and affinity purified onto, and directly crosslinked to, glutathione resin (Amersham) using disuccinimidyl suberate (DSS; Pierce). Serum was passed over the glutathione resin, washed, and eluted using 0.1M glycine, pH 2.5.

TRAP assays

TRAP assays used PAGE-purified oligonucleotides and were used based on the original description (16), but instead used 32P-g-ATP labeled TS primer and incorporated an internal control template and reverse primer (template: 5'-aatccgtcgagcagagttaaaaggccgagaagcgat-3'; rev: 5'-atcgcttctcggcctttt-3').

Immunofluorescence

HeLa cells grown on coverslips were fixed with 4% paraformadehyde in PBS, permeabilized with PBS/0.2% Triton-X100, and blocked in PBS/1% BSA. Primary antibodies (TCAB1 at 20ng/mL, p80-coilin (Abcam) at 20ng/mL, HA (3F10; Roche) at 20ng/mL) were incubated for 1 hr in PBS/1% BSA. Coverslips were washed 5 times with PBS for 2 min each. Secondary antibodies (at 1:1,000 dilutions and pre-adsorbed to prevent species crossreactivity; Jackson Immunoresearch) were incubated for 1 hr in PBS/1% BSA. Coverslips were again washed 5 times with PBS for 2 min each and mounted using Vectashield + DAPI (Vector labs).

TERC fluorescence in situ hybridization

A cocktail of three fluorescently-labeled antisense probes recognizing distinct regions of TERC were used to detect endogenous TERC in HeLa cells as previously described (17), (http://www.singerlab.org/protocols).

Telomere length analysis

HTC75 cell cultures were grown in DMEM/10% bovine calf serum/1% PS. After transduction with TCAB1 shRNA retroviruses or an empty shRNA vector control, each cell line was maintained in puromycin selection (2 ug/mL). After approximately 30 population doublings, each cell line was re-transduced with its original shRNA vector (or

empty vector control) to maintain knockdown. Isolated genomic DNA was restricted with RsaI and HinfI and fractionated as described previously (18). Membranes were prepared by Southern transfer and hybridized to a radioactively end-labeled $(ttaggg)_4$ oligonucleotide probe as described previously (19).

Results

To study dyskerin, we expressed tagged dyskerin protein at endogenous levels in the absence of competing endogenous protein (Fig. S1) and isolated dyskerin complexes using a dual affinity chromatography strategy. Purified dyskerin complexes were analyzed by SDS-PAGE and nanoLC-MS/MS for identification of co-purifying proteins (Fig. 1A,B). Dense peptide coverage was obtained for dyskerin and for the dyskerin-associated ATPases pontin and reptin (14). Each of the evolutionarily conserved dyskerin-binding proteins NHP2, NOP10 and GAR1 was detected, as were the dyskerin-associated proteins Nopp140 and NAF1, a nucleoplasmic factor required for assembly of H/ACA RNPs including telomerase (20) (Fig. 1B). In addition, this approach identified the WD40 repeat protein WDR79, a protein that had not been previously implicated in dyskerin or telomerase function.

We further characterized WDR79, hereafter referred to as TCAB1 (Fig. 1B, S2). Endogenous TCAB1 was specifically bound to Flag-dyskerin immunoprecipitated from Flag-dyskerin^{+shRNA} HeLa cells, as were endogenous pontin, NAF1, TERT and TERC (Fig. 1C). Interactions between TERT and dyskerin were disrupted by RNase A treatment of the extract, which degraded TERC. In contrast, dyskerin binding to TCAB1, NAF1 and pontin were not RNase A-sensitive, indicating that these associations occur through protein-protein contacts (Fig. 1C). Reciprocal immunoprecipitation of Flag-TCAB1 from Flag-TCAB1^{+shRNA} HeLa cells showed that Flag-TCAB1 not only associates with endogenous dyskerin, but also with TERT and TERC, the catalytic core of telomerase (Fig. 1D). Like the TERT-dyskerin association, TERT binding to Flag-TCAB1 was RNase A-sensitive, suggesting that the interaction of TCAB1 with telomerase is

dependent on TERC (Fig. 1D). Thus, TCAB1 interacts specifically with dyskerin, TERT and TERC, all three known components of active telomerase.

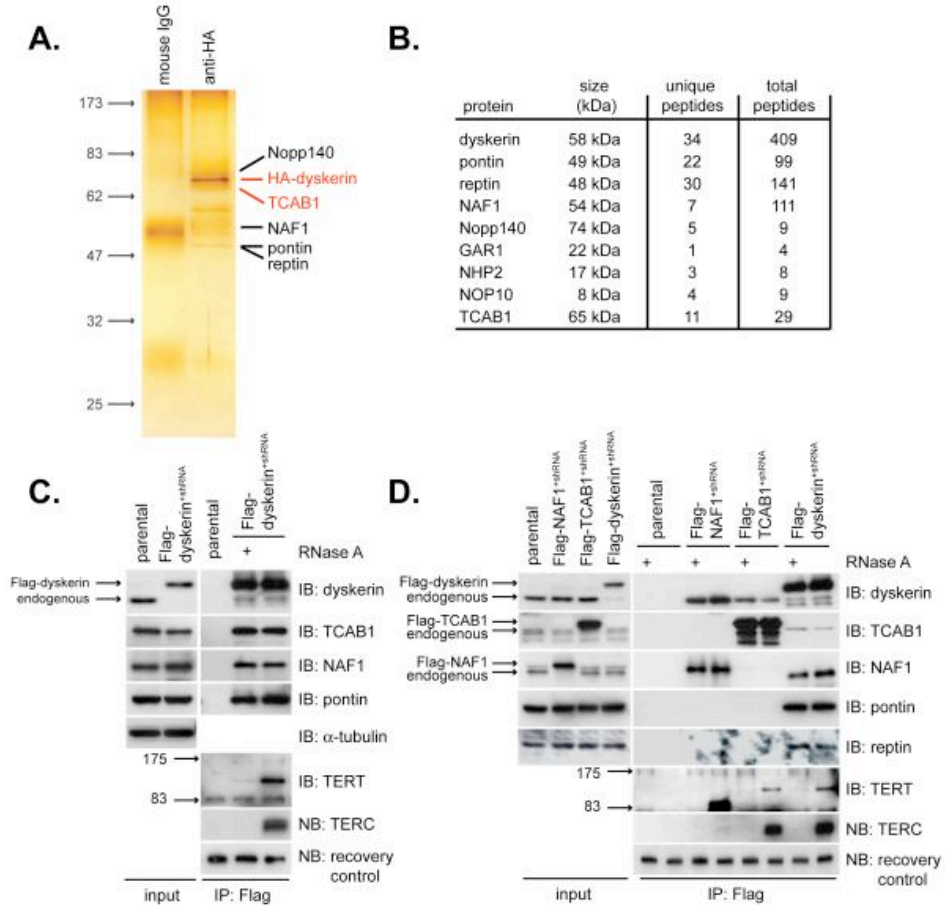


FIGURE. 1: Identification of TCAB1 as a dyskerin- and telomerase-interacting protein.

(A) Dual affinity-purified dyskerin complexes fractionated by SDS-PAGE and silver stained. (B) Unique and total peptides corresponding to dyskerin-associated proteins identified by nanoLC MS/MS (C) Flag-dyskerin interactions with endogenous TCAB1 and telomerase components and (D) Flag-TCAB1 and Flag-NAF1 interactions with endogenous telomerase components. Immunoprecipitation-western blot using extracts from Flag-dyskerin^{+shRNA} cells, Flag-TCAB1^{+shRNA} cells or Flag-NAF1^{+shRNA} cells. IB, immunoblot. NB, northern blot. parental, HeLa cells.

+, treatment of extracts with RNase A during immunoprecipitation. Recovery control, exogenous RNA spiked into samples after IP to control for differential RNA recovery.

Although TCAB1 associated with TERT, TERC and dyskerin, it did not interact with the assembly factors NAF1, pontin or reptin (Fig. 1D), suggesting that TCAB1 may be a component of the enzymatically active telomerase complex rather than a pre-telomerase complex. To test this hypothesis, we asked to what extent telomerase activity in cell extracts was associated with overexpressed TCAB1. Flag-tagged TCAB1, dyskerin and NAF1 were depleted from extracts by immunoprecipitation (Fig. 2A, lanes 14-21). Flag-TCAB1 and Flag-dyskerin immunoprecipitates were associated with high telomerase activity (Fig. 2A, lanes 12 and 13). Moreover, purification of either Flag-TCAB1 or Flag-dyskerin depleted these extracts of telomerase activity, indicating that both Flag-TCAB1 and Flag-dyskerin were associated with nearly all telomerase activity in the extract. In contrast, Flag-NAF1 bound only a small percentage of telomerase and did not deplete telomerase activity from cell extracts (Fig. 2A, lanes 1-8). Furthermore, immunoprecipitation of either Flag-TCAB1 or Flag-dyskerin depleted TERC but not unrelated RNAs such as U1 from cell extracts (Fig. 2A, lanes 14-21). Flag-TCAB1 and Flag-dyskerin were associated with similar amounts of endogenous TERT and TERC, further suggesting that TCAB1 and dyskerin reside together in the same active telomerase complexes (Fig 2A, lanes 24-25).

To understand the composition of endogenous telomerase, immunoprecipitations were performed using anti-TCAB1 and anti-NAF1 antibodies, each of which efficiently depleted its cognate protein from cell extracts (Fig. 2B, lanes 9-13). High telomerase activity was associated with TCAB1 immunoprecipitates and anti-TCAB1 antibodies quantitatively depleted telomerase activity from cell extracts (Fig. 2B, lanes 1-8, S4). In contrast, anti-NAF1 antibodies pulled down only a small percentage of telomerase activity. Assessing the TCAB1 immunoprecipitates for telomerase components revealed that TCAB1 interacts with endogenous dyskerin, TERT and TERC (Fig. 2B, lanes 14-16). TCAB1 immunoprecipitation effectively depleted cell extracts of TERC by northern blot (Fig. 2B, lanes 9-13, S4, S5). We conclude that TCAB1, like dyskerin, associates

stably with a vast majority of active telomerase and TERC, and therefore is a component of a human telomerase holoenzyme.

