CLARIFICATION OF THE ROLES OF THE TELOMERE BINDING PROTEIN TPP1

AND TELOMERASE RNA IN TELOMERASE TRAFFICKING

by

JIAN LI

(Under the Direction of Dr. Michael P. Terns and Dr. Rebecca M. Terns)

ABSTRACT

Telomerase is the ribonucleoprotein (RNP) that contains the telomerase reverse transcriptase (TERT) and the telomerase RNA component (TR). It maintains the length of telomeres and is closely associated with aging, cancer, and a few congenital diseases. In humans, the trafficking of telomerase includes the localization of telomerase to Cajal bodies and telomeres. This trafficking process has been shown to be critical in the functioning of telomerase. Our goal is to investigate the factors that affect telomerase trafficking in humans. We tried to clarify the individual roles of the telomere-located shelterin proteins TPP1 and TIN2 in telomerase trafficking. We postulated that TPP1 is the key recruiter for recruitment and we employed a tethering assay using the LacO-lacR system to investigate this hypothesis. We found that TPP1 is the major recruiter in telomerase localization to both telomeres and a non-telomeric site, while TIN2 appears to be expendable. Next, we investigated the molecular basis for the lack of localization of mouse TR to Cajal bodies in mouse cells. We examined the localization of human TR expressed in mouse cells and found that the inherent features of human human TR provide for recruitment of telomerase to Cajal bodies in mice. Taken together, this thesis reveals that telomerase trafficking is a highly regulated process controlled by multiple factors including telomere shelterin proteins and internal telomerase properties.

INDEX WORDS: telomerase, telomere, telomerase trafficking, TPP1, TIN2

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Chapter 1

INTRODUCTION AND LITERATURE REVIEWS

Human chromosomes have protective DNA-protein structures called telomeres that prevent chromosomal degradation as well as end-to-end fusions (1-3). The DNA portion of the telomere is comprised of repeats of the sequence TTAGGG (1-3). Due to the unidirectional replication of conventional DNA polymerases, some telomere sequences are lost after each round of DNA synthesis (4). Telomerase is a ribonucleoprotein (RNP) complex as it minimally contains a reverse transcriptase (TERT) protein and an RNA component (TR). The end of telomere can be elongated by telomerase reverse transcriptase using the RNA component as the template (5-7). Studies conducted over last two decades have indicated that human telomerase is related closely to aging, cancer, and some congenital diseases such as dyskeratosis congenita (DC) (7-9). The pace of the aging process of human penile smooth muscle cells is reduced after telomerase transcriptase over-expression (10). In cancer cells, telomerase has a much higher level of activity when compared to normal human somatic cells (8, 11, 12). Mutations in several telomerase subunits were also discovered in DC patients (13, 14). Telomerase is not always located at telomeres; it is only recruited to telomeres during the S phase of the cell (15, 16). Although the mechanism of how telomerase gets recruited to telomere remains unknown, the trafficking of telomerase is important for telomerase to function properly. Previous studies have shown that the disruption of telomerase trafficking affects telomere elongation and leads to certain diseases such as DC (17-19). Any of the factors involved in this trafficking process might

have an impact on telomerase function and contribute to potential therapeutic approaches in human cancer and aging (20-22).

Telomerase

TR

Telomerase RNA serves as the template for telomere repeat addition. Human telomerase RNA (hTR) has 451 nucleotides while mouse telomerase RNA (mTR) has 397 nucleotides (23, 24). The RNA molecule consists of a template and four distinct conserved structural domains: the pseudoknot domain, the CR4-CR5 domain, the box H/ACA domain, and the CR7 domain (25). Mouse telomerase RNA has a shorter template length compared to its human counterpart (26, 27). Extending the mTR template to resemble the length of hTR enhances telomerase activity (26, 27). Upstream of the template region, mTR doesn't have the P1 helix as does hTR. Mouse telomerase activity was higher when the mTR was constructed to carry the hTR P1 helix (27). Of the four conserved domains, the pseudoknot domain and the CR4-CR5 domain have been linked to telomerase activity as well as *in vivo* processing (24, 28-30). The Box H/ACA domain is associated with hTR accumulation and *in vivo* telomerase catalytic activity (31, 32). The CR7 domain encompasses a CAB (Cajal body) box that is considered to be important in telomerase trafficking (33). The CAB box is composed of four nucleotides (UGAG) and is located in the loop of the CR7 domain. Previous mutation research performed in human cancer cells has shown that the CAB box is essential for small Cajal body RNAs (scaRNAs) and hTR to accumulate at Cajal bodies (33). The CAB box is also associated with TCAB1 (Telomerase Cajal body protein 1), a telomerase subunit that was discovered to be crucial for the trafficking of telomerase and also for telomere maintenance (34-38).
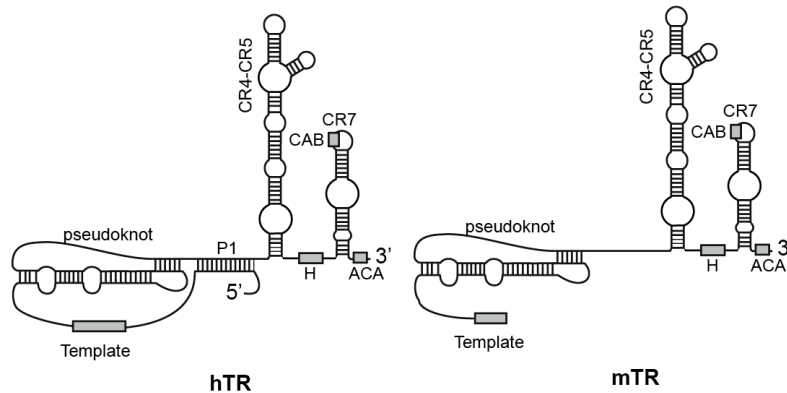
Figure 1: Proposed secondary structure of human telomerase RNA (hTR) and mouse telomerase RNA (mTR) [Figure modified from Chen et al., 2000]

TERT

Telomerase reverse transcriptase (TERT or hTERT in humans) is the catalytic subunit of telomerase. The enzyme is required not only in synthesizing the telomere DNA but also in telomerase trafficking (17, 39). It has a conserved reverse transcriptase (RT) motif located at the central region of the enzyme, an N terminal extension (NTE or TEN domain) of the protein that occupies about 400 amino acids, and a smaller C terminal extension (CTE) that contains about 200 amino acids (40). The N- terminal domain has been suggested to be important in telomerase processivity in human, yeast, and Tetrahymena (40-42). In the N-terminal domain, the telomerase RNA binding domain (TRBD) builds a close connection with the telomerase RNA component. It is considered to function in telomerase assembly and telomerase processivity (17, 39). The DAT (Dissociates Activities of Telomerase) domain also resides in the N terminal extension. hTERT-TRF2 fusion protein (carrying DAT mutant) could rescue the telomere-shortening caused by the DAT mutant of hTERT (43). Considering both the DAT mutants and

the fusion protein share similar telomerase activity *in vitro*, this observation suggests a recruitment function of DAT domain to interact with telomere (43).

Cajal bodies

As first reported by Spanish cytologist Ramón y Cajal, Cajal bodies are later discovered to be evolutionarily conserved, highly dynamic sub-nuclear compartments in plant and animal cells (44, 45). Cajal bodies are mostly found in cells that have high transcriptional activity, such as rapidly dividing cells (45). Many nuclear factors including components of splicing snRNPs, nucleolar snoRNPs, and their biogenesis enzymes accumulate in Cajal bodies. As a result, it has been predicted as a location for post-transcriptional RNP assembly and/or RNA modification in the cell (46-49). Coilin is a protein that is enriched in Cajal bodies and generally serves as a Cajal body marker protein (50). Other proteins that accumulate at Cajal bodies include: SMN (survival of motor neurons), TCAB1 (telomerase Cajal body protein 1), Nopp140 (a snoRNP chaperone), and Fibrillarin (a snoRNP protein) (36, 51-53). There is also a unique class of RNA found specifically in CBs called small Cajal body RNA (scaRNA) and this includes U85. This class of RNA is responsible for pseudouridylation of U2 splicesomal RNA (54, 55). Cajal bodies have been considered a site for telomerase biogenesis since SMN complex (a protein complex enriched in Cajal bodies) is known to interact with telomerase (37, 38). Both hTR and hTERT can be found at Cajal bodies and all three can be found at telomeres during the S phase (18, 39, 56). Disruption of telomerase localization at Cajal bodies by either mutation of hTR CAB box or depletion of TCAB1 resulted in a reduction of telomerase-telomere association (57, 58). These findings suggest that the Cajal bodies are crucial in telomerase trafficking.

Telomere shelterin proteins

A group of six telomere-binding proteins have been identified and classified as the shelterin complex. This includes: TRF1 (Telomere Repeat binding Factor 1), TRF2 (Telomere Repeat binding Factor 2), RAP1 (Repressor Activator Protein 1), POT1 (Protection Of Telomere protein 1), TIN2 (TRF1-Interacting protein 2), and TPP1 (TIN2 and POT1-interacting Protein 1) (59, 60). TRF1 and TRF2 directly bind to double strand telomeric DNA (61, 62). They are considered to be the negative regulators of telomeric length, since the over-expression of either one causes telomere shortening (61, 62). RAP1is tethered to telomere by an interaction with TRF2 and was predicted to be the negative regulator of telomere length in *vivo* since the knockdown of RAP1 resulted in longer telomeres (63-65). POT1 (Protection Of Telomere protein 1) binds to the 3' G rich single stranded overhangs of telomeres and aids in telomere extension when coupled with TPP1 (66-68). However POT1 is not required for telomerase trafficking since depletion of POT1 had little effect on telomerase-telomere association (69) Overexpression of epitope-tagged shelterin proteins in human cells revealed that the complex did not form or formed poorly when TIN2 or TPP1 were not present (70). As a result, shelterin proteins TIN2 and TPP1 were suggested to be key components in maintaining the stability of the complex (70). Recent studies have also discovered that the TPP1-TIN2 complex is essential in telomerase trafficking (69, 71). TIN2 was found to associate with TRF1 and TRF2 at the same
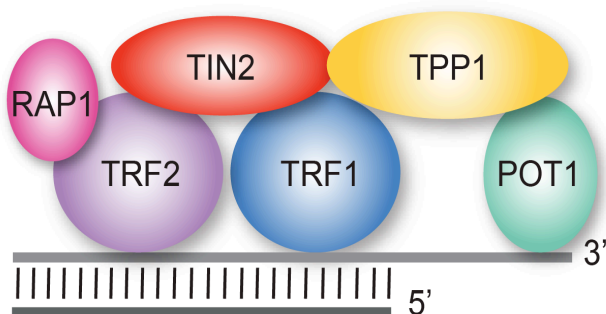
Figure 2: Schematic representation of telomere shelterin complex. *The six proteins that constitute the shelterin complex protect and regulate telomere. *Model indicates known interactions between shelterin components and telomere DNA***. [16166375, 18828880]**

time and serves to stabilize TRF2-RAP1-telomere complex (70). TPP1 does not bind to telomeres directly but links to telomeres via TIN2 or POT1 (70, 72). The TPP1 protein has a N-terminal OB fold domain, a recruitment domain for POT1, a serine/threonine rich domain, and a TIN2 interacting domain at the C- terminal end (72). The OB fold of TPP1 is a predicted oligonucleotide/oligosaccharide-binding fold (residue 87-240) that has been shown to regulate telomerase activity, interact with telomerase, and recruit telomerase (69, 71-73).
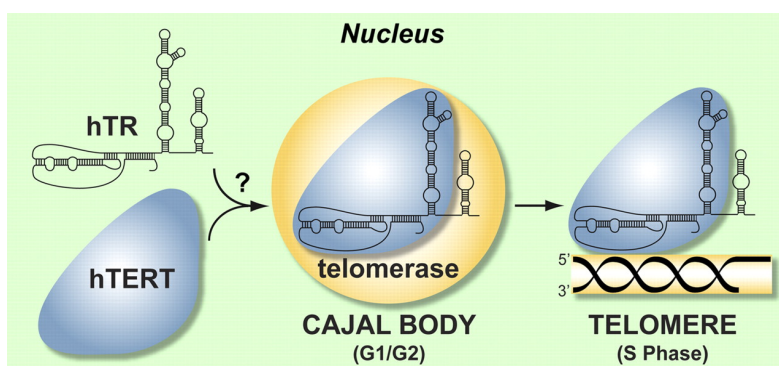
Telomerase trafficking pathway

Telomere extension can be impaired when the trafficking pathway of telomere is interrupted, even though the telomerase is catalytically active (57). This suggests that the telomerase trafficking pathway regulates telomere elongation by telomerase. The knowledge of telomerase trafficking is limited but it is known that telomerase can be found in Cajal bodies during the G1 and G2 phases of the cell cycle, as well as the telomere during the S phase (18, 56).