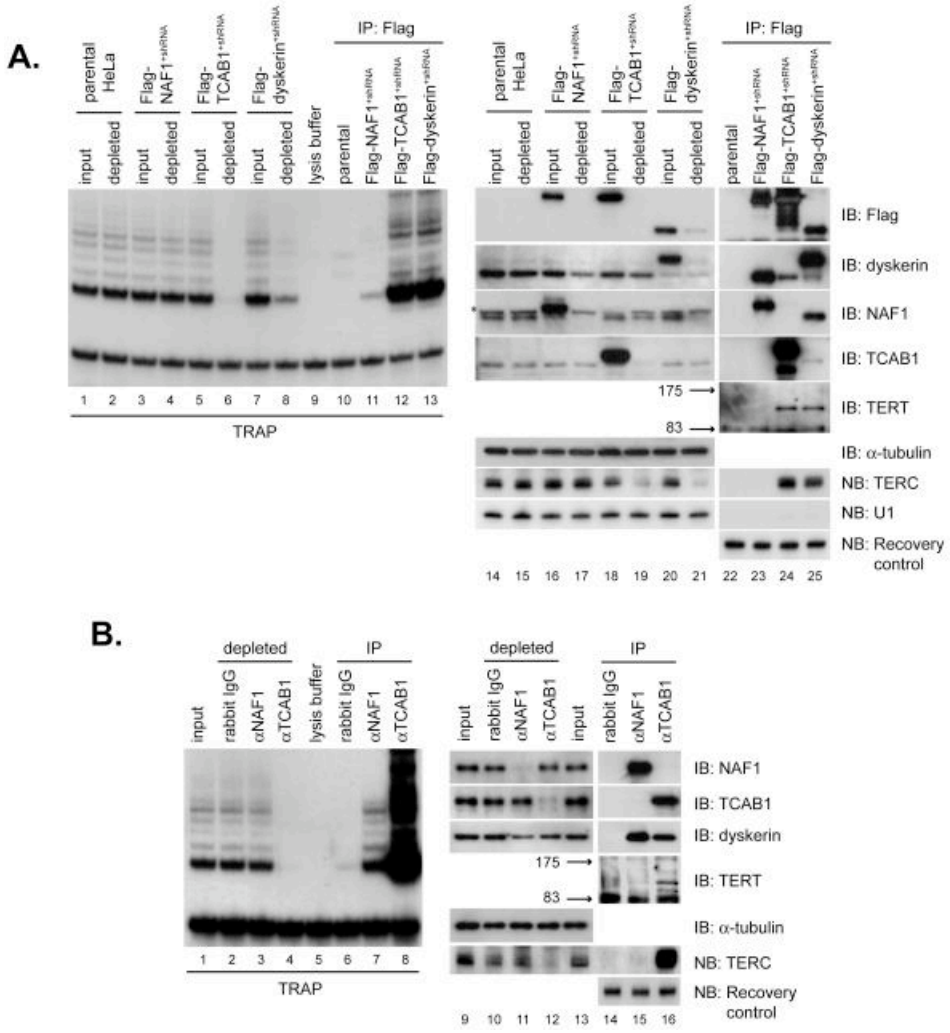


FIGURE 2: TCAB1 is a component of active telomerase. (A) Flag-TCAB1 or Flag-dyskerin immunoprecipitation (IP) quantitatively co-depletes telomerase activity and TERC from extracts. TRAP assays were performed on extracts before and after IP (lanes 1-8) and on each immunoprecipitated complex (lanes 10-13). Depletion of tagged proteins and depletion of endogenous TERC on extracts before and after IP (lanes 14-21). IP of tagged proteins and associated telomerase components (lanes 22-25). U1 splicing RNA, negative control. (B) IP of endogenous TCAB1 co-depletes telomerase activity and TERC. TRAP assays were performed on extracts before and

after each IP (lanes 1-4) and on the IP (lanes 6-8). Depletion of endogenous NAF1 or endogenous TCAB1 and depletion of TERC (lanes 9-13). Association of NAF1 and TCAB1 with telomerase components (lanes 14-16). Recovery control, exogenous RNA spiked into samples after IP to control for differential RNA recovery.

By immunofluorescence, stably overexpressed HA-tagged TCAB1 was distributed weakly throughout the nucleoplasm, but was strongly enriched within nuclear foci resembling Cajal bodies, sites of RNP processing shown to contain dyskerin and TERC (17,21,22). Immunofluorescence showed that the HA-TCAB1 foci overlapped with p80-coilin and were therefore Cajal bodies (Fig. 3A). Endogenous TCAB1 was also highly enriched in Cajal bodies, with smaller amounts distributed in the nucleoplasm (Fig. 3B). Whereas dyskerin accumulates in both Cajal bodies and nucleoli, the Cajal body-restricted localization of TCAB1 suggested that TCAB1 may function specifically with telomerase and with other non-coding RNAs found in Cajal bodies.

Immunoprecipitates of Flag-TCAB1 (Fig. 3C) and endogenous TCAB1 (Fig. 3D) were assayed for scaRNAs and representatives of other classes of nuclear non-coding RNAs by northern blot. Both overexpressed TCAB1 and endogenous TCAB1 specifically bound H/ACA scaRNAs, but associated with neither snoRNAs nor splicing RNAs. In contrast, Flag-dyskerin specifically bound both scaRNAs and H/ACA snoRNAs, while NAF1 did not associate stably with any non-coding RNAs studied. For each H/ACA scaRNA tested, a significant proportion was depleted from the extract by immunoprecipitation of TCAB1 (Fig. 3C, lanes 5-6; 3D, lanes 1-5). Thus, TCAB1 specifically binds scaRNAs, which share a CAB box sequence that controls Cajal body localization, and provides a potential mechanism to explain how scaRNAs, including TERC, are retained in Cajal bodies.

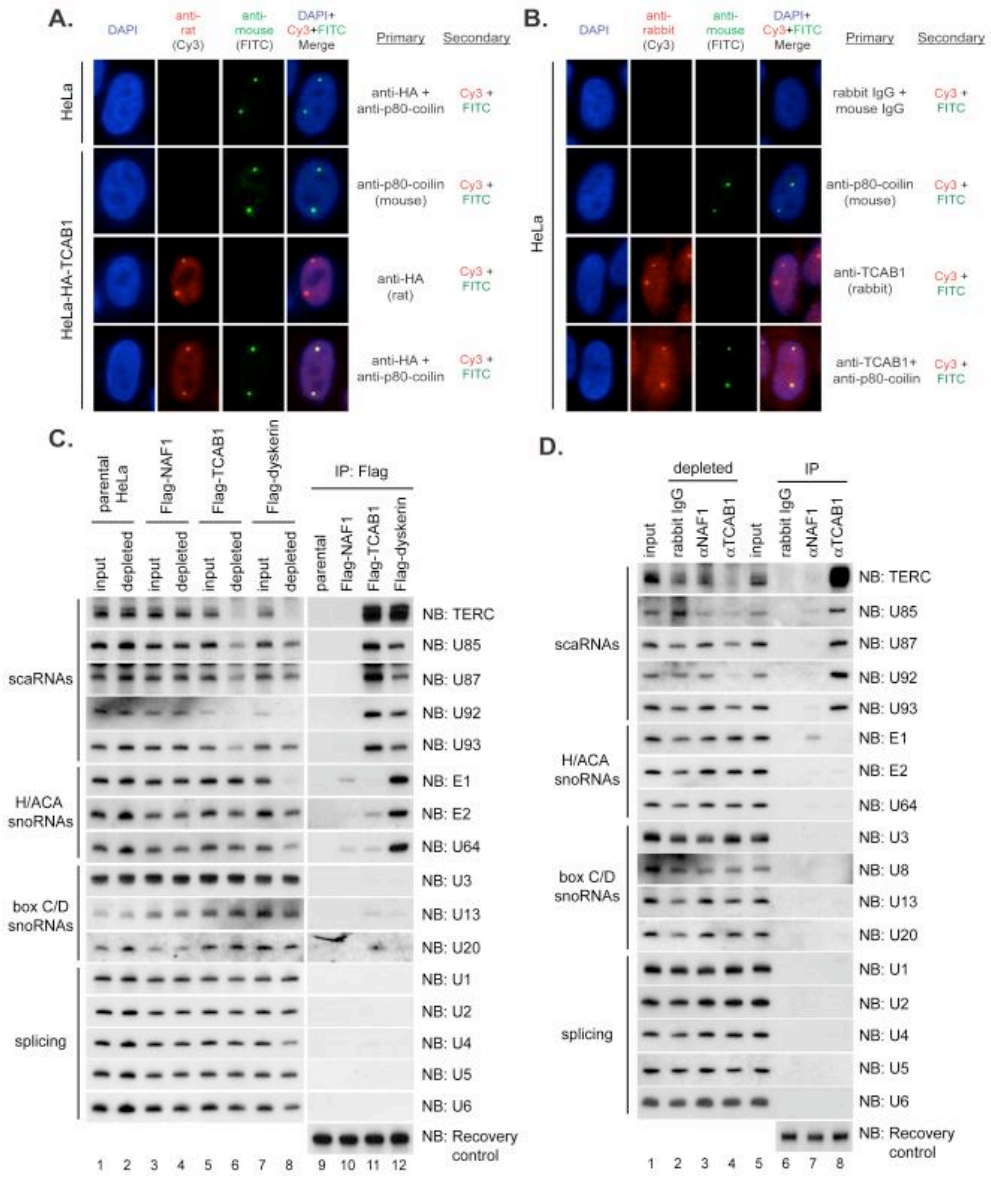


FIGURE 3: TCAB1 is enriched in Cajal bodies and associates specifically with small Cajal body RNAs (scaRNAs). (A) HA-TCAB1 colocalizes with the Cajal body marker p80-coilin. Immunofluorescence (IF) using anti-HA (rat) and anti-p80-coilin (mouse) antibodies on fixed HeLa cells stably overexpressing HA-TCAB1. (B) Co-localization of endogenous TCAB1 with p80-coilin. IF using anti-TCAB1 (rabbit) and anti-p80-coilin antibodies (mouse) on fixed HeLa cells. (C) Flag-TCAB1 associates specifically with scaRNAs by immunoprecipitation-northern blot. Cell

extract from parental HeLa cells, Flag-NAF1^{+shRNA} cells, Flag-TCAB1^{+shRNA} cells or Flag-dyskerin^{+shRNA} cells was incubated with Flag antibody beads. RNA was isolated from extracts before (input) and after (depleted) antibody treatment. Two µg total RNA was fractionated by urea-PAGE (lanes 1-8), representing 2% of the input. RNAs from IPs, lanes 9-12 (D) Endogenous TCAB1 associates specifically with scaRNAs. NAF1 antibodies, TCAB1 antibodies or IgG control were used in assays at the same scale as in (C). RNAs from input and depleted extracts, lanes 1-5. RNAs from IPs, lanes 6-8. Recovery control, exogenous RNA spiked into samples after IP to control for differential RNA recovery.

TCAB1 depletion using retroviral encoded shRNAs reduced neither telomerase activity nor TERC levels, in contrast to dyskerin depletion, which markedly diminished TERC and telomerase activity (Fig. 4A). Thus, TCAB1 may be required *in vivo* for a step after assembly of a catalytically competent telomerase complex. To determine the role of TCAB1 in Cajal body localization of telomerase, TERC localization was measured by RNA fluorescence *in situ* hybridization (FISH) in TCAB1-depleted HeLa cells. FISH revealed that TCAB1 knockdown significantly reduced the percentage of cells in which TERC was found in Cajal bodies (Fig. 4B,C) without affecting overall TERC RNA levels (Fig. 4A). Cajal bodies have been directly implicated in the delivery of TERC to telomeres during S phase (23,24,25). Therefore, we also examined the effect of TCAB1 knockdown on the localization of TERC to telomeres, using FISH for TERC and TRF2 immunofluorescence. Depletion of TCAB1 significantly reduced the presence of TERC at telomeres during S phase (Fig. 4B,C). Thus, TCAB1 is required for TERC localization in Cajal bodies and for delivery of TERC to telomeres during S phase.

To determine whether TCAB1 is required for telomere addition by telomerase, we first induced telomere elongation through TERC overexpression in HTC75 fibrosarcoma cells. Overexpression of wildtype TERC lengthened telomeres with cell passage, but telomere lengthening was inhibited in cells overexpressing a CAB box-mutant TERC that fails to accumulate in Cajal bodies, as previously shown (23). Depletion of TCAB1 in cells overexpressing wildtype TERC significantly inhibited telomere elongation, mimicking the effect of the CAB box mutation (Fig. 4D, S6). To determine whether

TCAB1 is required for telomere synthesis by endogenous telomerase, we assayed telomere lengths with serial passage in HTC75 cells transduced with TCAB1 shRNAs or with empty vector. Both shRNAs targeting TCAB1 led to progressive telomere shortening compared to the empty vector control (Fig. 4E, S7), indicating that TCAB1 is required for telomere synthesis in human cancer cells.