During G1 and G2 phases of the cell cycle of human cancer cells, human telomerase RNA (hTR) is observed in Cajal bodies (18, 56). The accumulation of hTR in Cajal bodies only occurs in telomerase positive cancer cells and not in telomerase-negative cells (17, 18, 39). Through ectopic expression, the other important component of telomerase (hTERT, YFP tagged) can also be found at Cajal bodies (39). A similar situation occurs in Xenopus oocytes: the Cajal

body is the site for the major trafficking pathway of telomerase based on the observation that microinjected TR could rapidly go to the Cajal bodies (74). As Cajal bodies appear to play a role in the assembly and maturation of small nuclear or nucleolar RNPs, they have been implicated as sites for telomerase biogenesis and trafficking of telomerase to telomere (33). In mouse cells (from various tissue sources) however, mTR (mouse telomerase RNA) does not correlate with any of the markers of Cajal bodies but instead accumulates at unique loci other than Cajal bodies (75).

During the S phase of the cell cycle, when the telomere replicates, both hTR and hTERT can be seen localizing to a subset of telomeres. This phenomenon peaks at middle S phase (18, 56). A portion of this telomere-associated hTR has Cajal bodies alongside (18, 56), suggesting that Cajal bodies might be carrying telomerase to telomere during this part of cell cycle. In contrast, mouse TR does not locate at Cajal bodies, yet it can be found at a subset of telomeres during S phase. This indicates a different pathway of telomerase trafficking to telomeres in mouse cells compared to the human cells (75).

Figure 3: Telomerase trafficking pathway. hTR and hTERT can be found at Cajal bodies. They can also reside at telomere during S phase (17).

Factors affecting telomerase trafficking

Trafficking to Cajal bodies

There are multiple factors affecting the recruitment of telomerase to Cajal bodies in human cells. TCAB1 (telomerase Cajal body protein 1) is a protein that is enriched in Cajal bodies and also a component of telomerase (36). Knockdown of TCAB1 did not affect telomerase activity or overall endogenous hTR levels in HeLa cells, but shortened telomere length. By labeling hTR and Cajal bodies in HeLa cells it was revealed that the knockdown of TCAB1 significantly reduced the number of cells with hTR-Cajal body associations (58). Therefore, TCAB1 is a factor required for telomerase association with CBs at telomeres (58).

TERT, the catalytic component of telomerase also plays a role in the trafficking of telomerase to Cajal bodies. In human normal somatic cells, which are originally TERT negative, expression of human TERT induced the accumulation of hTR at Cajal bodies (17). Depletion of hTERT in human cancer cells resulted in a loss of hTR association with Cajal bodies, while the overall hTR level remained the same (39).

In addition to TCAB1 and TERT, elements in the RNA component of telomerase also contribute to the telomerase-Cajal body association. Studies have shown that mutation of the CAB box within hTR alters the hTR-Cajal body association and impairs telomere length without affecting the biogenesis of active telomerase (33, 55). This suggests that the CAB box is critical in the trafficking of telomerase to Cajal bodies. Since the mutation of hTR CAB box also affects the interaction of the Sm proteins with hTR (76), some Sm proteins such as the SmB or SmD might be potential regulators of telomerase recruitment to Cajal bodies as well.

Trafficking to Telomeres

There are some key factors that contribute to the recruitment of telomerase to telomeres. TCAB1 and TERT not only play an important role in the localization of telomerase to Cajal bodies, but telomeres as well. hTR seldom goes to telomeres after the knockdown of TCAB1 and the exogenous hTERT in human normal somatic cells stimulates the association of hTR with telomeres, specifically in the S phase (17, 58).

The co-localization of hTR to a subset of telomeres in cells is generally observed in the S phase, particularly peaking at the middle of the S phase (18, 56). This phenomenon suggests that the cell cycle could be one of the affecting factors for recruitment of telomerase to telomeres as well. As a result, some cell cycle regulators such as cdk2-cyclin A and cdc 25 might be crucial in the process.

 In recent studies, it was discovered that the depletion of either TPP1 or TIN2 led to a striking loss of telomerase localization to telomeres in humans and mice (69, 77). This indicates that telomere shelterin protein could also be an important factor in telomerase trafficking pathways.

Overview

The general goal of this research is to investigate factors affecting telomerase trafficking in humans. The focus of the first work is to elucidate the role of telomere binding proteins TPP1 and TIN2 in the recruitment of telomerase. Previous work by our group showed that TPP1 along with TIN2, was required for the localization of telomerase to telomere (69). This study was not able to unmask the individual function of the shelterin complex components due to limitations in techniques used (RNA interference-induced depletion of TPP1 or TIN2 attenuates level of the

other) (69).TPP1 was able to pull down *in vitro* translated tagged TERT protein, suggesting that

it interacts with telomerase (72). Also, co-transfection of shRNA- resistant TPP1 restored hTR-

telomere association caused by the knockdown of TPP1 (69), suggesting a crucial role of TPP1

in telomerase trafficking. We hypothesize that telomerase is recruited to telomeres by TPP1 and

this process is independent of TIN2.

In chapter 2, we will describe the known interactions between the telomere shelterin

proteins and telomerase, particularly TPP1, TIN2 and telomerase. We will also show the role of

the TPP1-TIN2 complex in telomerase recruitment. We used a tethering assay to bridge TPP1 to

telomeres via TRF2 to circumvent TIN2 and studied changes in telomerase-telomere association,

after introduction of TPP1-TRF2 protein in the cell. To avoid possible secondary effects at

telomeres we also employed the LacR-LacO system (a system to link target proteins to the

chromosomes via the binding of the *lac* repressor protein to *lac* operator sequence) to test the

ability of TPP1 to traffick telomerase to a non-telomeric site. Our results indicate that TPP1 is a

major recruiter of telomerase and this recruitment is independent of TIN2. This discovery is one

step towards understanding the process of telomerase recruitment to telomeres and provides a

possible target for modification and regulation of this process.

Our second focus is to understand the telomerase trafficking pathways of humans by

comparison with telomerase trafficking pathways in mice. Surprisingly, while telomerase RNA

localizes to Cajal bodies in a variety of human cancer cells, it does not accumulate at Cajal

bodies in mouse cells (including several different somatic and cancer cell lines). Instead, it

resides in novel nuclear bodies of similar size and number as Cajal bodies (75). Considering the

factors affecting localization of telomerase to Cajal bodies, we postulated that either protein

interactions specific to cellular environment or specific telomerase RNA elements were

responsible for this trafficking difference between humans and mice. As localization of telomerase at Cajal bodies facilitates telomerase recruitment to telomeres in human cancer cells (57), it was hoped that this investigation would help us to further understand the role of Cajal bodies in telomerase trafficking in humans.

The study, which is described in the appendix, shows the detection of mouse telomerase RNA, Cajal bodies, and telomeres in mouse cells. We describe the trafficking process of mouse telomerase to both of the novel nuclear bodies and telomere. Differences between the trafficking of mouse and human telomerase RNA are also compared. To identify the major cause of the difference, we introduced hTR into the mouse cell line. We found that hTR localizes at Cajal bodies in mice. This result suggests that the inherent telomerase RNA elements determine recruitment of telomerase to Cajal bodies in mice. This work lays the foundation for further telomerase RNA structural studies and also reveals an alternative pathway for telomerase trafficking.

Overall, our work provides a better understanding of the regulation of telomerase recruitment through the identification of key factors including shelterin proteins and telomerase RNA structures. This work contributes to the current knowledge of telomerase trafficking pathways and the mechanisms behind it.

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Chapter 2

TPP1 RECRUITS TELOMERASE TO TELOMERES INDEPENDENTLY OF TIN2

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Abstract

Telomerase is recruited to telomeres during DNA replication to catalyze the extension of telomeres. The intranuclear mobilization of telomerase is critical for the activity of telomerase and the elongation of telomeres in human cells. Two proteins in the shelterin complex that are found at telomeres have been implicated in recruitmet of telomerase to telomeres. Here we used two distinct protein-tethering systems to clarify the individual roles of TPP1 and TIN2 in telomerase trafficking. We found that tethering the OB domain of TPP1 (known to mediate interacton with telomerase) to telomeres via TRF2 (independently of TIN2) resulted in recruitment of telomerase to telomeres. Expression of the TPP1-OB-TRF2 fusion protein also resulted in increased hTR-telomere association in cells depleted of TIN2. Using a second tethering system we also found that telomerase can be recruited to an internal site in chromosome 7 when TPP1 is tethered there via lacO-lacR interaction. Importantly, the accumulation of telomerase at this non-telomeric site is independent of TIN2. Together, our results suggest that TPP1, not TIN2, mediates the recruitment of telomerase to telomeres by the shelterin complex, and that the OB fold of TPP1 is sufficient for this process.

Introduction

Telomeres are the DNA repeats and associated proteins found at the ends of chromosomes (1, 2).

Due to the incomplete replication of chromosome ends by DNA polymerase, some telomere

sequence is lost with each round of DNA replication (3-6). Telomeres are elongated by

telomerase, a ribonucleoprotein complex that includes a reverse transcriptase (TERT) and an

RNA component (TR) (7). Using an RNA template found in TR, the reverse transcriptase

synthesizes the short telomeric repeats at the 3' end of telomeres and translocates the template

for the next reaction cycle (8). High telomerase activity is detected in most human tumor cell

lines while the majority of human normal fibroblast cells possess low telomerase activity only in

S phase (9-11). Also, the speed of the aging process in some human cells is reduced by

overexpression of telomerase (12). These studies suggest that telomerase is crucial in the process

of cancer and aging. Therefore, the ability to control the activity of telomerase could be

beneficial with regards to tumor inhibition and potentially to the regulation of the aging process.

Telomerase activity is modulated by regulated trafficking of the enzyme in cells. In cancer cells,

human telomerase RNA (hTR) resides in Cajal bodies, (subnuclear bodies that are associated

with the assembly and maturation of RNPs) during the G1 and G2 phases of the cell cycle (13,

14). Telomerase accumulates at telomeres specifically in S phase, which is the time DNA

synthesis and telomere elongation occur (14-17). Accumulation of hTR at Cajal bodies is

important in the localization of hTR to telomeres and regulates telomere extension (19).

Disruption of telomerase trafficking reduces the length of telomeres (18, 19). Several inherent

properties of telomerase are important for its trafficking to Cajal bodies and telomeres. The CAB

box motif (UGAG) within hTR has been shown to target telomerase to Cajal bodies. Mutation of

this motif disrupts hTR-Cajal body association (20, 21). Depletion of human reverse transcriptase also prevents accumulation of hTR at Cajal bodies as well as at telomeres, indicating that trafficking also depends on the presence of hTERT (22). Depletion of a telomerase subunit TCAB1 (Telomerase Cajal bodies protein 1) has been shown to prevent hTR-Cajal body associations and also hTR-telomere associations, without affecting catalytic activity of the enzyme *in vitro* (23). Mutations that occur in TCAB1 in dyskeratosis congenita patients disrupt telomerase recruitment to Cajal bodies and lead to telomere shortening (18). Proteins in the telomere shelterin complex have also been implicated in recruitment of telomerase to telomeres (35(24). There are six shelterin proteins that are associated with telomere end protection and telomere length regulation. (25)TPP1 interacts with the double-strand regions of telomeres (26)(27)(28) through interaction with TIN2, which binds to telomeres by association with TRF1 (29) or TRF2 (30). TPP1 associates with single stranded regions of telomeres through interaction with POT1 (31). TPP1 protects the telomere ends from forming TIFs (Telomere Dysfunction Induced Foci, DNA damage foci at dysfunctional telomeres enriched in DNA damage response proteins) (26, 28), and enhances the telomerase reaction cycle by stabilizing the telomeric primer extension and translocation function of telomerase (8, 32). Evidence suggests that TPP1 interacts with TERT. (33)Est3, a yeast telomerase regulating protein that shares high similarity with TPP1, interacts with the TEN domain (N-terminal domain) of telomerase reverse transcriptase (34). (35)In addition, TPP1 co-purifies with the tagged TERT component of telomerase (28, 36). Co-purification requires the OB fold of TPP1, a predicted oligonucleotide/oligosaccharide binding fold at the N terminus (residues 87-240) (28, 36).

The physical association between TPP1 and hTERT provided the first insight that TPP1 might play a role in the recruitment of telomerase to telomeres (28, 36). In ciliate the protein TEBP-β, a possible homolog of TPP1, was shown to be required in the recruitment of telomerase to telomeres (35). More recently, direct support for a role of TPP1 in recruiting human telomerase to telomeres was observed by our group. In human cells, it has been shown that the TPP1-TIN2 complex is required to recruit telomerase to telomeres (36). Furthermore, in mice, the knockdown of TPP1 in embryonic fibroblast cells resulted in a reduction of chromatin associated TERT (24). These results indicate that TPP1 is a potential recruiter to direct telomerase trafficking to telomeres. In this paper, we tried to bypass the requirement for TIN2 by tethering TPP1 to TRF2 so that TPP1 can bind to telomeres without TIN2. We report here that the TPP1-TRF2 fusion protein facilitates the localization of telomerase to telomeres. TPP1-TRF2 also rescues the loss of hTR-telomere association caused by the depletion of TIN2. To circumvent the possible damage to telomerase recruitment by telomere instability we applied the LacR-LacO system (lactose repressor protein binding to lactose operator array in the chromosome) to immobilize TPP1 to a site outside of telomere. We provide clear evidence that TPP1 can initiate the recruitment of hTR and hTERT to a non-telomeric site on the chromosome and, importantly, that this initiation for the trafficking of telomerase is TIN2 independent.