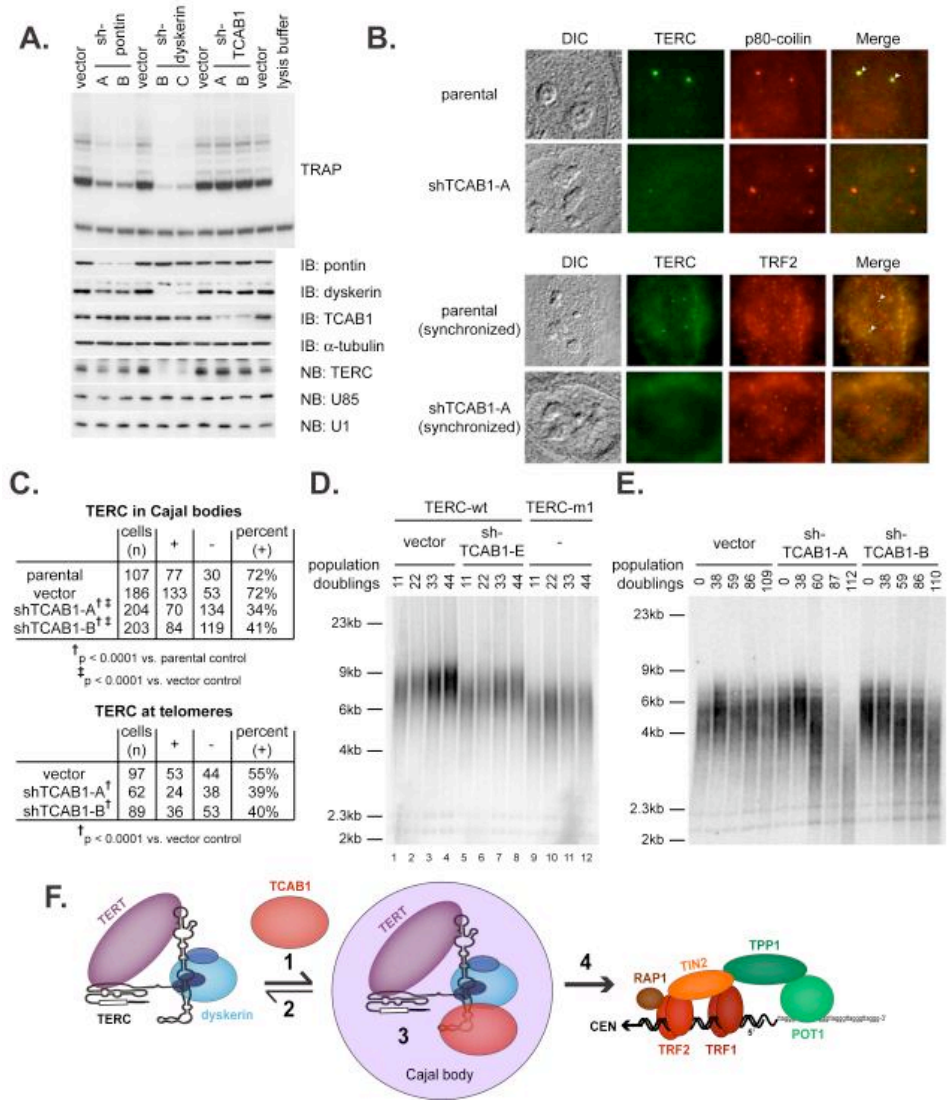


FIGURE 4: TCAB1 is essential for TERC localization to Cajal bodies and for telomere synthesis by telomerase. (A) HeLa cells were transduced with retroviruses expressing independent shRNA sequences targeting the indicated proteins or with

empty vector control. Telomerase activity was measured by TRAP assay. (B) TERC colocalization with p80-coilin was determined by RNA FISH for TERC (green) and immunofluorescence for p80-coilin (red)(top). Cells synchronized in S phase were assayed for TERC by RNA FISH (green) and for telomeres with TRF2 antibodies (red) to assess trafficking of TERC to telomeres (bottom). (C) Quantification of data in (B). Top: cells in which TERC colocalized with p80-coilin (+) versus cells in which TERC was not detected in Cajal bodies (-), $p<0.0001$ by Fisher's exact test. Bottom: cells in which TERC colocalized with telomeres (+) versus cells in which TERC was not detected at telomeres (-), $p<0.0001$ by Fisher's exact test. (D) Telomere lengths were measured by TRF Southern blot in HTC75 cells overexpressing wild-type TERC (lanes 1-8) or mutant TERC-m1 (lanes 9-12). Cells overexpressing wild-type TERC were transduced with shRNA retroviruses targeting TCAB1 or with empty vector. (E) Effect of TCAB1 depletion on endogenous telomerase was assessed by TRF Southern blot in parental HTC75 cells transduced with empty vector or retroviruses expressing independent TCAB1 shRNAs. Cells were counted at each passage and population doublings are indicated (F) Model for TCAB1 function in the telomere synthesis pathway.

Conclusions

Our data identify TCAB1 as a Cajal body-enriched protein that associates with TERC and other scaRNAs, and explains how TERC, and perhaps other scaRNAs, localize in Cajal bodies. TCAB1 functions as a telomerase holoenzyme component in the telomere synthesis pathway at a step after assembly of a minimal telomerase complex containing TERT, TERC and dyskerin. NAF1 and the ATPases pontin and reptin are required for assembly of the catalytically competent complex (14, 20). In contrast, TCAB1 stably associates with active telomerase enzyme and directs it through Cajal bodies to telomeres. In this manner, TCAB1 may act as a Cajal body-targeting or retention factor, may facilitate additional assembly steps of the enzyme in Cajal bodies, and/or may facilitate translocation of telomerase to telomeres (Fig. 4F). Once at telomeres, the activity of the telomerase holoenzyme may be enhanced by interaction

with the telomere binding proteins TPP1 and POT1 (26,27), as well as other factors that remain to be discovered.

Acknowledements

Supported by Medical Scientist Training Program Grant GM07365 (A.S.V), NCI CA104676 (M.P.T., R.M.T) with a Supplement to Promote Diversity in Health-Related Research (E.B.A.). This project has been funded in part with federal funds from the NCI under Contract NO1-CO-12400 (T.D.V). This work was supported by NCI CA111691 and CA125453 (S.E.A.) and by a Leukemia and Lymphoma Society SCOR grant (S.E.A).

References

1. Collins K. (2006) A novel RNA binding domain in tetrahymena telomerase p65 initiates hierarchical assembly of telomerase holoenzyme Nat Rev Mol Cell Biol.,7:2029-2036.
2. Meier UT. (2005) The many facets of H/ACA ribonucleoproteins Chromosoma., 114:1-14.
3. Matera AG, Terns RM, Terns MP. (2007) Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAsNat Rev Mol Cell Biol., 8:209-220.
4. Mitchell JR, Wood E, Collins K. (1999) A telomerase component is defective in the human disease dyskeratosis congenital. Nature., 402:551-555.
5. Mochizuki Y, He J, Kulkarni S, Bessler M, Mason PJ. (2004) Mouse dyskerin mutations affect accumulation of telomerase RNA and small nucleolar RNA, telomerase activity, and ribosomal RNA processing. Proc. Natl. Acad. Sci. U. S. A., 101:10756-10761.
6. Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. (2007) Protein composition of catalytically active human telomerase from immortal cells Science.;315:1850-3.

7. Kirwan M, Dokal I. (2008) Dyskeratosis congenita: a genetic disorder of many faces, Clin. Genet.;73:103-112.
8. Fu D, (2007) Collins K. Purification of human telomerase complexes identifies factors involved in telomerase biogenesis and telomere length regulation. Mol. Cell.;28:773-785.
9. Pogacic V, Dragon F, Filipowicz W. (2000) Human H/ACA small nucleolar RNPs and telomerase share evolutionarily conserved proteins NHP2 and NOP10 Mol. Cell. Biol., 20:9028-9040.
10. Dragon F, Pogacic V, Filipowicz , (2000) In vitro assembly of human H/ACA small nucleolar RNPs reveals unique features of U17 and telomerase RNAs W. Mol. Cell. Biol.; 20:3037-3048.
11. Snow BE, Erdmann N, Cruickshank J, Goldman H, Gill RM, Robinson MO, Harrington L. et al., (2003) Functional conservation of the telomerase protein Est1p in humans.Curr. Biol. 13:698-704.
12. Reichenbach P, Höss M, Azzalin CM, Nabholz M, Bucher P, Lingner (2003) A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. J. Curr. Biol.,13:568-74.
13. Schnapp G, Rodi HP, Rettig WJ, Schnapp A, Damm K. (1998) One-step affinity purification protocol for human telomerase. Nucleic Acids Res., 26:3311-13.
14. Venteicher AS, Meng Z, Mason PJ, Veenstra TD, Artandi SE. (2008) Identification of ATPases pontin and reptin as telomerase components essential for holoenzyme assembly. Cell.,132:945-57.
15. Dickins, R. A., Hemann, M. T., Zilfou, J. T., Simpson, D. R., Ibarra, I., Hannon, G. J., and Lowe, S. W. (2005). Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. Nat Genet *37*, 1289-1295
16. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994).

Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011-2015.

17. Zhu Y, Tomlinson RL, Lukowiak AA, Terns RM, Terns MP. (2004)Telomerase RNA accumulates in Cajal bodies in human cancer cells. Mol. Biol. Cell.;15:81-90.
18. Cristofari, G., and Lingner, J. (2006). Telomere length homeostasis requires that telomerase levels are limiting. Embo J *25*, 565-574.
19. Middleman, E. J., Choi, J., Venteicher, A. S., Cheung, P., and Artandi, S. E. (2006). Regulation of cellular immortalization and steady-state levels of the telomerase reverse transcriptase through its carboxy-terminal domain. Mol Cell Biol *26*, 2146-2159.
20. Hoareau-Aveilla C, Bonoli M, Caizergues-Ferrer M, Henry Y. (2006) hNaf1 is required for accumulation of human box H/ACA snoRNPs, scaRNPs, and telomerase.RNA.;12:832-840.
21. Meier UT, Blobel G. J. (1994) NAP57, a mammalian nucleolar protein with a putative homolog in yeast and bacteria. Cell Biol.;127:1505-1514.
22. Jady BE, Bertrand E, Kiss T. J. (2004) Human telomerase RNA and box H/ACA scaRNAs share a common Cajal body-specific localization signal. Cell Biol.;164:647-652.
23. Cristofari G, Adolf E, Reichenbach P, Sikora K, Terns RM, Terns MP, Lingner J. (2007)Human telomerase RNA accumulation in Cajal bodies facilitates telomerase recruitment to telomeres and telomere elongation. Mol. Cell.;27:882-889.
24. Jady BE, Richard P, Bertrand E, Kiss T. (2006) Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. Mol. Biol. Cell.;17:944-954.
25. Tomlinson RL, Ziegler TD, Supakorndej T, Terns RM, Terns MP. (2006) Cell cycle-regulated trafficking of human telomerase to telomeres. Mol. Biol. Cell.;17:955-965.

26. Wang F, Podell ER, Zaug AJ, Yang Y, Baciu P, Cech TR, Lei M. (2007) The POT1-TPP1 telomere complex is a telomerase processivity factor.Nature.;445:506-510.

27. Xin H, Liu D, Wan M, Safari A, Kim H, Sun W, O'Connor MS, Songyang Z. (2007) TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase.Nature. 445:559-562.

Supplemental Chapter III:

Processive and Distributive Extension of Human Telomeres by Telomerase Under Homeostatic and Non-equilibrium Conditions

---

Yong Zhao[1], Eladio Abreu[2], Jinyong Kim[1], Guido Stadler[1], Ugur Eskiocak[1], Michael P. Terns[2], Rebecca M. Terns[2], Jerry W. Shay[1] and Woodring E. Wright[1*]

Submitted to Molecular Cell

Abstract

Specific information about how telomerase acts *in vivo* is necessary for understanding telomere dynamics in human tumor cells. Our results imply that under homeostatic telomere length-maintenance conditions only one molecule of telomerase acts at each telomere during every cell division and processively adds ~60 nt to each end. In contrast, multiple molecules of telomerase act at each telomere when telomeres are elongating (non-equilibrium conditions). Telomerase extension is less processive during the first few weeks following the reversal of long-term treatment with the telomerase inhibitor GRN163L, a time when Cajal bodies fail to deliver telomerase RNA to telomeres. This result implies that processing of telomerase by Cajal bodies may affect its processivity. Overexpressed telomerase is also less processive than the endogenously expressed telomerase. These findings reveal two major distinct extension modes adopted by telomerase *in vivo*.