Materials and Methods

Plasmids

shTIN2 was generated as described (37). TPP1-OB-TRF2 plasmid and TPP1-ΔOB-TRF2

plasmids are a gift from S. Zhou (Baylor College of Medicine, Houston). Cherry-lacR was

prepared as described (38), pBS-U1-hTR, 3XFLAG-hTERT and Wild type TPP1 plasmid with

the 3XFLAG tag from both the N and C terminals were from J. Lingner (EPF of Lausanne,

Switzerland) (19). To generate TPP1-LacR-GFP and TPP1-ΔOB -LacR-GFP, LacI-NLS was

fused with C-terminus of TPP1-LacR-GFP or TPP1-ΔOB -LacR-GFP cloned into pN3-EGFP

(Clontech) as a HindIII-SalI fragment.

Cell culture, Transfection and Drug selection

E1 (Super-telomerase HeLa) cells were prepared as described (39) and LacO cells were

generated from HeLa cells with 256 lactose operator arrays incorporated into the 7th chromosome

(38). Both were cultured in DMEM (Mediatech, Herndon, VA) and supplemented with 10% fetal

bovine serum (FBS) (Mediatech) at 37˚C with 5% CO_2. For single transfection, cells were

transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions

using 4 μg of DNA. The DNA ratio is 5:1 for co-transfection of shTIN2(3.33 μg) and TPP1-OB-

TRF2 (0.66 μg) or TPP1-ΔOB-TRF2 (0.66 μg) plasmid. The DNA ratio is 5:1:5:1 for co-

transfection of TPP1-LacR-GFP/ TPP1-ΔOB -LacR-GFP (3.33 μg), Cherry-lacR (0.66 μg), pBS-

U1-hTR (3.33 μg) and 3XFlag-hTERT (0.66 μg). Cells are harvested after 24 hours of

transfection and followed by fixation or drug selection using 1 μg/mL puromycin.

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

For FLAG labeling, all the cells were treated with cold 0.5% Triton X-100 in CSK buffer (100

mM NaCl. 300 mM sucrose. 3 mM MgCl2. 10 mM PIPES (pH 6.8)), followed by a 4%

formaldehyde fixation. After washing 3 times in 1X PBS, the cells were permeablized in 0.2%

triton X-100 in 1X PBS. To prepare for telomere DNA FISH, the cells were then immersed in

70% formamide in 2X SSC for 10 minutes at 80°C and then rinsed 3 times in 1X PBS at room

temperature. Then, after a one-hour blocking in 3% BSA, the cells were subsequently incubated

with the Anti-FLAG antibody (mouse, Sigma-Aldrich, St. Louis, MO) with a dilution of 1:500 in

3% BSA. Cells were washed again 3 times with 1X PBS then incubated with the secondary

antibody of either AMCA-conjugated goat anti-rabbit IgG or Cy5-conjugated goat anti-rabbit

IgG (Jackson Immuno Research Laboratories, West Grove, PA) with a dilution of 1:50 in 0.5%

PBST. For hTR and Telomere FISH, all the cells were washed with 1X PBS then fixed again

with 4% formaldehyde. A pre-hybridization process and hybridization process using a mixture of

three oligonucleotide hTR probes and telomere probes (Qiagen, Valencia, CA) were performed

as described before (14, 16).

Microscopy

The images of the cells were acquired using the Zeiss Axioskop 2 Mot Plus fluorescence

microscope (63x objective, Carl Zeiss Microimaging, Thornwood, NY) and the connecting Orca-

ER camera (Hamamatsu, Bridgewater, NJ). All the images were analyzed with the IPLab

Spectrum software.

Results

***TRF2-tethered TPP1 OB fold facilitates telomerase localization to telomere***

TPP1, and particularly the OB fold, has been shown to physically interact with telomerase and affect telomerase-telomere association (28, 36). To test the possibility that the OB domain of TPP1 plays a direct role in the recruitment of telomerase, we tethered the OB domain of telomeres by expressing it as a fusion protein with TRF2. We examined the number of hTR-telomere associations per cell in cells expressing the tethered TPP1 OB domain by FISH using probes complementary to hTR and telomere as has been described previously (16, 40). A FLAG tag incorporated in the fusion protein allowed us to confirm that the OB domain was tethered at telomeres (Fig.4A). Expression of the TPP1-OB-TRF2 increased hTR-telomere association was enhanced by almost 2 fold per cell (Fig. 4A and B, panel 3), from 3.9 (mean, SEM =0.12) associations per cell in parental cells to 7.4 associations per cell (mean, SEM =0.07) in cells expressing the TPP1-OB-TRF2. On the other hand, expression of untethered full-length TPP1 did not produce increased localization of hTR to telomeres, and in fact resulted in decreased associations (which could reflect competition for telomerase binding) (Fig 4A and 4B). Cells expressing a tethered fusion of full length TPP1 lacking the OB domain failed to induce localization and exhibited similar numbers of associations per cell as the parental group (4.0 mean, SEM =0.10) hTR-telomere associations per cell (Fig. 4A and B). Taken together, these results directly implicate the OB fold of TPP1 in the recruitment of hTR to telomeres.
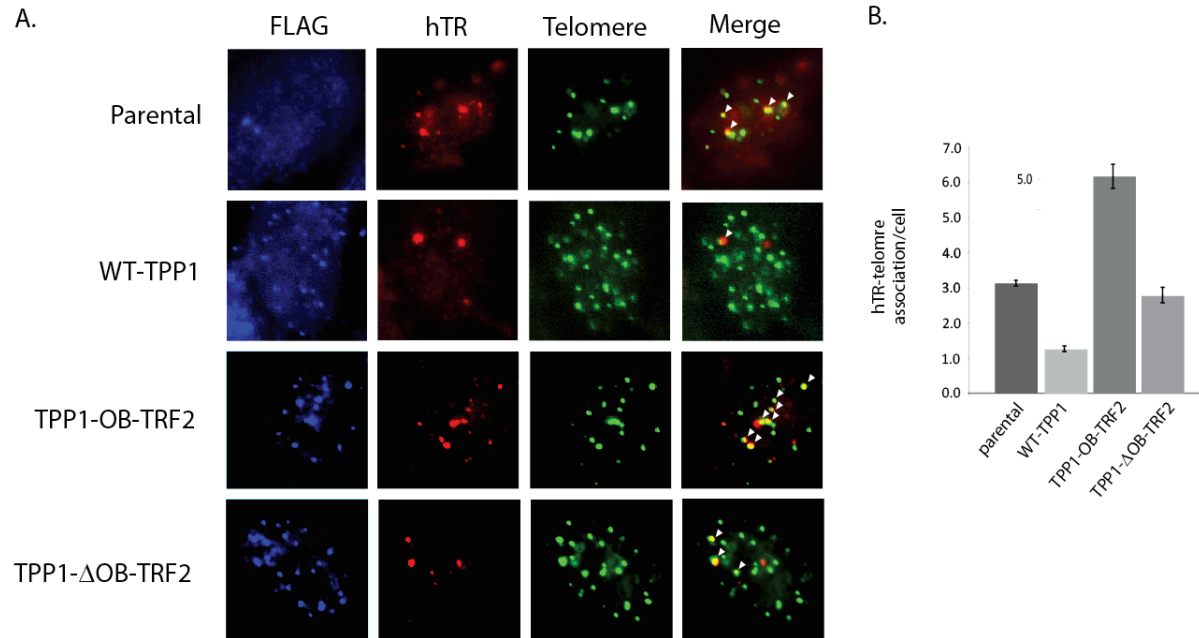
Figure 4. TPP1-OB-TRF2 mediates the localization of hTR to telomeres. *A. FLAG-tagged TPP1 constructs (blue) were detected by IF and hTR (red) and telomeres (green) were probed by FISH in parental E1 HeLa cells, and cells expressing untethered TPP1 (TPP1), and the tethered TPP1 OB domain (TPP1-OB-TRF2), and TPP1 lacking the OB domain (TPP1-ΔOB-TRF2). Merge panels show hTR (red) and telomere (green) signals with arrowheads denoting colocalizations. B. The average number of hTR-telomere associations per cell was quantified in 200 cells in 2 experiments for each experimental group and calculated the means and standard errors are shown.*

TPP1 OB fold rescues the loss of hTR localization caused by the depletion of TIN2

To determine whether the observed recruitment of telomerase by the telomere-tethered OB domain of TPP1 is dependent on TIN2, we examined localization following knockdown of TIN2. Expression of shRNAs against TIN2 reduced TIN2 protein levels in the TPP1-expressing cell lines (Fig. 5C). Telomere associations were scored in cells co-expressing the TPP1 fusion
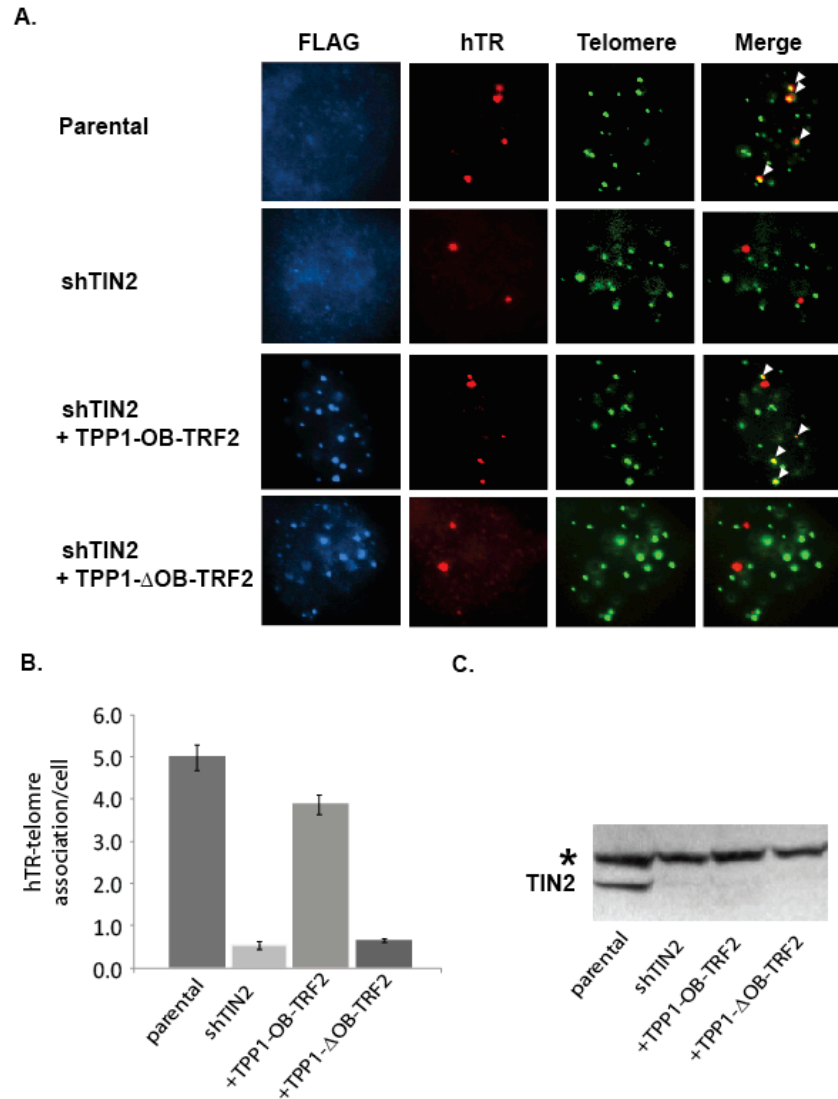
Figure 5. TPP1-OB-TRF2 induces hTR-telomere association in the absence of TIN2. *A.*

FLAG-tagged TPP1 constructs (blue) were detected by IF and hTR (red) and telomeres (green)

were detected by FISH in parental E1 HeLa cells, and cells expressing small hairpin RNA

against TIN2 (shTIN2), and TIN2 knockdown plus TPP1-ΔOB-TRF2 (shTIN2+TPP1-OB-TRF2),

and TIN2 knockdown plus TPP1-ΔOB-TRF2 (shTIN2+TPP1-ΔOB-TRF2). Merge panels show

hTR (red) and telomere (green) signals with arrowheads denoting colocalizations. B. The

average number of hTR-telomere associations per cell was quantified in 200 cells in 2

experiments for each experimental group and calculated the means and standard errors are

shown. C. The level of TIN2 protein in each experimental group was assayed by TIN2

immunoblot analysis.The asterisk indicates a lower mobility non-specific band.

proteins (detected by FLAG IF) (Fig. 5A). As was observed previously (36), knockdown of

TIN2 in the parental cells reduced hTR-telomere association by about 10 fold compared to the

untreated group (Fig. 5A and B), from 5.0 associations per cell (mean, SEM=0.30) of parental

group to 0.5 associations per cell (mean, SEM=0.09) of TPP1-OB-TRF2 group. However, we

found that TRF2-tethered TPP1 OB fold can mediate telomerase recruitment in the absence of

TPP1, rescuing this hTR-telomere associations by 8 fold to an average of 3.9 associations per

cell (SEM=0.23, Fig. 5A and 5B). TPP1 lacking the OB fold fail to induce hTR-telomere

association (mean = 0.7, SEM=0.04) (Fig. 5A and B). The result indicates that TPP1 can recruit

telomerase in the absence of TIN2 when anchored to telomeres by TRF2.