Introduction

Linear eukaryotic chromosomes are capped by telomeres, a functional complex consisting of repetitive DNA and associated proteins. Telomeres maintain chromosome integrity by preventing DNA degradation, end-to-end fusion and illegitimate recombination (3, 11). Because of the unidirectional nature of DNA polymerases and additional processing events, human telomeres shorten at ~50-200 base pairs per cell division(14). Eventually, critically short telomeres trigger replicative senescence or apoptosis(28). Most tumor cells and germ line cells overcome this proliferative limit by activating telomerase, a ribonucleoprotein with reverse transcriptase activity that catalyzes de novo repeat addition using sequences in the integral telomerase RNA (hTR/hTERC) as a template(4, 13, 22).

In human cancer cells, telomere length is maintained at a steady state (e.g. telomere addition and loss are in homeostatic balance). Under length-maintenance conditions, telomerase adds ~50-60 nt to each telomere end during every cell cycle to counteract intrinsic telomere shortening rates (39). However, the molecular

characteristics of telomerase extension on a single telomere are unknown. Telomerase complexes isolated from human cells are modestly processive *in vitro*, capable of adding several telomeric repeats to a given primer in a single recruitment event (7). This extension processivity can be synergistically enhanced by heterodimers of POT1 and TPP1, components of a six-protein shelterin complex at telomeres (37). In the presence of an optimal concentration of POT/TPP1, telomerase processively adds an average of 48 nt of telomeric repeats (23), suggesting that one round of telomerase recruitment and extension might be sufficient to account for the ~60 nt of synthesis seen at individual telomeres in cancer cells under maintenance conditions. Experimental demonstration *in vivo*, however, is lacking.

Under nonequilibrium conditions, in which telomere length has been experimentally manipulated, there is a general consensus that telomerase preferentially extends the shortest telomere (15, 27, 30). While telomerase action is nonprocessive on *S. cerevisiae* telomeres with normal length, the processivity is increased at critically short telomeres (6). This enhancement of telomerase processivity is proposed to rapidly elongate critically short telomeres. Telomerase does not act on every telomere in each cell cycle in *S. cerevisiae* (32). In human cancer cells in which telomerase does act on every telomere, the mechanism underlying the preferential extension of a particularly short telomere remains to be elucidated.

Telomerase extension is coupled with telomere replication in S phase (39). Both hTR and hTERT have been found associated with telomeres during S phase (21, 34). Assembly of catalytically active telomerase requires the chaperones Hsp23 and Hsp90 (12), and also requires association with Cajal bodies before it can be delivered to hTR foci at telomeres and produce telomere elongation (9, 36). Two components of the telomeric shelterin complex, TIN2 and TPP1 have been identified as factors required for recruiting hTR foci to telomeres (1). In contrast to these observations in human cells, mouse telomerase RNA (mTR) does not localize to Cajal bodies but resides in different nuclear foci, but nonetheless is found in foci on a subset of telomeres during replication (33). The nature of this association of hTR foci with telomeres during S phase remains to be determined.

The low abundance of telomerase in human cancer cells and its transient binding to telomeres have presented a significant challenge in studying telomerase action *in vivo.* In this work, we investigated telomerase extension under different conditions using techniques that allow us to examine telomere elongation during a single cell cycle. Our results suggest that under telomere length-maintenance conditions, every telomere was elongated by a single telomerase molecule that processively added ~60 nt to the 3' overhang (processive action). Reduction of hTERT levels by shRNA demonstrated that telomerase abundance was limiting and reducing levels of telomerase reduced the fraction of extended ends without apparently affecting processivity. The absence of non-extended ends under conditions where telomerase is not in excess suggests a telomerase/telomere prepositioning step that ensures that all ends get extended but only by a single molecule. When telomeres are lengthening (recovery after telomeres had been artificially shortened or after overexpression of an exogenous telomerase) telomeres appear to be elongated as a result of multiple telomerase molecules acting on each end to produce longer extensions (distributive action). Long term treatment with the telomerase inhibitor GRN163L (Imetelstat) resulted in the disappearance of hTR/telomere co-localization without changing total Cajal body number or hTR/Cajal body co-localization. This lack of co-localization persisted during the first few weeks following removal of the inhibitor, corresponding to a period in which telomerase activity was restored but telomerase processivity was greatly reduced, suggesting that Cajal bodies are modifying telomerase to make it more processive. These observations demonstrate two major modes of telomerase action, processive and distributive, and reveal additional steps (prepositioning of telomerase at telomeres under length-maintenance conditions and the modification of processivity in Cajal bodies) in telomerase action, and expand our understanding of the multiple mechanisms regulating the ability of telomerase to maintain telomeres in tumor cells.

EXPERIMENTAL PROCEDURES

Cell Culture and GRN163L treatment

Hela cervical carcinoma, H1299 lung adenocarcinoma were cultured at 37ºC in 5% CO_2 in 4:1 DMEM:Medium 199 containing 10% calf serum (Hyclone, Logan, UT). Hela cells with short telomeres were obtained by continuously treating cells with 2 μM GRN163L (Geron Corp., Menlo Park, CA) for about 3 months.

Cell Cycle synchronization

Hela and H1299 cells were synchronized at G1/S by double thymidine block. Briefly, exponentially growing cells were treated with 2 mM thymidine for 19 hr followed by washing with PBS (3 times). Cells were then cultured in fresh medium for 9 hr, 2 mM thymidine was added for 16 hr, followed by washing with PBS. Cells were released to pre-warmed fresh medium containing 100 μM BrdU for 4 hr, harvested and genomic DNA purified using Blood&Cell culture Midi kit (QIAGENE, Valencia CA). Cells were treated with GRN163L 3 days before synchronization started and the same concentration of GRN163L was included in all medium used for synchronization. Cells were always allowed to attach for 4 hours before GRN163L addition to avoid effects on spreading (20).

shRNA knockdown of hTERT

The retroviral shRNA for human hTERT was generous gift from Dr. Hahn (24). 15 μg of proviral hTERT shRNA plasmid was cotransfected into 293FT cells with 7.5 μg each of the packaging plasmids pPAX2 and pMD2G using calcium phosphate. Viral supernatants were collected at 24-48, 48-72 and 72-96 hrs, filtered, and used to infect Hela cells.

Detection of expression level of hTERT mRNA

Total RNA was prepared using the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA by using First-strand cDNA kit (Roche). All Real-Time PCR reactions were performed in a 20 µl mixture containing cDNA preparation, 2× Reaction Buffer (Roche), 4 mM $MgCl_2$, 1 µM of hTERT primers (Forward 5'-GGGGTCACTCAGGACAGC-3' and Reverse 5'-TCTTGAAGTCTGAGGGCAGTG-3'), 0.2 mM dNTPs mix and 0.1 µl of Universal probe #37. GAPDH was used as internal control for all experiments. The CT value from each template was calculated using the Roche 480 software. The Roche 480 software calculated crossing points (CT) based on the first maximum of the second derivative of the amplification curve of template. Relative expression level of hTERT mRNA was calibrated by GAPDH mRNA to minimize experimental variation.

Overexpression of hTERT into Hela cells

Hela cells were infected with pBabeHygro virus containing the hTERT cDNA. hTERT overexpression was confirmed by PCR and TRAP assay.

Lagging Daughter Overhang Analysis

CsCl gradient separation of leading versus lagging telomeres was performed as described (39). The DSN (Duplex Specific Nuclease)assay on lagging daughters was performed as described (38) with minor modification. Briefly, purified lagging telomere DNA was digested with DSN at 37ºC for 2 hr. Overhangs were resolved on 1% denature agarose gel followed by transfer to H**y**bond-XL^+ membrane using capillary transfer. The membrane was air-dried and DNA was fixed by UV cross-linking. A high specific activity C-rich probe (19) was hybridized to the G-rich overhangs at 42 ºC.

Estimation of Telomerase Activity

The telomeric repeat amplification protocol (TRAP) was used to measure the telomerase activity(31). Samples were resolved by PAGE and scanned using a Typhoon

PhosphorImager scanner system (Molecular Dynamics, GE Healthcare, Piscataway, NJ). The telomerase products (6 bp ladder) and the 36 bp internal control (IC) bands were quantified using the Imagequant software provided by manufacturer. Relative telomerase activity was calculated as the intensity ratio of the TRAP ladder to that of the IC band.

Telomere Length Measurement

The average length of telomeres were measured as described (39).

Fluorescence in situ hybridization (FISH) and Immunofluorescence (IF) Detection of hTR, Telomeres and Cajal Bodies

Detection of hTR and telomeres via FISH was performed essentially as described (34). Briefly, 25 ng/coverslip of each of three Cy3 conjugated DNA probes complementary to different regions of human telomerase RNA (**17**) and 20 ng/coverslip of an Oregon Green conjugated telomere DNA probe (**22**) was used. Following FISH, Cajal bodies were detected by IF with the primary antibodies against the Cajal-body marker protein, coilin and Cy5 conjugated secondary antibody (Jackson ImmunoResearch laboratories, West Grove, PA) as described (34). Cells were mounted in Prolong Gold (Invitrogen) and microscopy was performed using a Zeiss Axioskop 2 Mot Plus fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were acquired at 63x (Plan Apochromat objectives, numerical aperture 1.4) using a cooled charge-coupled device ORCA-ER digital camera (Hamamatsu photonics, Bridgewater, NJ) and IPLab Spectrum software (BioVision Technologies, Inc., Exton, PA.)

RESULTS

Telomerase action is processive in human cancer cells

Telomerase action during a single S phase in the presence of BrdU can be examined by first purifying telomeres replicated by lagging strand synthesis on a first CsCl gradient, and then analyzing the density of their overhangs on a second CsCl gradient (Fig. 1A). While unextended overhangs (thymidine only) band at low density, the overhangs extended by telomerase incorporate BrdU in the new synthesized telomeric repeats and shift to a higher density (intermediate density) depending upon how many repeats are added. The ratio of intermediate versus low density overhangs indicates the fraction of telomeres extended by telomerase. The density of the intermediate peak is proportional to the ratio of BrdU to thymidine in the overhangs (supplementary Fig. S1A). Therefore the average length of telomerase addition products (BrdU containing repeats) can be calculated by combining the BrdU/thymidine ratio and the total lagging overhang length. Using this approach we found that under telomere length-maintenance conditions in human H1299 adenocarcinoma and Hela cervical carcinoma cells, telomerase extends most telomere ends by adding ~60 nt to each overhang during every cell cycle (39).

The action of vertebrate telomerase *in vivo* was examined to determine whether the extension of each telomere represented the result of a single processive event by one molecule (processive) versus repetitive additions of smaller amounts by a multiple telomerase molecules (distributive). The telomerase inhibitor GRN163L, a synthetic lipid-conjugated 13-mer oligonucleotide with an extremely high affinity for the template region of hTR (17), binds essentially irreversibly to the active site template and blocks activity (18). If telomerase action is processive the inactivation of individual telomerase molecules by a partially inhibitory dose will decrease the fraction of extended ends without affecting the length of the extension products. However, if many telomerase molecules each add a small amount to each end (distributive action), a decreased fraction of active molecules would initially reduce the size of the extension products and would only affect the fraction of extended ends at high levels of inhibition. These two possibilities can be distinguished based on the predicted change in the density of lagging overhangs on CsCl gradients (Fig. 1B).

Treatment with 0.5 or 1 μM GRN163L reduced telomerase activity in H1299 lung adenocarcinoma cells by roughly 50% and 70%, respectively (Fig. 1C). Cells growing in

different concentrations of GRN163L were synchronized at G1/S and released into S for 4 hours (middle of S phase) in the presence of BrdU. In the absence of GRN163L, no thymidine-only containing lagging overhangs were present in H1299 lung adenocarcinoma cells labeled for four hours with BrdU, indicating that essentially 100% of the telomeres had been elongated by telomerase (Fig. 1D top). In the presence of increasing inhibition by GRN163L, the density of the extended ends did not change; the fraction of ends at the intermediate density decreased while the fraction of unlabeled ends increased (Fig. 1D middle and bottom). These results are not consistent with multiple molecules producing the extension and indicate that a single molecule of telomerase processively adds the full amount to each end. Our results do not address whether or not this single molecule is a dimer or multimer or whether the same molecule is extending both leading and lagging daughter telomeres.

In H1299 cells the intermediate density peak is half-way between the thymidine only and fully BrdU substituted overhangs, implying an equal number of thymidine and BrdU containing repeats (supplementary Fig. S1A). The total overhang size of lagging daughter telomeres was approximately 130 nt (supplementary Fig. S1B). Lagging strand overhangs prior to telomerase elongation were thus about 65 nt (thymidine-only segment) and telomerase addition produced the remaining 65 nt (BrdU substituted segment). H1299 telomeres shortened at a rate of about 55 bp/PD (base pairs per population doubling) when the majority of telomerase activity (>90%) is inhibited (supplementary Fig. S1C, D). The addition of ~65 nt to every telomere end by telomerase is roughly consistent with the amount that would need to be added to counteract telomere shortening.

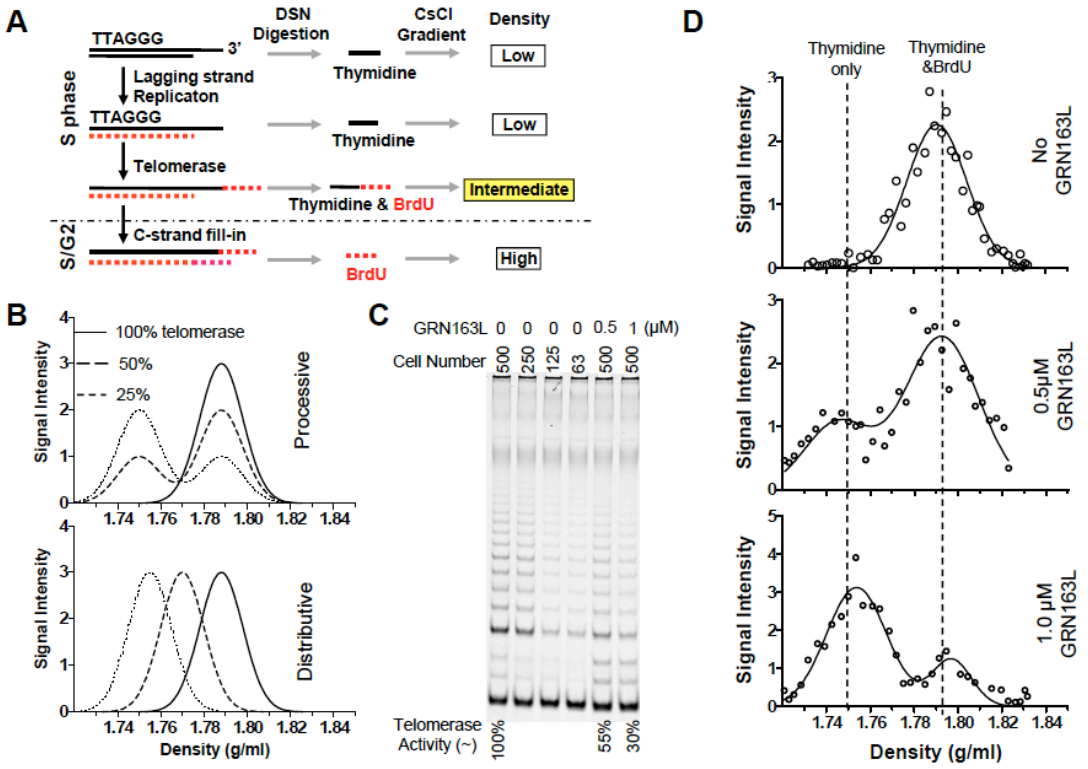
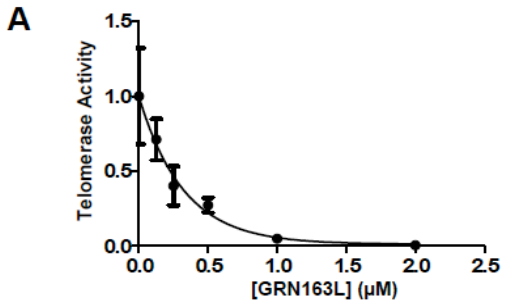


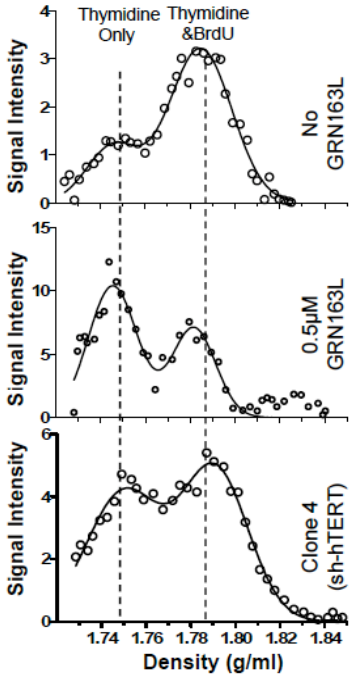
FIGURE 1. Telomerase action is processive in H1299 cells. (A) Strategy to study telomerase action on lagging daughter telomeres. Extended lagging overhangs have higher density than unextended overhangs because of the incorporation of BrdU by telomerase. (B) Predicted results for processive and distributive manner of telomerase action when 50% and 75% of activity was inhibited. (C) TRAP assay for telomerase activity in the cells with and without GRN163L treatment. 0.5 and 1.0 μM of GRN163L were used to obtain approximately 50% and 70% of inhibition. (D) CsCl overhang assay of telomere extension in H1299 cells with and without treatment of GRN163L that partially inhibit telomerase activity. See also Figure S1.

The generality of this observation was examined in Hela cervical carcinoma cells. Only about 70% of Hela cells express telomerase at any given time (5), and 70% of telomere ends were extended by telomerase during each cell cycle (Fig. 2C top). Similar to H1299 cells, inhibition by GRN163L resulted in a decrease in the fraction of extended ends without changing their density (Fig. 2C middle). The extended fraction was roughly

proportional to the fraction of active molecules (Fig. 2 A, C, and supplemental Fig. S2 A, B) for both Hela and H1299, suggesting that the number of active telomerase molecules was limiting. The rate of telomere shortening in 0.25 and 0.5μM GRN163L (Fig. 2D and supplementary Fig. S2B) was inversely-proportional to the fraction of extended telomeres.

In addition to GRN163L-mediated telomerase inhibition, we also examined the effects of reducing telomerase by depleting hTERT levels using shRNA in Hela cells. Two clones with about 50% reduction of telomerase mRNA levels (Fig. 2B) were examined for their effects on telomere extension. The decrease in telomerase changed the ratio of extended versus unextended overhangs but not the length of the extension products (Fig. 2C bottom panel, Fig. 2E and supplementary Fig. S2C), reinforcing the conclusion that telomerase action is processive and further suggesting that one and only one extension event occurs on each telomere.





D

Table 1: Telomerase action responding to GRN163L treatment in Hela cells

GRN163L (μM)	Telomerase activity (TRAP)	Fraction of Extended telomeres (experimental)	Telomere Shortening Rate (bp/PD)
0	100%	70%	0
0.25	~50%	**33%**	16.8
0.5	~25%	**23%**	30

E

Table 2: Telomerase action responding to Knockdown of hTERT in Hela cells

Clone number	Telomerase activity(TRAP)	hTERT level (qPCR)	Fraction of Extended telomeres	Telomere Shortening Rate (bp/PD)
4	~20%	48%	**34%**	23
10	~33%	52%	**48%**	20

FIGURE 2. Processive extension of telomere by telomerase in Hela cells.
(A) Dose-response curve for the inhibition of telomerase activity by GRN163L (assayed by TRAP). ±SD of three independent experiments. (B) q-PCR determination of mRNA level of hTERT in clones 4 and 10 after sh-hTERT virus infection and drug selection. (C) CsCl overhang assays of telomere extension on lagging daughter in normal Hela cells (top) and in cells with telomerase partially inhibited by GRN163L (middle) or sh-hTERT (bottom). The Y-axis represents an arbitrary scale within each experiment that best displays the variation between the different fractions. (D) Summary of data on telomerase action and telomere shortening in response to partial inhibition of telomerase by GRN163L. (E) Summary of data on telomerase action and telomere shortening in response to knockdown of hTERT. It is worth noting that both telomerase activity assayed by TRAP and qPCR of hTERT mRNA are both PCR based and of limited precision, and thus minor differences between the data in 2D and 2E are not significant. See also Figure S2.

More than one round of telomerase extension occurs on each telomere when telomerase is overexpressed in Hela cells

The number of catalytically active telomerase molecules in human cancer cells is low, ranging from 50 to several hundred per cell ((8) and our unpublished data), and limiting for telomere length homeostasis (10, 26). Overexpressing hTERT in Hela cells produced a 10-fold increase in TRAP activity and elongated telomeres at 120 nt/PD (Fig. 3 A, B). The intermediate peak of telomerase extension products in these cells banded at a density of 1.808, indicating that 73% of each overhang was BrdU substituted. With 60 nt due to the initial thymidine containing overhang (generated by lagging DNA replication) , the size of the BrdU containing telomerase extension products would be about 160 nt to produce the overall 73% fraction. This is roughly equal to the observed

telomere elongation rate (120 nt) plus that required for compensating for the rate of telomere shortening during each division (44 nt) (supplementary Fig. S2B).

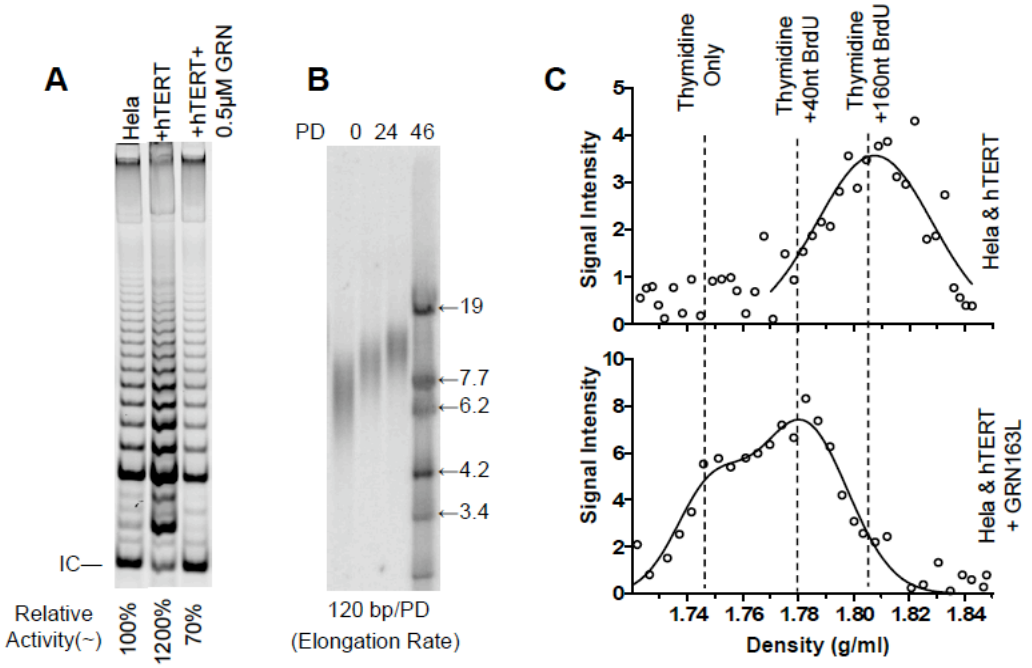


FIGURE 3. DISTRIBUTIVE EXTENSION OF TELOMERE BY TELOMERASE IN HELA CELLS WITH OVEREXPRESSED HTERT. (A) A PARTIAL INHIBITION OF TELOMERASE ACTIVITY BY GRN163L IN CELLS WITH OVEREXPRESSED TELOMERASE. TELOMERASE ACTIVITY WAS ASSAYED BY TRAP. (B) TELOMERES ARE ELONGATED BY OVEREXPRESSED TELOMERASE. TELOMERE LENGTH WAS ASSAYED BY TRF. (C) CSCL OVERHANG ASSAY SHOWING TELOMERE EXTENSION IN CELLS WITH OVEREXPRESSED TELOMERASE (TOP) AND INHIBITORY EFFECT OF GRN163L ON TELOMERASE ACTION (BOTTOM).