LacR tethered TPP1 OB fold recruits telomerase to a non-telomeric site

Studies of the interaction between shelterin components and telomerase using RNA interference

can harm telomere by disrupting the shelterin complex or triggering DNA damage responses. *In*

vitro experiments showed that depletion of any of the shelterin proteins, particularly TPP1 and

TIN2, resulted in the breakdown of the shelterin complex (27). Also it is well established that

RNAi-mediated depletion of TPP1 and TIN2 lead to TIFs and DNA damage which complicates

analysis. To circumvent the damage to telomeres, we took advantage of the LacO-LacR system

to study interactions between shelterin proteins and telomerase at an engineered LacO site on

chromosome 7 outside of telomere. The LacR protein can bind tightly to the LacO array on the

chromosome. As a result, via tethering the target protein to LacR, the target protein can be linked

to the LacO site (indicated by Cherry-LacR) of the chromosome (41) (Fig. 6). Here we tethered

TPP1 or TPP1 ΔOB fold with GFP-LacR to make a TPP1-LacR-GFP fusion protein and asked

the question whether this TPP1 fusion protein can initiate the hTR recruitment to the non-
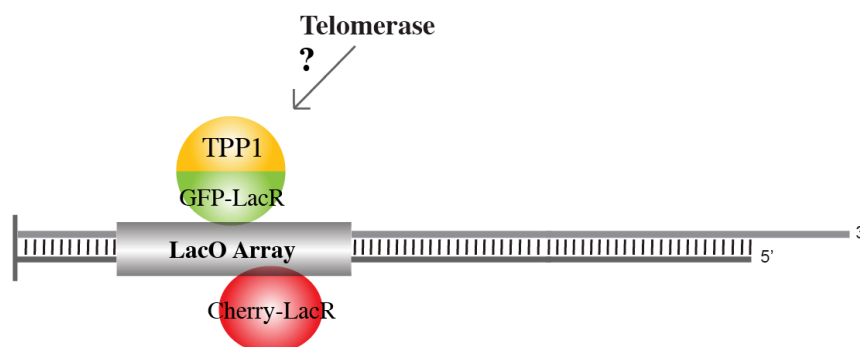
Figure 6. Scheme showing the LacR-LacO method to immobilize TPP1 to a non-telomeric

site. *256 copies of the LacO (lactose operator) array were incorporated into the P arm of the 7ᵗʰ*

chromosome of HeLa cell away from telomere; Cherry-LacR (lactose repressor protein) fusion

protein is overexpressed to indicate the location of the specific LacO site; TPP1-LacR-GFP

fusion protein is also co-expressed with Cherry-LacR and is immobilized to the LacO array

*through LacR; telomerase is overexpressed in the cell and is detected for its localization.*

telomeric site LacO. As we expected, both TPP1-LacR-GFP and TPP1-LacR-GFP lacking the

OB fold could be seen at telomere (showed the punctuate telomere like pattern in the nucleus) as

well as the LacO array (one of the GFP fusion protein foci colocalized with the Cherry-LacR foci

in the cell) (Fig. 7). By co-expression of telomerase, Cherry-LacR, and the GFP fusion proteins

in the LacO cells (HeLa cells that have LacO arrays incorporated into the chromosome), we

observed hTR localization with respect to the LacO site (Fig. 6). We found that hTR

accumulated at the LacO site at a much higher frequency with the presence of LacR-GFP-TPP1

(53% of the cells have hTR-LacO colocalization, out of 100 cells) than cells in other groups

which either lack the TPP1 OB fold (3% of the cells has hTR-LacO colocalization, out of 100

cells) or lack the GFP-fusion protein (2% of the cells has hTR-LacO colocalization, 100 cells)

(Fig. 7A and B.). This suggests that TPP1, particularly the OB fold, can recruit hTR to the LacO

site.
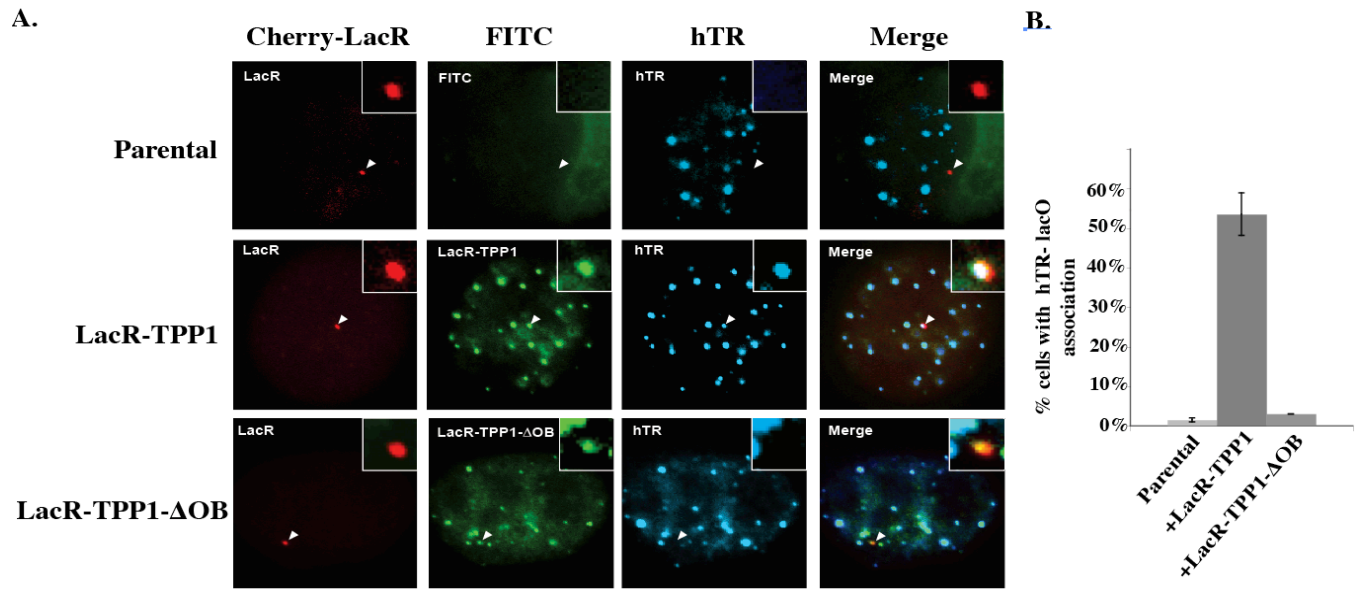
Figure 7. TPP1-LacR-GFP initiates localization of hTR to LacO site. *A. FISH detection of*

hTR in LacO cells of parental group (no GFP protein expressed), the TPP1-LacR-GFP group or

the TPP1-ΔOB-LacR-GFP group. HeLa LacO cells were transfected with Cherry-LacR,

telomerase, and different GFP fusion protein (no GFP fusion protein in the control parental

group) plasmids. Red indicates Cherry-LacR, green indicates TPP1 fusion protein, and blue

indicates the hTR. Arrowheads denote the colocalization of red, green and blue panels. Insets

show an enhanced magnification of the foci indicated by the arrow. B. Statistical analysis to

show the average number of hTR-Cherry-LacR associations per cell in each group represented

in panel A. Error bars on each column that represent different groups in panel A were calculated

using standard errors.

The recruitment of hTR to telomeres depends on the presence of hTERT (22). Thus, we wanted to know whether hTERT could also be recruited to the LacO site by the same TPP1-LacR-GFP fusion protein. After co-expression of telomerase (with FLAG tagged hTERT), Cherry-LacR, and the GFP fusion proteins in the LacO cells, we used IF to track the location of FLAG tagged hTERT to see if it locates at the LacO site. As expected, hTERT was found at the LacO site when TPP1-LacR-GFP was present (49% of the cells has hTR-LacO colocalization, 100 cells) (Fig. 8, panel 2). In cells that lacked the TPP1 OB fold (1% of the cells has hTR-LacO colocalization, 100 cells) or lacked the GFP-fusion protein (3% of the cells has hTR-LacO colocalization, 100 cells), the hTR-LacO association was rare (Fig. 8, panel 1 and 3). With these observations, it is clear that the LacR tethered TPP1, and more specifically the OB fold, can recruit telomerase to a non-telomeric site.
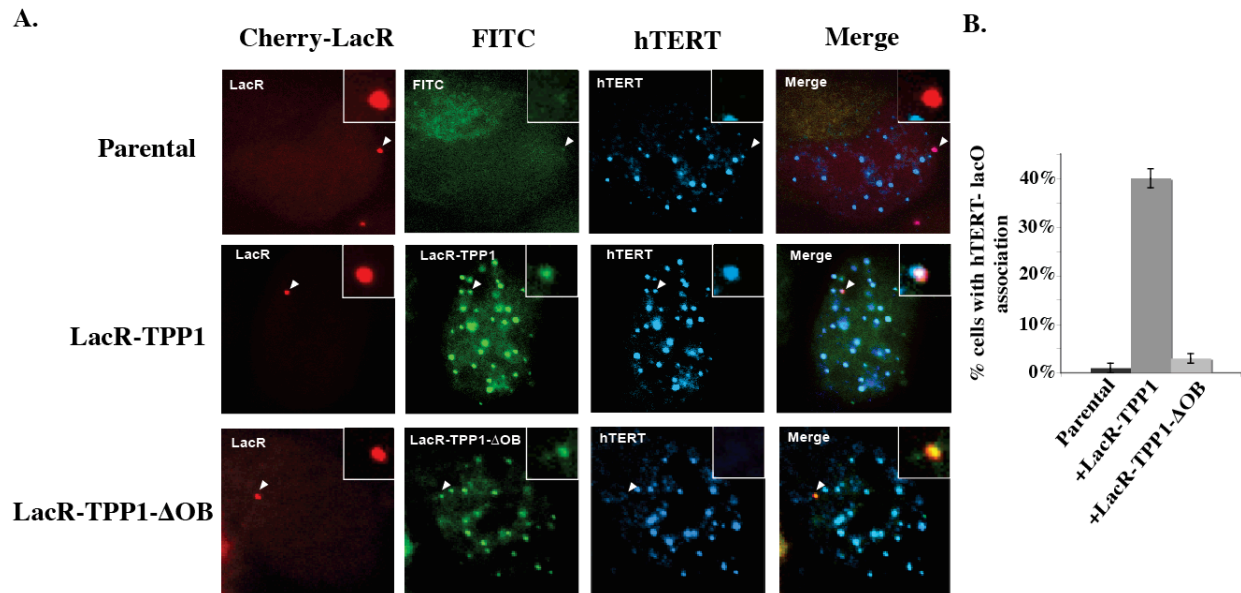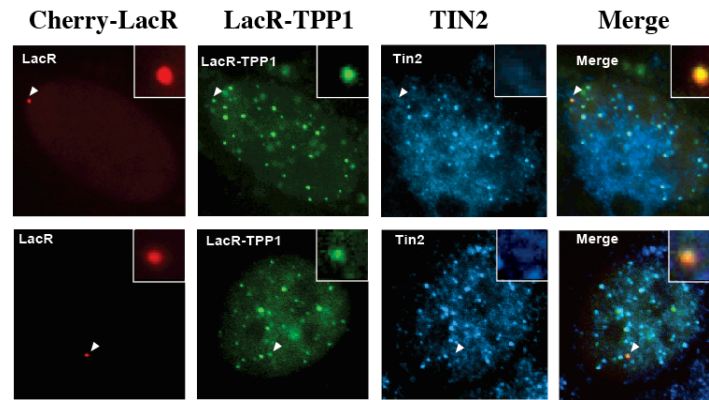

Figure 8. TPP1-LacR-GFP initiates localization of hTERT to LacO site. *A. HeLa LacO cells expressing TPP1-LacR-GFP or the TPP1-ΔOB-LacR-GFP. HeLa LacO cells were transfected with Cherry-LacR, telomerase, and different GFP fusion protein (no GFP fusion protein in the control parental group) plasmids, and IF is applied to trace the location of hTERT. Red*

indicates Cherry-LacR, green indicates the TPP1 fusion protein, and blue indicates the hTERT.