Treating hTERT-overexpressing Hela cells with 0.5 μM GRN163L inhibited telomerase activity to about the level of normal Hela cells (Fig. 3A). In contrast to the results under length maintenance conditions (Fig. 2), this inhibition resulted in a shift of the extension products to a lower CsCl denstity with only a small decrease of the fraction of ends that had been extended (Fig. 3C). This indicates that overexpressed telomerase is acting in a distributive fashion (Fig. 1B), where several different telomerase molecules act on each end during this period of telomere elongation. In addition, the shifted peak of

the extension products (40 nt) after GRN163L inhibition is less than that observed with endogenous telomerase (Figs. 1 & 2), suggesting an altered processivity of the overexpressed exogenous enzyme. Due to technical limitations, we are unable to formally determine how many telomerase molecules were involved in the additions at each telomere.

Taken together, this data indicates that under telomere length maintenance conditions each telomere is extended once and only once and there is not enough excess telomerase available for a second round of extension before the open configuration closes to make the end inaccessible. Overexpression of hTERT either modifies the open configuration or simply provides enough extra telomerase so that multiple rounds of extension by different molecules can occur.

Multiple telomerase molecules act on artificially shortened telomeres

Overexpression of the hTERT cDNA is a physiologically abnormal method to elongate telomeres. Telomere elongation using only the endogenous telomerase occurs following removal of a telomerase inhibitor in tumor cells in which telomeres have been shortened during prolonged treatment with the telomerase inhibitor (16). After 59 population doublings (PDs) in 2 μM GRN163L, the average length of Hela telomeres dropped from 6.5 kb to about 4 kb (Fig. 4A). After removing GRN163L, telomeres grew back to their original size over about 40 PD, indicating that telomerase had been extending the ends more than during length-maintenance conditions. Telomerase activity in cells released from GRN163L treatment returned to the same level as untreated Hela cells within one week (less than 5 PDs), and remained constant thereafter (Fig. 4B and our unpublished data). In contrast to normal Hela cells where only 70% telomeres were extended (Fig. 2C), all of the telomeres (100%) were elongated by telomerase during a single cell cycle in cells 22 PD following the removal of GRN163L (Fig. 4C top). This is consistent with the disappearance of the fraction of Hela cells transiently not expressing telomerase that is present in the parental cultures with longer telomeres. The density of

the intermediate peak increased from 50% BrdU at maintenance to 67% BrdU. The total overall length for lagging overhangs in these cells was 200 nt (Fig. 5B), demonstrating that telomerase had added ~135 nt (200×67%) to a thymidine containing initial overhang of ~65 nt at each telomere. In response to the decreased number of active telomerase molecules in the presence of GRN163L (supplementary Fig. S3), the intermediate peak shifted to lower density (Fig. 4C), indicating distributive action in which multiple molecules of telomerase act on single telomere (Fig. 1B).

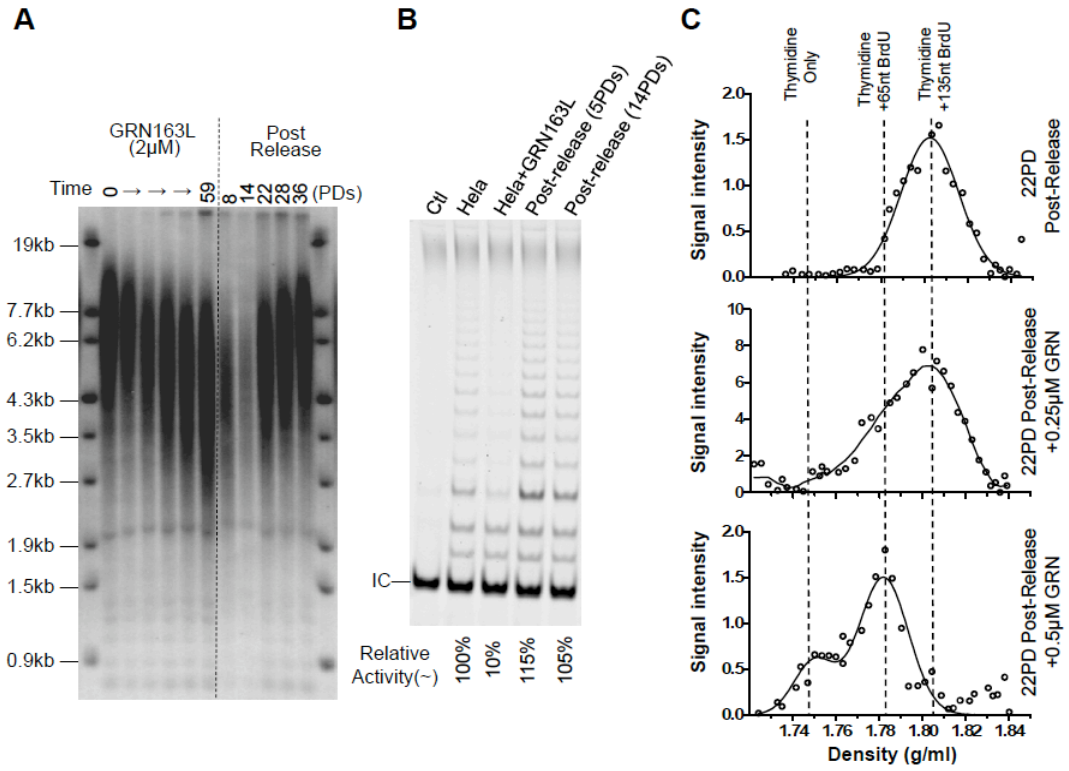


FIGURE 4. Distributive extension of telomere by endogenous telomerase in Hela cells with artificially shortened telomeres. (A) Telomere shortening induced by GRN163L treatment and gradual recovery in length after a removal of drug. (B) Telomerase activity rapidly returns to normal level and remains steady thereafter. (C) CsCl overhang assays of telomerase extension in cells with artificially shortened telomeres 22 PD after release (top). Telomerase action was assayed on CsCl gradients (middle and bottom) in different inhibitory concentrations of GRN163L. See also Figure S3.

Less processive telomerase is associated with less hTR co-localizing to telomeres

The above data was obtained 22 PD following GRN163L reversal, when telomeres were elongating. However, we did not observe telomere elongation at earlier PD (Fig 4A) despite rapidly restored telomerase activity (by 5 PDs, Fig. 4B). During this initial period telomerase extension products banded at a density of 1.775, which represents 35% BrdU substitution. Total lagging daughter overhangs were about 85 nt (Fig. 5B), thus telomerase had added only 30 nt (85×35%) to each telomere. Although we don't know how many telomerase molecules participated in this 30 nt addition, it is clear that under these conditions telomerase is much less processive than in normal Hela cells. Since Cajal bodies are involved in the processing and positioning of telomerase at telomeres(9, 21, 34) ,we examined whether changes in Cajal body or hTR positioning might be involved in this changed telomerase behavior.

Co-localization of hTR with one or more telomeres was found in ~13% of normal unsynchronized Hela cells (Fig. 5C). Treatment with GRN163L resulted in the progressive loss of hTR/telomere co-localization, dropping to ~6% after one week and ~2% after two weeks, where it remained at all times analyzed up to 12 weeks (Fig. 5C and Supplemental Fig. S4). No change was found in either the number of Cajal bodies/cell or the non-telomeric co-localization of hTR with Cajal bodies. This very low level of co-localization of hTR foci to telomeres was also present in cells newly released from GRN163L (5 PDs after release). The rapid inhibition of telomerase by GRN163L and its rapid recovery following GRN163L removal suggests that the slow change in co-localization is not directly related to the actual inhibition of telomerase by GRN163L. The number of hTR foci co-localizing with telomeres returned to normal by 20 PD, which coincides with the time that telomerase extension products changed from being short (~85) to ~200 nt (Fig. 5B) and telomeres began elongating (Fig. 4B). It has been reported that TCAB1 and TPP1 are directly involved in Cajal body mediated delivery of hTR/telomerase onto telomeres (1, 36). We examined the levels of TCAB1 and TPP1 during this period. TPP1 displayed no change in expression upon GRN163L treatment.

There was a minor ~15% decrease of TCAB1 in early released cells and a minor ~20% increase in late released cells (Fig. S4).

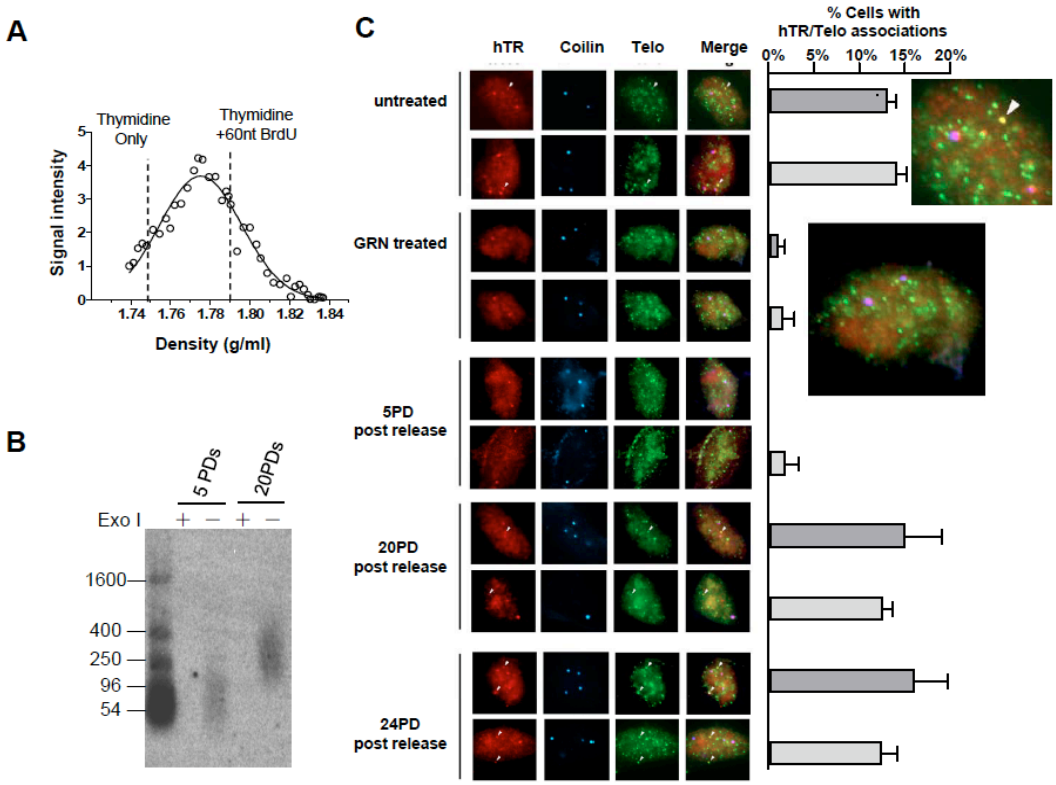


FIGURE 5. Less processive extension is associated with an absence of hTR foci on telomeres. (A) Reduced telomere extension (lower density of lagging overhangs on CsCl gradient) in cells released for 5 PD from GRN163L treatment. (B) Size of lagging overhang length for the cells released from GRN163L for 5 and 20 PDs. (C) Fluorescence micrographs (left panel) showing the intra-nuclear localization of hTR (red), telomeres (green), and Cajal bodies (visualized by immunofluorescence via coilin antibodies, blue) in cells treated as indicated. Plots (right panel) show the mean percentage of cells with hTR-telomere co-localizations observed in 5-15 fields of cells ± standard error. The results of two separate experiments are shown (dark grey and light grey). See also Figure S4.