Arrowheads denote the colocalization of red, green and blue panels. Insets show an enhanced

magnification of the foci indicated by the arrow. B. Statistical analysis to show the average

number of hTERT-Cherry-LacR associations per cell in each group represented in panel A.

Error bars on each column that represent different groups in panel A were calculated using

standard errors.

The recruitment of telomerase by TPP1 is independent of TIN2

We showed above that TIN2 serves indirectly in telomerase recruitment to telomeres, as

TPP1-TRF2 rescues the loss of hTR-telomere association by depletion of TIN2 (Fig.5). Since we

now show that LacR tethered TPP1 proteins can recruit telomerase to the non-telomeric site, we

next hypothesize that TIN2 is absent when telomerase-LacO colocalization occurs. Similarly to

what we described above, the cells overexpressing telomerase were co-transfected with Cherry-

LacR and GFP-TPP1. As we were unable to label TIN2 and telomerase at the same time, we

determined the percentage of TIN2-LacO association since we knew that about 40% of LacO

cells had hTERT-LacO colocalization. From our examination, it seems that TIN2 seldom occurs

at the LacO site (only 2 cells had TIN2-LacO association, 2 repeat experiments, out of 100 cells)

(Fig.9, upper and lower panels). Considering the observation that above 40% of the LacO sites

have telomerase colocalization while almost none of the LacO sites have the presence of TIN2,

our data suggests that TPP1 can recruit telomerase without the assistance of TIN2.

Cherry-LacR	LacR-TPP1	TIN2	Merge

Figure 9. TPP1-LacR-GFP initiates localization of telomerase to LacO site independent of

TIN2. *TPP1-LacR-GFP, Cherry LacR, and telomerase were transfected into LacO cells. IF is*

applied for the labeling of TIN2. Red indicates Cherry-LacR, green indicates TPP1-LacR-GFP

proteins, and blue indicates the TIN2. Arrowheads indicate the same location of Cherry-LacR in

the cell of different panels. Insets show an enhanced magnification of the foci indicated by the

arrow. Upper and lower panel are two different cells.

Discussion

Telomerase trafficking appears to be a highly regulated process determined by multiple factors such as the cell cycle, specific telomerase-associated components (telomerase reverse transcriptase and TCAB1), and specific telomere binding proteins (14, 22, 23, 40). Among six telomere shelterin proteins, TPP1 has been found to interact with telomerase physically and functionally (28, 31, 42). A recent study revealed the essential role of the TPP1-TIN2 complex in telomerase recruitment although the individual effect of the complex needs to be clarified (28, 36). In this study, using tethering techniques, we showed that TPP1 plays a key role in telomerase recruitment to telomeres (Fig.4). Furthermore, our results suggest that TIN2 is only indirectly required for telomerase recruitment via facilitating TPPI association to telomeres. We demonstrated that TPP1, when attached to a non-telomeric site, could induce trafficking of both hTR and hTERT to this same, non-telomeric location (Fig.7 and 8). We also observed that the recruitment of telomerase to the non-telomeric location was independent of TIN2 (Fig. 9). All these results indicate that TPP1, rather than TIN2, is the major telomere shelterin protein involved in regulating the trafficking of telomerase to telomeres.

Current studies of the interactions between telomerase and the TPP1-TIN2 complex haven't been able to clarify the role of the individual shelterin proteins. Abreu *et al*. showed that TPP1-TIN2 complex was required for telomerase recruitment to telomeres using RNAi. However, since the depletion of TPP1 or TIN2 attenuates each other, it is hard to determine which is the actual recruiter of telomerase (36). Previous research has utilized TRF2 fusion-protein tethering assays to study the role of the components of telomerase transcriptase in telomerase recruitment (43). We took advantage of this telomere-tethering assay to link TPP1 to telomere via TRF2 instead of

TIN2 to avoid the possible effects of TIN2 on TPP1-telomerase interaction. TPP1 and TIN2 are key components in the shelterin protein complex and the depletion of either one has destructive effects on the whole complex and also activates the telomere DNA damage response to form TIFs (telomere dysfunction-induced foci) (27, 44). Considering this it is challenging to unmask the individual function of telomerase recruitment under the limitation of RNAi. Early research has made use of the LacO-LacR system to explore the *cis* functions of TRF1 and TRF2 on telomere (45). We also employed this system to immobilize TPP1 to a non-telomeric LacO site to study telomerase recruitment by TPP1 without causing possible damage to telomeres (43). We observed that TPP1, and more specifically the OB fold, has a positive role on recruiting telomerase to both sites (Fig. 4, 7, 8). These results suggest that TPP1 is the major regulator in telomerase recruitment to telomere. Studies in the ciliate *Stylonychia lemnae* have shown that telomerase is recruited to telomeres by TEBPβ, a homolog of TPP1 (35). The Est 3 protein in yeast that shares structural and functional similarities with TPP1 also interacts directly with TERT (34). In humans, TPP1 alone has little impact in regard to the regulation of telomerase activity in either human or medaka fish (42). However TPP1, when tethered with other DNA binding proteins such as TRF2 and POT1, is capable of recruiting telomerase and enhancing telomerase activity (Fig.1; (32). We find that expression of a TPP1-TRF2 fusion protein enhances hTR-telomere association *in vivo* (Fig.1). Also, *in vitro* experiments found that the TPP1-POT1 complex can enhance the binding of telomerase to different telomeric DNA primers (32). Collectively, there is strong experimental support for a direct and conserved role for TPP1 in mediating telomerase recruitment to telomeres.

TIN2 has been implicated for its involvement in telomerase-telomere interaction in many ways. TIN2 bridges TPP1 to double strand telomere and regulates telomere length in a telomerase-dependent manner (29, 46). TIN2 was found to be associated with telomerase since anti-FLAG-TIN2 immunoprecipitantion could bring down hTR from human cells (47, 48). Studies on the human genetic disease, Dyskeratosis Congenita (DC) also found that the DC mutant in TIN2 broke the TIN2-telomerase association (47, 48). However, our finding that the recruitment of telomerase by TPP1 is independent of TIN2 (Fig.5 and 9) suggests an indirect role of TIN2 in telomerase recruitment (through mediating TPP1 interaction along the length of telomeres). Although our results show that TIN2 is not required for TPP1-telomerase association, TIN2 very likely regulates this association. Dong *et al.* reported that deletion of the TIN2 binding domain of TPP1 reduced TPP1-telomerase association by half (48). TPP1-TIN2 binding is mediated by the C-terminal region of TPP1 called TID (TIN2 interacting domain) (28). TIN2 is likely to allosterically change the ability of TPP1 to interact with telomerase via binding to TID domain. Besides TIN2, other telomere-binding proteins merit further investigation to see if they have a regulatory effect on TPP1-telomerase interaction or directly interact with telomerase. For example, a recent study found that UPF1, a nucleic acid-dependent ATPase and 5'-to-3'helicase, physically interacts with the TPP1 OB fold and telomerase (49). In this paper, our study on the effect of TPP1 on telomerase recruitment gives new insight into the interaction between shelterin proteins and telomerase and also provides further questions for future research on telomerase trafficking and function.

Acknowledgements

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Chapter 3

DISCUSSION

TPP1 as the main recruitment factor for telomerase to telomere

Previous research indicated that the shelterin proteins at telomeres can interact with telomerase and might induce the recruitment of telomerase to telomere. In the budding yeast *Saccharomyces cerevisiae*, the telomeric DNA-binding protein Cdc13 has been reported to interact with the telomerase-associated Est1 protein (1). In the ciliate Stylonychia lemnae, telomere end binding protein TEBPβ can recruit telomerase to telomere in a phosphorylated fashion (2). All the evidence shows that before acting on a telomere, telomerase might first interact with the shelterin proteins. Research on human HeLa cells recently revealed that TIN2 and TPP1 are both required for localization of telomerase to telomere, although POT1 is not needed (3). However, when TPP1 was discovered to physically interact with TERT, doubts arose as to whether TIN2 only served as the supporter for TPP1 or the one that actually recruited telomerase (4). In this research, we attempted to clarify this question and we found that TPP1, when tethered to TRF2 for bridging to telomere, could facilitate the accumulation of telomerase at telomere and can maintain this accumulation even when TIN2 is depleted (chapter 2). TPP1, when immobilized to an ectopic site of telomere, can also induce localization of telomerase to this novel site and this phenomenon is independent of TIN2. All these effects are specific to the TPP1 OB fold in contrast to a mutant group lacking this important domain (chapter 2). The findings in this research all point to the conclusion that TPP1, rather than TIN2, is the major shelterin component for telomerase recruitment. However, since we haven't investigated other shelterin components, we cannot exclude the possibility of their effort in recruiting telomerase.

TPP1 recruits telomerase to telomere and S phase

The trafficking of telomerase is a dynamic process. In the S phase, telomerase can be found to locate at telomere, which is not surprising because telomerase activity is restricted to the S phase in intact cells and this period is the time for DNA replication and telomere extension (5, 6). In this work, although we showed that TPP1, specifically the OB domain, could induce telomerase recruitment, we have not yet examined if this process in fact occurs during the S phase. Further research tracking the localization of telomerase and the cell cycle phase at the same time can address this question. If TPP1 recruitment of telomerase is cell cycle dependent and limited to the S phase, then another question would be what character on TPP1 can be turned on and off during cell cycle in order to achieve the recruitment. First, the ciliate telomere end forms a G-quadruplex structure and the phosphorylation of telomere binding protein TEBP-β associates with the unfolding of this structure during the S phase (7). Further study revealed that the phosphorylated form of TEBP-β is necessary for telomerase recruitment in order to unfold the G-quadruplex of telomere (2). Considering that TEBP-β is a likely homologue of TPP1, it would be interesting to know if TPP1 is in its phosphorylated form while TPP1 is inducing the recruitment of human telomerase during the S phase. Since the TPP1 OB fold is crucial in telomerase recruitment, it's possible that phosphorylated TPP1 causes a conformational change in TPP1 to expose the OB domain. Secondly, depletion of a protein called UPF1 (eukaryotic up-frameshift 1) leads to human cell cycle arrest in early S phase (8). Interestingly, this protein UPF1 was recently found to also interact with TPP1 and telomerase and its interaction with telomerase was dependent upon ATR, the upstream protein that controls the phosphorylation of UFP1 (9). Thus UPF1, ATR, or other UPF1-regulating proteins may induce telomerase recruitment by TPP1 when the S phase comes. Thirdly, some cyclin-dependent kinases must be considered as a

possible trigger for the S phase-specific telomerase trafficking event. For example, TEBP-β in ciliates is phosphorylated by Cdk2, and Cdc13 in budding yeast is phosphorylated by Cdk1 (7, 10). Regarding the cruicial role of Cdk proteins in regulating the cell cycle (4, 11), it is not surprising to think that this protein kinase family may be the one to initialize the cell cycle-regulated telomerase trafficking as well.

Interaction between TPP1 and telomerase

Much evidence points to the conclusion that TPP1 physically and functionally interacts with telomerase (4, 11). Both full-length TPP1 and TPP1 OB folds were able to pull down HA-TERT proteins, indicating that in the TPP1 protein, the OB fold domain is the major domain to interact with components of telomerase (4). In telomerase, the TEN domain of TERT has been implicated as the one to interact with TPP1 because mutation in the TEN domain prevents activation of TPP1 to telomerase activity (11). Considering the observation that the TEN domain is insufficient in this process, other components of telomerase and other shelterin proteins are also possibly involved in the inducing or maintaining of TPP1-telomerase interaction. Until now we still don't know whether TPP1-telomerase interaction is direct or mediated by unknown components. The fore-mentioned UPF1 was also found to be a telomere-binding protein that could physically interact with telomerase as well as TPP1. Interestingly, both telomerase and UPF1 associate with the OB fold of TPP1 (9). Although there are a couple of possibilities on how TPP1, telomerase, and UPF1 interact with each other, it is obvious that they are forming a complex, at least during the S phase, to extend the telomere end. Maybe when the S phase arrives, UPF1 is phosphorylated by ATR and then changes the conformation of the OB fold on TPP1 to attract telomerase. Or, possibly the phosphorylated UPF1 recruits telomerase directly

and at the same time depends on the availability of the OB fold in TPP1. It's also possible that both have an interaction site with telomerase. In this research, although we haven't addressed this question, tracking the location of UPF1 in relation to the LacR-TPP1-associated telomerase could give us some hint. Depletion of any of the three components and analysis of the interaction of the other two can also be done to examine the relationship between these members.

hTR is able to form active telomerase and target mouse telomere

Research in the mouse system has discovered that mouse telomerase RNA follows a different pathway than human telomerase RNA and that telomerase does not accumulate in the Cajal bodies but can still localize to telomere. However, when human telomerase RNA was introduced in mouse cells, it localized to mouse Cajal bodies as well as telomeres (12). Human and mouse telomerase share high similarities in both the RNA and the reverse transcriptase (13). Human telomerase RNA (hTR) interacts with mouse reverse transcriptase (mTERT) properly (*in vitro* and in *vivo*) in the mouse cells (14, 15). The hTR-mTERT combination even shows higher activity than the combination of mTR and mTERT (14). These results indicate that the heterologous hTR we expressed in mouse cells can replace mTR to form functionally active telomerase with mTERT. Therefore, it's not a surprise to find the recruitment of hTR at telomeres in the mouse system. When hTR was co-expressed with mouse telomerase transcriptase in human VA13 cells (an cell line that lacks hTR and hTERT expression), it was observed that hTR was not forming any foci in the cell nor did it go to telomeres (16). While hTR and mTERT can form functionally active telomerase *in vitro* and *in vivo* in mouse, it would be interesting to know why this combination cannot direct telomerase to telomeres in human VA13 cells. One possibility is that there might be interactions between celluluar components and

TERT needed in the VA13 cells in order to recruit telomerase to telomere. Considering the role of Cajal bodies in telomerase trafficking it would also be interesting to know if hTR and mTERT can recruit telomerase in the Cajal bodies of VA13 as well.