DISCUSSION

Telomerase extension occurs at most telomeres during a single cell cycle in human cancer cells (39). In this study, our data indicates that under telomere length-maintenance conditions the extension on each telomere is carried out by one molecule of telomerase that processively adds ~60 nt to the end in both H1299 and Hela cells. This demonstrates that human telomerase is processive *in vivo*. The mechanism of telomerase action changes when telomeres elongate (either following overexpression of hTERT or after reversing the inhibition telomerase that produced shortened telomeres). Rather than increasing the processivity of telomerase so that each molecule adds many more repeats, telomerase becomes distributive, with multiple molecules acting in succession to produce greater numbers of extensions. Based on the behavior of telomerase in yeast (6) many authors have speculated that telomerase processivity is increased on short mammalian telomeres. Our results establish this is not the case. However, multiple differences (length, rates of shortening per division, fraction of extended ends, timing of replication during S phase) make mammalian telomere length control very different from that in yeast. Our results demonstrate that processivity is not increased on very short telomeres, but that the mode of elongation changes from a processive to a distributive one. These results elucidate different mechanisms of telomerase action under steady state and non-equilibrium conditions. In a cell under non-equilibrium conditions, containing both very long or maintenance-length and some much shorter telomeres, telomerase may be extending the maintenance-length telomeres with a single processive molecule while using multiple molecules in succession to preferentially elongate the very short telomeres. Mutational analysis has identified domains that affect the processivity of telomerase *in vitro*, but the ability of wild-type telomerase to exhibit changes in processivity *in vivo* has not been previously demonstrated in mammals. We also find that the endogenous telomerase is transiently less processive after cells have been released from long-term exposure to the telomerase inhibitor GRN163L, and also that exogenous overexpressed telomerase is less processive.

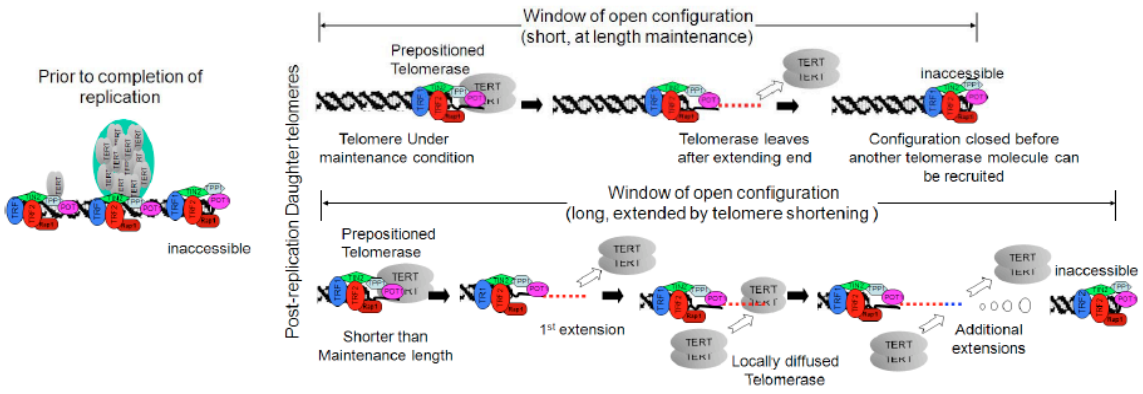


Figure 6. Model for telomerase action under steady and non-equilibrium condition. A prepositioned telomerase occupies the overhang at the start of the open configuration, and the open configuration closes before a second molecule can access the end. If the open configuration window is extended, additional molecules can be recruited. See also Figure S5.

Processivity and hTR foci

The reduction in processivity that occurs following the reversal of long-term GRN163L inhibition (Fig. 5A) is accompanied by changes in Cajal body-mediated trafficking of telomerase. Although no change in Cajal body number per cell or co-localization of hTR with Cajal bodies was seen, there was a large reduction in the number of hTR foci that co-localized with telomeres. The mechanism by which GRN163L produces this change is unclear, but it must be indirect since it requires several weeks while the direct inhibition of telomerase action by GRN163L in culture only requires a few days. Similar effects have been seen following depletion of TCAB1, the factor that brings telomerase to Cajal bodies. TCAB1 depleted cells show a decrease in hTR/telomere co-localization that is associated with telomere shortening (36). This is consistent with our observation of a decrease in telomerase processivity following a reduction in hTR-telomere co-localization, so that it is now insufficient to compensate for the rate of telomere shortening. However, an estimated 90% knockdown of TCAB1 only resulted in a 30% reduction of hTR at telomeres (36). Although we observed a small (~15%) decrease in TCAB1 expression in cells newly released from GRN163L and a similarly small increase (~20%) after 20 PD (Fig. S5), we do not believe these small

changes are sufficient to explain the very large (6-fold) reduction of hTR at telomeres that we observed. This suggests the existence of additional factors in addition to TCAB1 that contribute to the regulation of telomerase localization to telomeres and its extension processivity.

Telomerase activity in extracts was rapidly restored within a few days of GRN163L removal but the decrease in processivity persisted for several weeks (Fig. 4B and 5A). The timing of the return to a normal processivity coincided with the time that hTR foci reappeared (Fig. 5B,C). It is unclear why delivery of hTR foci to telomeres should affect processivity. *In vitro*, telomerase processivity is increased by interactions with both TPP1 and Pot1 (23), and TPP1 is required for hTR co-localization at telomeres (1). However, we observed no change of the expression level of TPP1 upon treatment with GRN163L (Fig. S5), suggesting that in addition to facilitating specific TPP1/Pot1 interactions that might affect processivity, there may be posttranslational modifications, post-transcriptional modifications (hTR), conformational changes, or associations with other factors that occur in Cajal bodies that affect the processivity of the telomerase delivered to telomeres in hTR foci. These changes may be removed or altered by extraction and thus not affect the *in vitro* activity or processivity of telomerase in cell lysates.

The number of catalytically active telomerase molecules per tumor cell has been estimated to be less than 100 (8), while the number of telomere ends following replication in an aneuploid cancer cell with 70-80 chromosomes is several hundred. One role for Cajal bodies may be to coordinate the successive delivery of this limited number of molecules to different telomeres during S-phase (10, 21). The decreased processivity we observed following reversal of long-term exposure to GRN163L suggests that passage through Cajal bodies and delivery to telomeres may have additional effects.

Because of its high specificity and efficiency in inhibition of telomerase activity both *in vitro* and *in vivo*, GRN163L (Imetelstat) is now in phase II clinical trials (29). If similar effects on hTR localization/processivity occur in patients, it has significant implications on dosing schedules. The requirements for achieving adequate drug levels to continuously suppress telomerase activity may be much greater than that needed to simply sustain the lack of hTR/telomere co-localization. Preclinical *in vitro* studies have

found that telomere length rapidly recovers following removal of inhibition, suggesting that 4-5 days of telomerase inhibition followed by 2-3 days of telomere elongation would result in very little overall shortening. However, if lack of telomerase co-localization is sustained for a few extra days, recovery of telomerase activity between doses would be ineffective in elongating telomeres and the rate of shortening would only show minor differences under a less frequent dosing schedule.

Processivity and distributive action in cells overexpressing hTERT

Many molecules of telomerase successively elongate telomeres in cells soon after the overexpression of telomerase (distributive action). Inhibition by GRN163L results in the amount of extension progressively decreasing, and when a significant fraction of non-extended ends appears the amount added to the remainder is only ~40 nt compared to the ~65 nt added under length-maintenance conditions. The reasons for this decreased processivity of individual molecules remain unresolved. The accuracy of the TRAP assay and the variability of inhibition under different experimental conditions make it unreliable to distinguish whether the actual inhibition achieved by 0.5 μM GRN163L in the cells overexpressing telomerase was 75% versus 90%. If telomerase were inhibited by 75% in this experiment, the probability of a non-extended end would be 75% after one cycle of recruitment, 56% ($.75^2$) after two cycles, and 34% ($.7^4$) after 4 cycles. If one estimates that the thymidine-only peak in cells overexpressing hTERT and inhibited by 0.5 μM GRN163L (Fig. 3C) represents ~30% of the total, this would be consistent with ~4 different molecules each adding 40 nt to the ends to produce the observed 160 nt extension. Whether these multiple recruitments occur within the same time window of open configuration present before overexpression or whether the massive overexperession of the catalytic subunit is altering the open configuration by interacting with other factors is unknown. We cannot formally exclude the possibility that some of the decreased addition length results from overexpressed telomerase causing degradation of the 3' end.

Prepositioning of Telomerase at Telomeric Overhangs

Under telomere length maintenance conditions, our data indicates that every telomere is extended by telomerase only once in both H1299 and Hela cells. This observation has important implications for the molecular mechanisms by which telomerase interacts with the protein complex at the 3' overhang and the nature of the open configuration during which telomerase is capable of extending the end. TRF1 and TRF2 (at least GFP-fusion protein versions) appear to have telomere binding half-lives of seconds to minutes (25), suggesting that telomerase recruited to the telomere by binding to shelterin components may not be stably bound but could be in constant flux, resulting in an increased diffusible local concentration of telomerase at the telomeres. Whether the distribution of telomerase is uniform over the telomere or biased towards the base or terminus (35) is unknown in mammals. If telomerase were simply interacting with factors at the end of the telomere based upon diffusion (from a high local concentration due to interaction with telomeric proteins or delivery of multiple telomerase molecules to the double-stranded region of the telomere by Cajal bodies (1)), one would expect a Gaussian distribution of elongation products. A Gaussian distribution of the recruitment of diffusible telomerase to the ends could produce an average of one elongation event per telomere, but it should produce a distribution in which the average might be one but some ends would be extended by more than one molecule and others not at all. This was not observed. Thus special conditions must be present to narrow the distribution to prevent a significant fraction of either non-extended ends or ends extended several times. One possibility is that the time it takes for telomerase to add ~60 nt is long in comparison to the time of the open configuration during which telomerase could be recruited to act on the end. Under these circumstances, the open configuration would have closed before a second telomerase molecule could be recruited to act, and the ability of more than one molecule to act would be minimized. A diffusion-limited recruitment of telomerase could thus yield the observed result as long as the abundance of telomerase in the vicinity of the end of the telomere was in sufficient excess to ensure that virtually every end recruited a

molecule. However, the hTERT depletion results (Fig. 2D) demonstrate that the concentration of telomerase was limiting (not in excess) in these experiments. Depletion of hTERT by only ~50% resulted in a roughly 50% decrease in the fraction of ends that were extended. A high enough local concentration of telomerase to ensure that a Gaussian distribution did not result in any detectable non-extended ends should only show extremely small changes resulting from a 50% decrease in the amount of telomerase. Because of these considerations, we propose the working hypothesis that there is a specific prepositioning step at the overhang that ensures that following the completion of replication every end both gets extended but also gets extended by only one molecule of telomerase under length-maintenance conditions. This prepositioning step could be separate from the delivery of multiple telomerase molecules to the telomere by Cajal bodies. It would not distinguish between an active telomerase molecule and one with GRN163L bound to its active site, thus the prepositioning of an inhibited telomerase molecule would prevent a second uninhibited molecule from being subsequently recruited before the open configuration closed. The probability of being able to preposition telomerase would be directly proportional to amount of telomerase present under length homeostasis.

One of several possible models for the prepositioning step is that the closed configuration would have Pot1 sequestering the end in a TRF1/2-Tin2-TPP1-Shelterin complex (2). The open configuration would be initiated by binding of the locally diffusible or a specifically transferred telomerase molecule to this terminal TPP1-Pot1, either in competition with the sequestered end or causing a conformational change that caused the sequestered end to be released. This could produce the observed result if the residency time (the time it took telomerase to elongate the telomere) were longer than the potential time of the open configuration for initiating extension, since all elongating ends would close before a second elongation event could occur. Since our data indicates that only a single molecule of telomerase elongates each end at maintenance, this would imply that telomerase is released from its TPP1-Pot1 interaction after it processively adds ~60 nt. Under length-maintenance conditions, this could provide the signal for closing the open configuration by allowing the freed Pot1 to reestablish sequestration of the end.

Many other detailed hypotheses for the nature of these steps are possible, and our working model is only presented as a framework for understanding the concept of prepositioning.

Telomerase action in stem cells

It is important to know whether the mechanisms we have found for telomerase action in cancer cells also apply to stem cells. We thus performed our telomerase action assays using human Embryonic Stem cells (hES). Technical limitations restricted the assays to unsynchronized cells. Similar to what we observed in human cancer cells, telomerase extends most (70%) telomeres during a single cell cycle in hES cells and C-rich Fill-in is uncoupled from telomerase elongation of the G-rich strand (Fig. S5). Although at present we are unable to determine the extension processivity of telomerase in hES cells due to our inability to synchronize the cells without altering their pleuripotency, it appears that the general principles of telomerase regulation are shared by cancer and stem cells.

Major modes of telomerase action

Figure 6 presents a model that summarizes the two major modes of telomerase action under homeostatic versus disequilibrium length conditions. Prior to the completion of replication, multiple telomerase molecules processed by Cajal bodies are delivered to the telomere. Whether these remain in a packaged structure or are dispersed along the length of the telomere is unknown. Passage of the replication fork and processing makes the 3' overhang accessible for telomerase action. Under length maintenance conditions the end is extended by a single molecule of telomerase that processively adds ~60 nt to the end. A prepositioning step ensures that the frequency of elongation is not solely due to diffusion-limited interactions due to a high local concentration of telomerase but is limited to a single extension reaction before the open configuration closes. When telomeres are shorter than their maintenance size, the length of the open configuration is extended, possibly due to lesser amounts of TRF1 and TRF2 binding to the double

stranded regions. Additional telomerase molecules are then able to diffuse to the overhang from high local concentrations and additional extensions take place. Thus, multiple rounds of extension could occur until the closed condition became reestablished. In addition to these two major modes of telomere elongation, we have identified two situations in which the processivity of telomerase is decreased; in cells overexpressing hTERT and following a treatment that interferes with Cajal-body delivery of hTR foci to telomeres. The detailed understanding of the mechanisms underlying all of these different ways in which telomerase acts on telomeres will greatly contribute to our knowledge of how telomerase functions in human cancer cells and what steps might be subject to manipulation for therapeutic purposes.

ACKNOWLEDGMENTS

This work was supported by AG01228 from the National Institute on Aging (WEW), CA104676 from the National Cancer Institute (MPT and RMT), the American Federation for Aging Research (YZ) and a Ruth L. Kirschstein National Research Service Award (F31GM087949) for Individual Predoctoral Fellows from the National Institutes of Health (EA). We thank Prof. Songyang Zhou from Baylor College of Medicine for providing the pCMV-TPP1 plasmid.

References

1. Abreu, E., E. Aritonovska, P. Reichenbach, G. Cristofari, B. Culp, R. M. Terns, J. Lingner, and M. P. Terns. 2010. TIN2-tethered TPP1 recruits human telomerase to telomeres in vivo. Mol Cell Biol 30:2971-2982.
2. Bianchi, A., and D. Shore. 2008. How telomerase reaches its end: mechanism of telomerase regulation by the telomeric complex. Mol Cell 31:153-165.
3. Blackburn, E. H. 2001. Switching and signaling at the telomere. Cell 106:661-673.
4. Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright. 1998. Extension of life-

span by introduction of telomerase into normal human cells. Science 279:349-352.

5. Bryan, T. M., A. Englezou, M. A. Dunham, and R. R. Reddel. 1998. Telomere length dynamics in telomerase-positive immortal human cell populations. Exp Cell Res 239:370-378.
6. Chang, M., M. Arneric, and J. Lingner. 2007. Telomerase repeat addition processivity is increased at critically short telomeres in a Tel1-dependent manner in Saccharomyces cerevisiae. Genes Dev 21:2485-2494.
7. Chen, J. L., and C. W. Greider. 2003. Determinants in mammalian telomerase RNA that mediate enzyme processivity and cross-species incompatibility. EMBO J 22:304-314.
8. Cohen, S. B., M. E. Graham, G. O. Lovrecz, N. Bache, P. J. Robinson, and R. R. Reddel. 2007. Protein composition of catalytically active human telomerase from immortal cells. Science 315:1850-1853.
9. Cristofari, G., E. Adolf, P. Reichenbach, K. Sikora, R. M. Terns, M. P. Terns, and J. Lingner. 2007. Human telomerase RNA accumulation in Cajal bodies facilitates telomerase recruitment to telomeres and telomere elongation. Mol Cell 27:882-889.
10. Cristofari, G., and J. Lingner. 2006. Telomere length homeostasis requires that telomerase levels are limiting. EMBO J 25:565-574.
11. de Lange, T. 2005. Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev 19:2100-2110.
12. Forsythe, H. L., J. L. Jarvis, J. W. Turner, L. W. Elmore, and S. E. Holt. 2001. Stable association of hsp90 and p23, but Not hsp70, with active human telomerase. J Biol Chem 276:15571-15574.
13. Greider, C. W., and E. H. Blackburn. 1989. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature 337:331-337.
14. Harley, C. B., A. B. Futcher, and C. W. Greider. 1990. Telomeres shorten during ageing of human fibroblasts. Nature 345:458-460.

15. Hemann, M. T., M. A. Strong, L. Y. Hao, and C. W. Greider. 2001. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell 107:67-77.
16. Herbert, B., A. E. Pitts, S. I. Baker, S. E. Hamilton, W. E. Wright, J. W. Shay, and D. R. Corey. 1999. Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. Proc Natl Acad Sci U S A 96:14276-14281.
17. Herbert, B. S., G. C. Gellert, A. Hochreiter, K. Pongracz, W. E. Wright, D. Zielinska, A. C. Chin, C. B. Harley, J. W. Shay, and S. M. Gryaznov. 2005. Lipid modification of GRN163, an N3'-->P5' thio-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition. Oncogene 24:5262-5268.
18. Herbert, B. S., K. Pongracz, J. W. Shay, and S. M. Gryaznov. 2002. Oligonucleotide N3'-->P5' phosphoramidates as efficient telomerase inhibitors. Oncogene 21:638-642.
19. Herbert, B. S., J. W. Shay, and W. E. Wright. 2003. Analysis of telomeres and telomerase. Curr Protoc Cell Biol Chapter 18:Unit 18 16.
20. Jackson, S. R., C. H. Zhu, V. Paulson, L. Watkins, Z. G. Dikmen, S. M. Gryaznov, W. E. Wright, and J. W. Shay. 2007. Antiadhesive effects of GRN163L--an oligonucleotide N3'->P5' thio-phosphoramidate targeting telomerase. Cancer Res 67:1121-1129.
21. Jady, B. E., P. Richard, E. Bertrand, and T. Kiss. 2006. Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. Mol Biol Cell 17:944-954.
22. Kim, N. W., M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011-2015.
23. Latrick, C. M., and T. R. Cech. 2010. POT1-TPP1 enhances telomerase processivity by slowing primer dissociation and aiding translocation. EMBO J 29:924-933.

24. Masutomi, K., E. Y. Yu, S. Khurts, I. Ben-Porath, J. L. Currier, G. B. Metz, M. W. Brooks, S. Kaneko, S. Murakami, J. A. DeCaprio, R. A. Weinberg, S. A. Stewart, and W. C. Hahn. 2003. Telomerase maintains telomere structure in normal human cells. Cell 114:241-253.
25. Mattern, K. A., S. J. Swiggers, A. L. Nigg, B. Lowenberg, A. B. Houtsmuller, and J. M. Zijlmans. 2004. Dynamics of protein binding to telomeres in living cells: implications for telomere structure and function. Mol Cell Biol 24:5587-5594.
26. McChesney, P. A., D. L. Aisner, B. C. Frank, W. E. Wright, and J. W. Shay. 2000. Telomere dynamics in cells with introduced telomerase: a rapid assay for telomerase activity on telomeres. Mol Cell Biol Res Commun 3:312-318.
27. Ouellette, M. M., M. Liao, B. S. Herbert, M. Johnson, S. E. Holt, H. S. Liss, J. W. Shay, and W. E. Wright. 2000. Subsenescent telomere lengths in fibroblasts immortalized by limiting amounts of telomerase. J Biol Chem 275:10072-10076.
28. Palm, W., and T. de Lange. 2008. How shelterin protects mammalian telomeres. Annu Rev Genet 42:301-334.
29. Roth, A., C. B. Harley, and G. M. Baerlocher. 2010. Imetelstat (GRN163L)--telomerase-based cancer therapy. Recent Results Cancer Res 184:221-234.
30. Samper, E., J. M. Flores, and M. A. Blasco. 2001. Restoration of telomerase activity rescues chromosomal instability and premature aging in Terc-/- mice with short telomeres. EMBO Rep 2:800-807.
31. Shay, J. W., and S. Bacchetti. 1997. A survey of telomerase activity in human cancer. Eur J Cancer 33:787-791.
32. Teixeira, M. T., M. Arneric, P. Sperisen, and J. Lingner. 2004. Telomere length homeostasis is achieved via a switch between telomerase- extendible and -nonextendible states. Cell 117:323-335.
33. Tomlinson, R. L., J. Li, B. R. Culp, R. M. Terns, and M. P. Terns. 2010. A Cajal body-independent pathway for telomerase trafficking in mice. Exp Cell Res.
34. Tomlinson, R. L., T. D. Ziegler, T. Supakorndej, R. M. Terns, and M. P. Terns. 2006. Cell cycle-regulated trafficking of human telomerase to telomeres. Mol Biol Cell 17:955-965.

35. Vega, L. R., M. K. Mateyak, and V. A. Zakian. 2003. Getting to the end: telomerase access in yeast and humans. Nat Rev Mol Cell Biol 4:948-959.
36. Venteicher, A. S., E. B. Abreu, Z. Meng, K. E. McCann, R. M. Terns, T. D. Veenstra, M. P. Terns, and S. E. Artandi. 2009. A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. Science 323:644-648.
37. Wang, F., E. R. Podell, A. J. Zaug, Y. Yang, P. Baciu, T. R. Cech, and M. Lei. 2007. The POT1-TPP1 telomere complex is a telomerase processivity factor. Nature 445:506-510.
38. Zhao, Y., H. Hoshiyama, J. W. Shay, and W. E. Wright. 2008. Quantitative telomeric overhang determination using a double-strand specific nuclease. Nucleic Acids Res 36:e14.
39. Zhao, Y., A. J. Sfeir, Y. Zou, C. M. Buseman, T. T. Chow, J. W. Shay, and W. E. Wright. 2009. Telomere extension occurs at most chromosome ends and is uncoupled from fill-in in human cancer cells. Cell 138:463-475.

SUPPLEMENTAL INFORMATION

Supplemental data including five figures can be found online.