Inherent RNA structure determines Cajal body localization in mouse

To understand the difference of Cajal body accumulation of telomerase between humans and mice, we looked into the possible mechanism behind it. Multiple factors determine the localization of telomerase to Cajal bodies in human systems. A protein uniquely enriched in Cajal bodies called TCAB1 can prevent hTR from associating with Cajal bodies when TCAB is depleted (17). Considering the fact that TCAB1 is conserved between species, it is not likely to cause the difference in Cajal body localization of telomerase in this situation (18). TERT is another factor found to be a requirement in this process because overexpression of hTERT induced accumulation of hTR in Cajal bodies and depletion of it led to the loss of hTR-Cajal body associations (19). In our research we found that it is the inherent RNA structure that determines the accumulation of TR in Cajal bodies (12). In the secondary structure of hTR, the CAB box motif with a sequence of UGAG has been shown to be required for targeting hTR to Cajal bodies in the human system (20, 21). This motif was found to be very conserved between species and its structures were very identical between the human and the mouse so this might not be the cause (22, 23). One obvious difference between mouse and human telomerase RNA is the 5' terminal sequence. hTR has 45 nucleotides upstream of the template region, and part of this sequence base paired with the 5' pseudoknot domain of the RNA to form the P1 stem. The mouse TR only has 2 nucleotides upstream of the template and thus no P1 stem could be formed (24). The CR4-CR5 domain of human telomerase RNA was found to be able to bind with

hTERT and affect the hTR-hTERT interaction (25). As a result the difference of this domain between two species might also affect telomerase activity and localization to Cajal bodies. More experiments are yet to be done to figure out further information on the effects of structural differences between both telomerase RNAs on telomerase recruitment to mouse Cajal bodies.

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Supplemental Chapter 1

A CAJAL BODY-INDEPENDENT PATHWAY FOR TELOMERASE TRAFFICKING IN
MICE

Abstract

The intranuclear trafficking of human telomerase involves a dynamic interplay between multiple nuclear sites, most notably Cajal bodies and telomeres. Cajal bodies are proposed to serve as sites of telomerase maturation, storage, and assembly, as well as to function in the cell cycle-regulated delivery of telomerase to telomeres in human cells. Here, we find that telomerase RNA does not localize to Cajal bodies in mouse cells, and instead resides in separate nuclear foci throughout much of the cell cycle. However, as in humans, mouse telomerase RNA (mTR) localizes to subsets of telomeres specifically during S phase. The localization of mTR to telomeres in mouse cells does not require coilin-containing Cajal bodies, as mTR is found at telomeres at similar frequencies in cells from wild-type and coilin knockout mice. At the same time, we find that human TR localizes to Cajal bodies (as well as telomeres) in mouse cells, indicating that the distinct trafficking of mTR is attributable to an intrinsic property of the RNA (rather than a difference in the mouse cell environment such as the properties of mouse Cajal bodies). We also find that during S phase, mTR foci coalesce into short chains, with at least one of the conjoined mTR foci co-localizing with a telomere. These findings point to a novel, Cajal body-independent pathway for telomerase biogenesis and trafficking in mice.

Introduction

Telomeres are capping structures that comprise the physical ends of eukaryotic chromosomes. In vertebrates, they consist of tandemly repeated arrays of TTAGGG and a number of associated proteins [1, 2]. Telomeres serve protective functions, preventing the ends of chromosomes from being recognized as double stranded DNA breaks [3] and serving as barriers to the loss of genetic information that results from the inability of DNA polymerases to fully replicate the ends of linear DNA. However, a portion of the telomere is lost with each cell division in most adult human somatic cells, and eventually telomere attrition triggers the cell to stop dividing and enter a state of proliferative senescence or undergo apoptosis. Increasing numbers of human telomere shortening diseases are being recognized and investigated [4-6].

Telomerase is the specialized reverse transcriptase that synthesizes telomeres and combats telomere erosion [7]. The telomerase enzyme is minimally comprised of two essential components: telomerase RNA (TR), which provides the template for repeat addition, and telomerase reverse transcriptase (TERT), which synthesizes the repeats. In humans, telomeres are synthesized early in development [8, 9]. Telomerase activity is not detected in most adult somatic cells, but notably, the enzyme is active in over 90% of human cancers and is responsible for the prolonged proliferative capacity of these cells [10-12]. (However, telomerase activity is readily detected in murine somatic cells [13-16].) Insights into the biogenesis and regulation of this enzyme therefore have the potential to lead to the development of novel anti-cancer therapeutics [17, 18].

A recent series of studies has revealed important aspects of the trafficking of the telomerase ribonucleoprotein complex (RNP) critical to the biogenesis and function of the enzyme in human cells [19-25]. Throughout interphase, human TR is found in Cajal bodies [21,

23], dynamic nuclear structures that have been implicated in the biogenesis of various cellular RNPs (reviewed in [26-28]). Localization of hTR to Cajal bodies is important for function of the telomerase enzyme [19, 24]. Accumulation of hTR in Cajal bodies occurs in cancer cells where telomerase activity is present, but not telomerase-negative normal cells [22, 23, 25], consistent with the hypothesis that Cajal bodies serve as sites of telomerase assembly. Human TERT is detected in distinct nucleoplasmic foci, separate from Cajal bodies by immunofluorescence (IF) [23], though exogenously expressed GFP-tagged, hTERT can be detected within Cajal bodies [25] as well as nucleoli [29-31]. During S phase, both human TR and TERT move to foci found immediately adjacent to Cajal bodies, and then, peaking in mid-S phase when human telomeres are replicated and synthesized [32-34], both hTR and hTERT associate with subsets of telomeres [19, 21-24]. These findings indicate that Cajal bodies are important for telomerase biogenesis and may act to deliver telomerase to the telomere.

The trafficking of telomerase components has emerged as a key process in the biogenesis and function of the enzyme, and the factors important for trafficking are being defined. The localization of human TR to both Cajal bodies and telomeres depends on TERT [22], suggesting that assembly and trafficking are tightly linked processes. The trafficking of telomerase to both Cajal bodies and telomeres depends on a newly identified telomerase component, called TCAB1 (Telomerase Cajal-body protein 1; also called WDR79) [24, 35]. There is also evidence that the core telomere binding protein, TPP1 is critical for telomerase-telomere associations [36-40]. However, much remains to be learned regarding the factors and mechanisms that influence the biogenesis and trafficking of telomerase.

To gain a better understanding of the regulation of telomerase trafficking, we initiated studies in the mouse model system where genetic approaches have contributed to our

understanding of basic telomere and telomerase biology, particularly with regard to cancer and aging [41-45]. Here, we report a fluorescence in situ hybridization (FISH) procedure specific for detection of mouse telomerase RNA (mTR) and the characterization of mTR localization patterns in cultured mouse cell lines using this approach. Unexpectedly, we found that mTR does not co-localize with coilin or other Cajal body markers, and instead, is found in distinct nuclear foci. However, a fraction of the mTR localizes to telomeres selectively during S phase of the cell cycle. In many cases, we observe chains of connected mTR foci during S phase, and frequently find that at least one of the foci in a chain co-localizes with a telomere. Our findings suggest an alternative mechanism for recruitment of telomerase RNA to telomeres during S phase in murine cells, which involves the convergence of non-Cajal body mTR foci at telomeres.

Materials and Methods

Cell culture and transfection

MEF-26 (WT), MEF-42 (coilin -/-), MEF-14 (mTR -/-) (a gift of Carol Greider, Johns Hopkins University, Baltimore, MD), 3T3 (ATCC, Manassas, VA), n2a (a gift of Brian Condie, University of Georgia, Athens, GA), c2c12 (ATCC), and A9 cells were grown on coverslips in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (FCS) (Mediatech) at 37˚C with 5% CO_2. For hTR overexpression, cells were transfected with pBS-U1-hTR [19] (gift of J. Lingner, EPF of Lausanne, Switzerland) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

FISH probes

All probes were synthesized by Qiagen (Valencia, CA) as follows: mTR 117-169 (probe 1), CT*CCCGGCGAACCT*GGAGCTCCTGCGCT*GACGTTTGTTTTT*GAGGCTCGGGT*A; mTR 296-342 (probe 2), CT*CGGGGACCAGT*TCCATTCCTGT*CCTTGCGGCGCT*CGCCCGGCCT*G; mTR 224-282 (probe 3), GT*GCCCCGCGGCT*GACAGAGGCGAGCT*CTTCGCGGCGGCAGCGGAGT*CCTAAG ACCCT*A; mTR 57-104 (probe 4), CT*CTGCAGGTCT*GGACTTTCCT*GGCCCGCTGGAAGT*CAGCGAGAAAT*A; U3 (probe 5), TT*CAGAGAAACTTCTCT*AGTAACACACTAT*AGAACTGATCCCT*GAAAGTATAGT *C; mU85 (probe 6), AT*TACCAAAGATCT*GTGTGTCATCT*CTCAGTGGCCAT*GACACAGCTAAGT*C; telomere (probe 7),

CT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*A;

hTR 128-183 (probe 8),

GCT*GACATTTTT*TGTTTGCTCT*AGAATGAACGGT*GGAAGGCGGCA; hTR 331-393

(probe 9),

CT*CCGTTCCTCTTCCT*GCGGCCTGAAAGGCCT*GAACCTCGCCCT*CGCCCCCGAG

T*G; hTR 393-449 (probe10),

AT*GTGTGAGCCGAGT*CCTGGGTGCACGT*CCCACAGCTCAGGGAAT*CGCGCCGC

GCT*C. T* indicates aminoallyl-modified thymidines. All mTR and hTR probes (probes 1-4

and 8-10 above) were conjugated with cy3 mono-functional reactive dye according to the

manufacturers protocol (GE Healthcare, Little Chalfront, Buckinghamshire, United Kingdom).

The remaining probes were labeled with Alexa Fluor 647 or Oregon green dye according to the

manufacturers protocol (Molecular Probes/Invitrogen, Carlsbad, CA).

mTR and hTR FISH

FISH was performed essentially as described [23, 25]. Hybridizations were carried out overnight

at 37˚C. 200 ng of each of two of the cy3-labeled mTR probes (most often probes 1 and 2) or 25

ng of each of the three cy3-labeled hTR probes (probes 8, 9 and 10) above were used per

coverslip. 5-10 ng Oregon green or Alexa Fluor 647 U3 (probe 5), 100 ng Oregon green mU85

(probe 6), or 10 ng Oregon green telomere (probe 7) were also included in the hybridization

when indicated. Cells were rinsed twice in 50% formamide, 2X SSC for 30 minutes at 37˚C.

Cells were subsequently washed once in 50% formamide, 2X SSC, 0.1% NP-40 for 30 minutes

at 37˚C. The coverslips were mounted in Prolong gold mounting media (Molecular

Probes/Invitrogen), cured at room temperature for 1 hour and stored at -20˚C until microscope

analysis.

RNAse treatment

After fixation and permeabilization, cells were rehydrated in 1X PBS containing 1.5 mM MgCl$_2$ at room temperature for 5 min. Then the cells were incubated with RNAse A (0.2 mg/ml in 1X PBS containing 1.5 mM MgCl$_2$) at 37°C for 2 h. mTR FISH was performed after the cells were washed 3 times with 1X PBS and once with 50% formamide, 2X SSC.

Indirect Immunofluorescence (IF)

Following FISH, cells were washed three times with 1X PBS. Next, cells were incubated with one of the following primary antibodies at the indicated dilution for 1 hour at room temperature: rabbit anti-Nopp140 (1:1,000, a gift from U. Thomas Meier, Albert Einstein College of Medicine, New York, NY), rabbit anti-TRF1 (1:500), rabbit anti-TPP1 (1:500), or rabbit anti-Tin2 (1:500) (all 3 a gift from Susan Smith, Skirball Institute, New York, NY). Cells were washed three times in 1X PBS and then incubated with secondary antibody (1:100 Cy2 conjugated goat anti-rabbit IgG (H+L), Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. All antibodies were diluted in 0.05% Tween-20 in PBS (PBST). Cells were then subjected to three final 1X PBS washes and mounted as above. For the following antibodies, IF was performed prior to FISH analysis: mouse anti-p80 coilin (1:1,000), mouse anti-fibrillarin (17C12, 1:1,000), mouse anti-SMN (2B1, 1:100, Novus Biologicals, Littleton, CO). Briefly, cells were grown on coverslips overnight and then washed one time in 1X PBS. Cells were fixed in 4% formaldehyde in 1X PBS for 10 minutes at room temperature. Cells were rinsed twice in 1X PBS and blocked in 1% BSA (Sigma-Aldrich, St. Louis, MO) in 1X PBS for 1 hour at room temperature. Cells were then incubated with the antibodies at the indicated dilutions for 1 hour at room temperature followed by incubation with a cy2-conjugated goat anti-mouse secondary antibody (1:100, Jackson Immunoresearch

Laboratories) for 1 hour at room temperature. These antibodies were diluted in 1% BSA in 1X

PBS. Following three 1X PBS washes, cells were again fixed in 4% formaldehyde in 1X PBS

for 10 minutes at room temperature. Cells were rinsed twice in 1X PBS and once in 50%

formamide, 2X SSC prior to FISH analysis.

S phase synchronization

Synchronous populations of MEF and 3T3 cells were obtained by double thymidine block. Cells

were treated with 2 or 5 mM thymidine (Sigma-Aldrich) for 16 hours. Cells were released by

rinsing twice with 1X PBS and replacing the normal growth media for 8 hours. Cells were

retreated with 2 or 5 mM thymidine for another 16 hours. At various time points after release,

cells were fixed and analyzed by PCNA staining or FACS analysis to verify cell cycle phase.

PCNA (proliferating cell nuclear antigen) staining

To distinguish S phase cells, cells on coverslips were rinsed once in 1X PBS and fixed for 10

minutes at room temperature in 4% formaldehyde, 10% acetic acid in 1X PBS. Cells were rinsed

twice in 1X PBS and permeablized in 70% ethanol at 4˚C overnight. The cells were incubated

with mouse anti-PCNA antibody (PC10, 1:2500, Abcam, Cambridge, MA) for 1 hour at room

temperature. Cells were rinsed 3 times in 1X PBS and then incubated with secondary antibody

(1:100, AMCA-conjugated goat anti-mouse IgG (H+L), Jackson Immunoresearch Laboratories)

for 1 hour at room temperature. Cells were rinsed 3 times in 1X PBS and fixed in 4%

formaldehyde in 1X PBS for 10 minutes at room temperature. Cells were rinsed twice in 1X

PBS and once in 50% formamide, 2X SSC prior to FISH analysis. Both antibodies were diluted

in PBST.

FACS (fluorescence activated cell sorting) analysis

Cells were collected and resuspended to a single cell suspension in 0.5 mL 1X PBS. The cell

suspension was transferred to a tube containing 4.5 mL of 70% ethanol and stored at -20˚C until

analysis (at least overnight). On the day of FACS analysis, the ethanol suspended cells were

centrifuged for 5 minutes at 200 x g. The pellet was washed once in 5 mL of 1X PBS,

centrifuged again for 5 minutes at 200 x g and resuspended in a solution of 0.02 mg/mL

propidium iodide (Sigma-Aldrich), 0.1% Triton X-100, and 2 mg DNase-free RNAse A in 1X

PBS. The cells were stained in this solution for 30 minutes at 37˚C. FACS analysis was

performed using a FACSCALIBUR scanner (Becton Dickinson Biosciences, San Jose, CA) and

data was analyzed using FlowJo software (Tree Star, Inc., Stanford, CA).

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 objective,

numerical aperture 1.4) using a cooled charge-coupled device Orca-ER camera (Hamamatsu,

Bridgewater, NJ) and IPLab Spectrum software.

Results

Specific detection of mouse telomerase RNA in cultured mouse cells

We developed a FISH procedure for the detection of mouse telomerase RNA (mTR) in order to examine its subcellular localization in mouse cells (where telomerase is active). We designed four probes against different regions of mTR; the regions encompassed by the probes are indicated in Figure 1A. (See Materials and Methods for probe sequences.) Figure 1B shows the results of hybridization with a combination of probe 1 and probe 2 in cultured mouse embryonic fibroblast (MEF) cells. Approximately 2/3 of the MEF cells displayed 1-3 small, clearly defined, spherical nuclear foci (Figure 1B). Hybridization with each of the four individual mTR probes gave similar localization patterns, and maximal signal was obtained with combinations of two probes (data not shown). The combination of probes 1 and 2 was used throughout the remainder of this work.
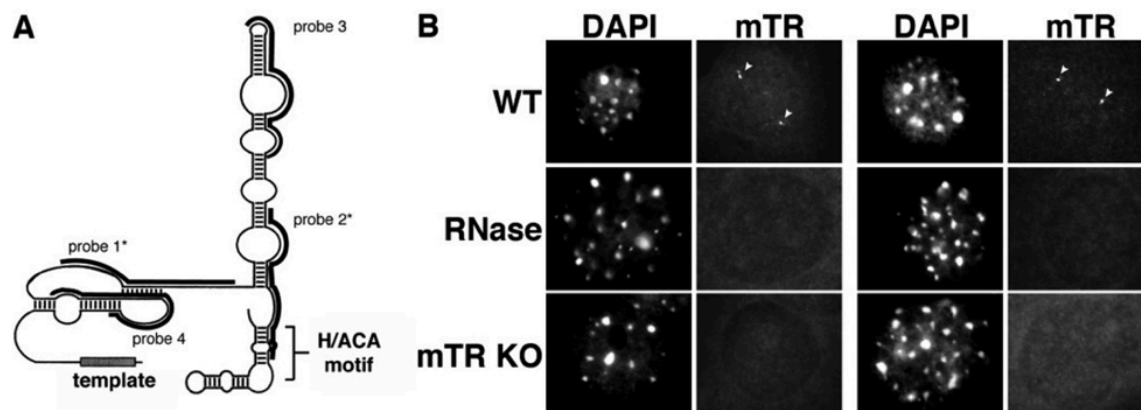
Figure 1. Mouse telomerase RNA is found in small spherical foci within the nuclei of cultured mouse cell lines. A. Schematic structure of mTR. The predicted secondary structure of mTR is shown [70]. Black bars indicate the regions encompassed by each oligonucleotide probe. Asterisks denote the two probes (probes 1 and 2) used throughout this manuscript. B. FISH procedure specifically detects the presence of mTR. mTR FISH was performed on wild

type MEF cells (WT, RNAse panels) or MEF cells derived from mTR -/- mice (mTR KO panels).

Arrowheads denote intranuclear mTR foci present in the WT cells (WT panels), which are lost

upon treatment of cells with RNAse A prior to FISH (RNAse panels). DAPI was used as a

nuclear stain. Scale bar, 10 microns.

The results of control experiments indicate that the procedure is specific for detection of

mTR. First, mTR signal was lost upon treatment of the cells with RNAse A prior to

hybridization with the mTR probes (Figure 1B). As controls, we also tested the RNAse-treated

cells with probes against U3 snoRNA (positive control for treatment) and the telomere repeat

(negative control for treatment). As expected, the U3 signals were eliminated or severely

reduced in the RNAse treated cells, while telomere signals were virtually unaffected (data not

shown). In addition, mTR foci were not observed in MEF cells derived from mTR knockout

mice (Figure 1B). Finally, the foci were not observed in HeLa (human cervical carcinoma) cells

using the mTR FISH probes (data not shown), further indicating the specificity of the procedure

for detection of mTR.

### mTR does not localize to Cajal bodies in mouse cells

Telomerase RNA localizes to Cajal bodies throughout interphase in human cell lines [20,

25]. To determine if the mTR foci observed in the MEF cells correspond to Cajal bodies, we

combined mTR FISH with immunofluorescence (IF) using antibodies against coilin, a marker

protein of Cajal bodies [46]. Surprisingly, the mTR foci present in the MEF cells did not co-

localize with coilin (Figure 2, MEF-26). We also found that mTR is localized in foci distinct

from Cajal bodies in other mouse cell lines representing various tissue sources (Figure 2).
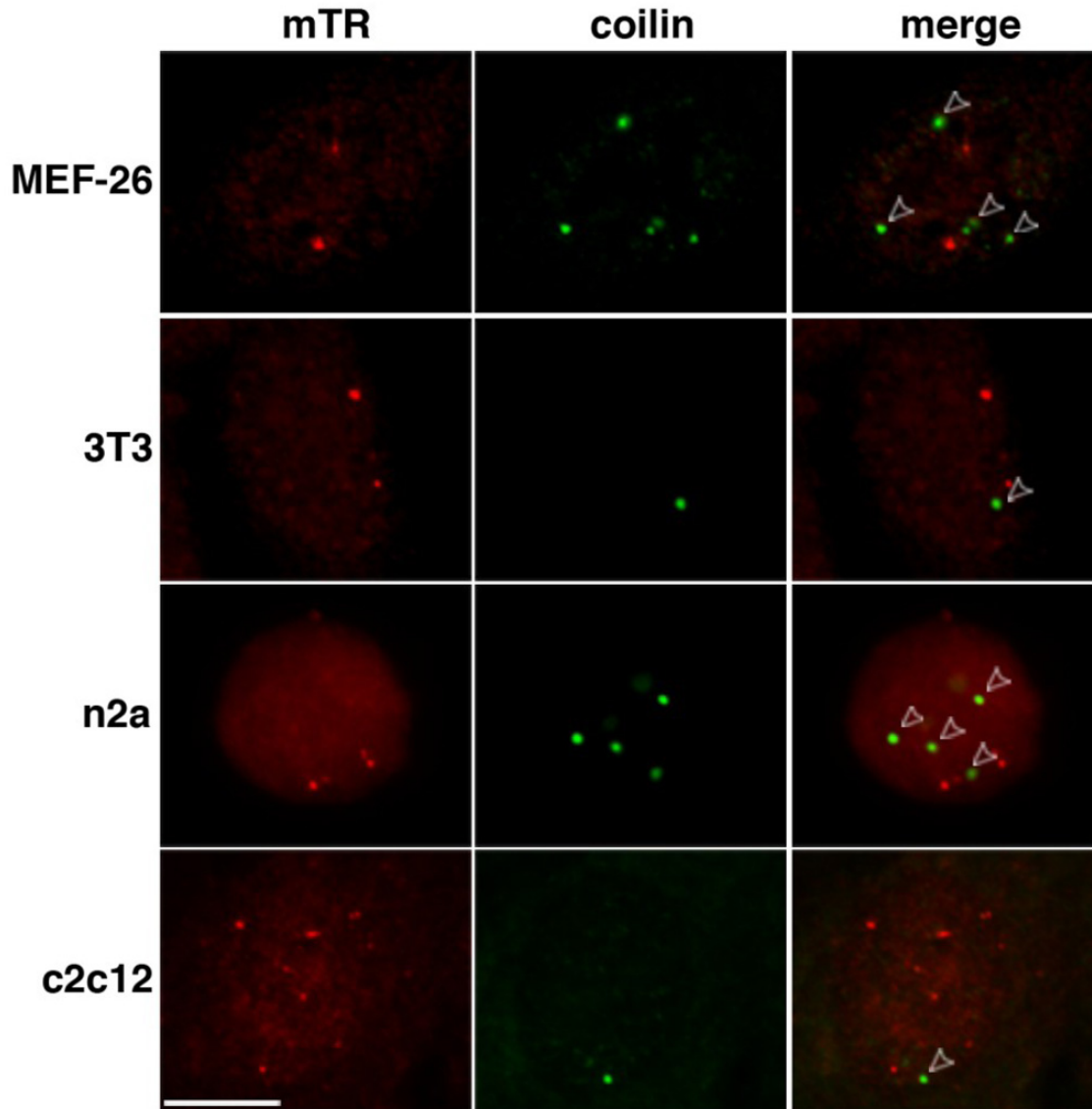
Figure 2. mTR foci do not colocalize with coilin in a variety of mouse cell lines. *Mouse embryonic fibroblast (MEF-26, 3T3), n2a neuroblastoma, and c2c12 myoblast cell lines were co-analyzed for mTR (detected by FISH, red) and coilin (marker protein for Cajal bodies, detected by IF, green). Merge panels indicate an overlay of mTR and coilin panels. Open arrowheads point to Cajal bodies that do not overlap with mTR foci. Scale bar, 10 microns.*

We further tested for co-localization of mTR with a series of additional molecules characteristically found in Cajal bodies. U85, a small Cajal body (sca)RNA, and SMN, the survival of motor neurons protein are prototypical markers for Cajal bodies, whereas Nopp140 localizes to both Cajal bodies and nucleoli in mammalian cells [47-50]. mTR did not co-localize with these additional Cajal body markers in the MEF cells (Figure 3). We also tested whether mTR co-localized with the snoRNP protein fibrillarin or U3 snoRNA (markers with distributions similar to Nopp140), and found no significant overlap (data not shown).

In addition, we examined mTR localization in MEF cells derived from coilin knockout mice [51] and found no reduction in mTR foci (Figure 3B). In these MEFs where coilin is not expressed, standard components of Cajal bodies such as U85, SMN and Nopp140 can be found in distinct nucleoplasmic foci termed residual Cajal bodies [51, 52]. mTR also did not co-localize with any of these residual Cajal body markers in the coilin knockout MEFs (data not shown). Taken together, our data unexpectedly indicate that mTR accumulates in nuclear structures distinct from Cajal bodies in cultured mouse cells.
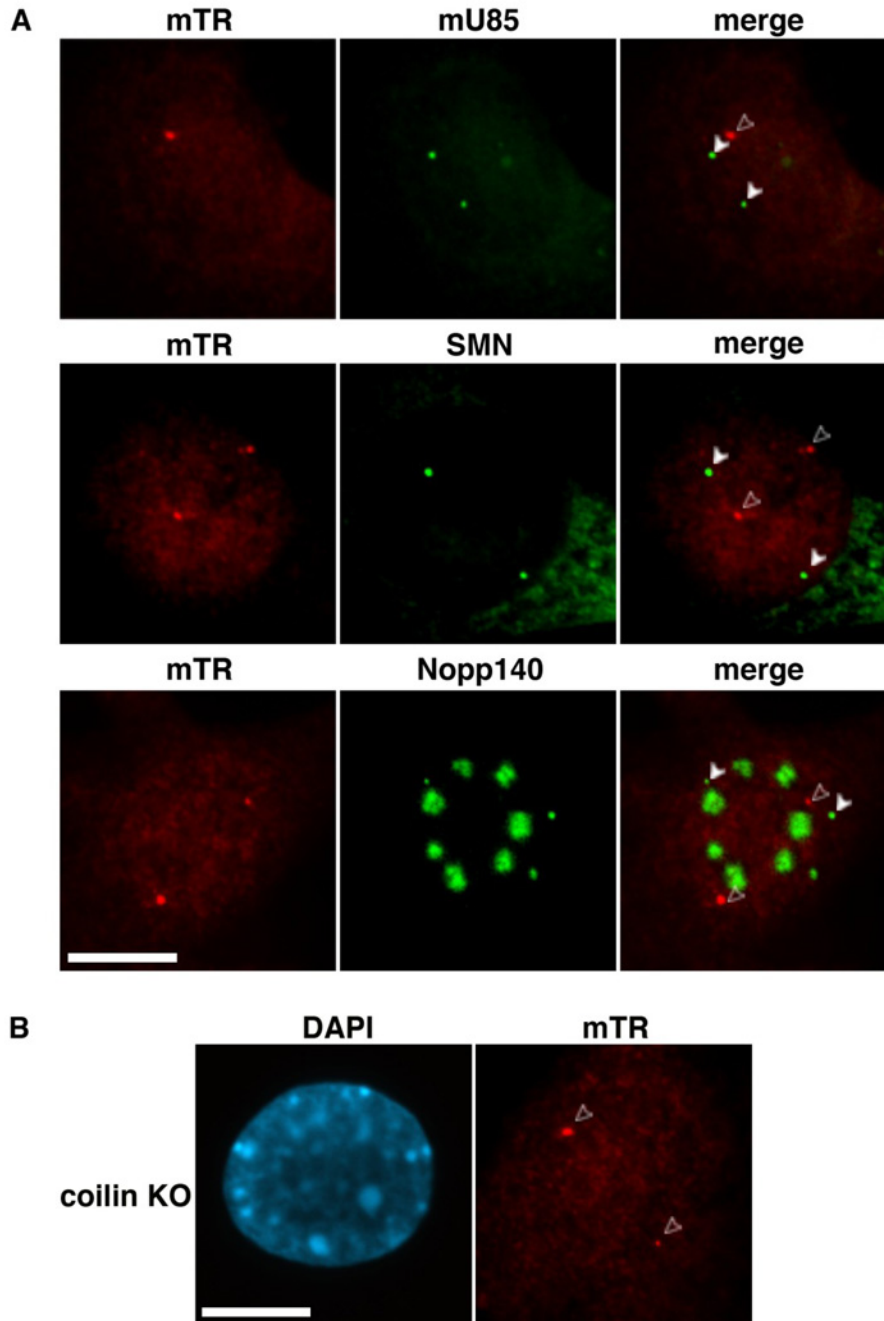
Figure 3. mTR resides in foci separate from Cajal bodies. *A. mTR foci do not correspond to known markers for Cajal bodies. mTR FISH (red, mTR panels) was performed in tandem with one of three markers for Cajal bodies: U85 scaRNA (top row, detected by FISH), SMN (middle row, detected by IF, signal present in Cajal bodies and cytoplasm), or Nopp140 (bottom row, detected by IF, signal present in Cajal bodies and nucleoli). Arrowheads denote Cajal bodies;*

open arrowheads point to mTR foci that do not localize to Cajal bodies. B. mTR localizes to intranuclear foci in MEF cells derived from coilin knockout (KO) mice. mTR FISH was performed on coilin KO MEF cells. Open arrowheads point to mTR foci. DAPI was used to stain the DNA. Scale bars, 10 microns.

mTR is found at a subset of telomeres in S phase cells

Having found that mTR does not localize to Cajal bodies, we investigated whether any of the mTR foci correspond to telomeres. When mTR FISH was combined with IF using antibodies against the telomere binding proteins TRF1, TIN2, or TPP1, or with FISH using a probe directed against the telomeric repeats, we found that mTR co-localized with a subset of telomeres in approximately 10% of the MEF (and 3T3) cells with distinct mTR foci (Figure 4 and data not shown). This is similar to the percentage of cells in which TR-telomere associations are observed in unsynchronized human cancer cell lines, where TR localizes to telomeres specifically in S phase [21-23]. In order to see if telomere localization was an S phase-specific event in the mouse cells, we stained the MEF and 3T3 cells with antibodies against PCNA (proliferating cell nuclear antigen). PCNA is expressed by interphase cells, but specifically associates with chromatin in cells undergoing DNA replication [53]. Based on established PCNA staining patterns, we were able to distinguish which cells were in S phase and to discern the S sub-phase as early, mid, or late (Figure 4A). We found that mTR-telomere associations were restricted to S phase cells and occurred throughout S phase (Figure 4A).
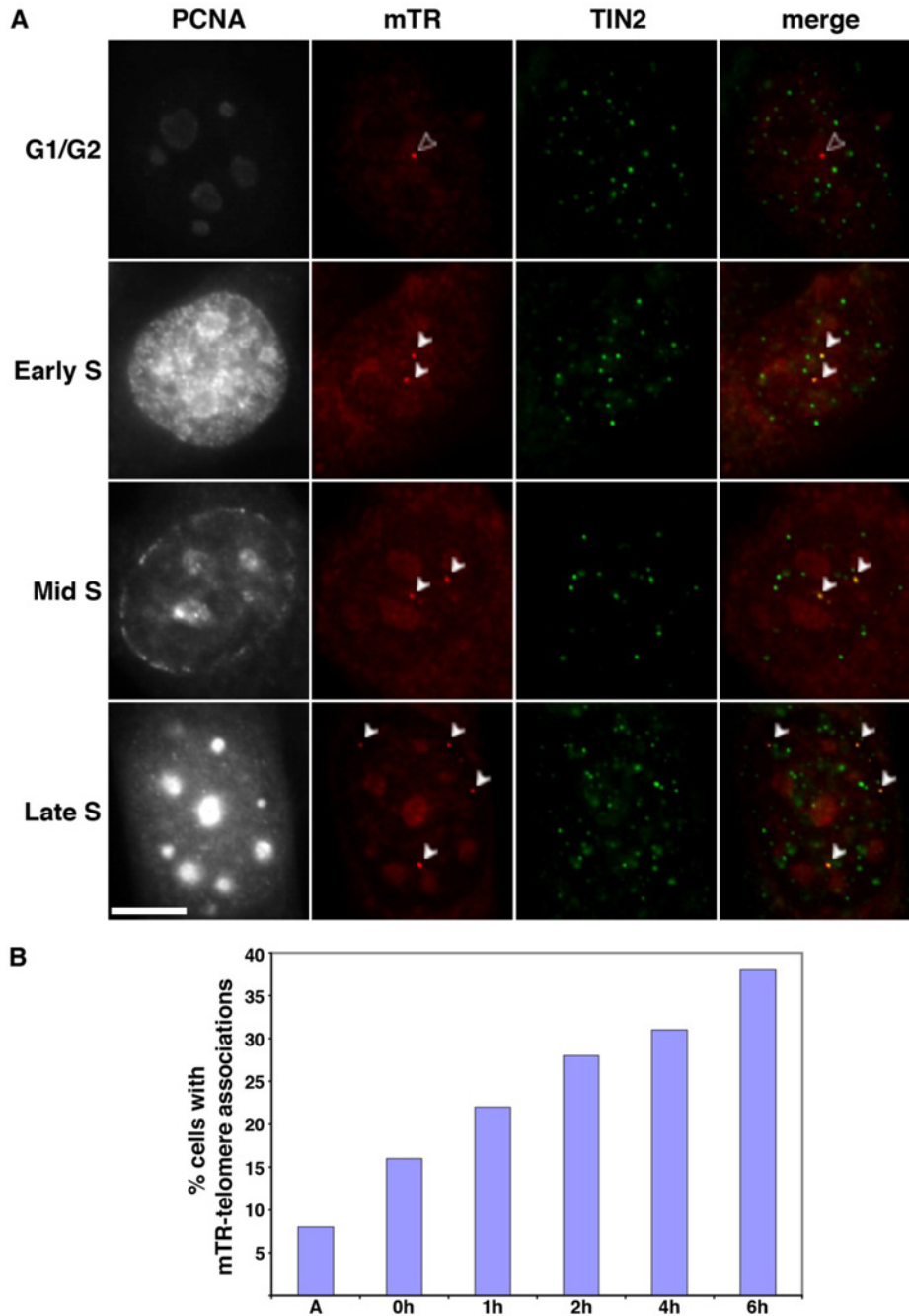
Figure 4. mTR localizes to subsets of telomeres in S phase. *A. Localization of mTR to telomeres occurs throughout S phase. mTR FISH (red) and TIN2 IF (green, marker for telomeres) were performed on 3T3 cells at various stages of the cell cycle. PCNA staining was performed to identify S phase cells, as well as S sub-phase (i.e. early, mid, or, late, as indicated). Merge panels show a superimposition of mTR and TIN2 panels, yellow indicates an overlap of*

signal. The foci where mTR and telomeres colocalize are indicated by white arrowheads. Open arrowheads (in G1/G2 panel) denotes mTR foci that do not overlap with a telomere. Scale bar, 10 microns. B. The frequency of mTR-telomere associations increases as cells progress through S phase. The percentage of 3T3 cells with mTR foci that display mTR at the telomere (assessed via TIN2) is plotted relative to time in hours (h) after release from a double thymidine block. A, asynchronous cells. Data was collected from multiple slides prepared as part of at least two separate experiments.

To further investigate the S phase-specific localization of mTR to telomeres, we synchronized 3T3 and MEF cells using a double thymidine treatment and analyzed mTR localization at various points in the cell cycle (Figure 4, Supplemental Figure 1A, and MEF data not shown). We found that mTR-telomere associations peaked in S phase (I don't think we have evidence showing 6h is the late S phase), when co-localization was observed mostly in 38% of the 3T3 cells with mTR foci (Figure 4B, 6h post release from the double thymidine block, 60 of 157 cells). As can be seen in Figure 4B, the frequency of telomere association gradually increased as cells progressed through S phase. At G1/S (0h post release), mTR was found at at least one telomere in 16% of cells (18 of 113 cells with mTR foci). In early S (1h and 2h post release) and mid S (4h post release), the percentage increased steadily from 22% (1h post release, 38 of 174 cells with mTR foci) to 28% (2h post release, 55 of 196 cells with mTR foci) to 31% (4h post release, 50 of 162 cells with mTR foci). (same as above, not sure about the accuracy of mentioning the hours related to specific stage in S phase) The frequencies of mTR-telomere associations observed were similar when determined relative to two different telomere markers (TIN2 and TPP1). Synchronized MEF cells behaved similarly to the 3T3 cells (data not

shown). Our data indicate that mouse TR localizes to telomeres specifically during S phase and

that co-localization of mTR and telomeres increases over the course of S phase.

Recent reports have implicated Cajal bodies in the delivery of telomerase to telomeres in

human cancer cells [19, 21, 23, 54]. However, we did not observe associations of mTR with

Cajal bodies in cultured mouse cells (Figures 2 and 3), suggesting that Cajal bodies may not be

necessary for delivery of mTR to the telomere. To further address a potential requirement for

Cajal bodies in telomerase recruitment to telomeres in mouse cells, we performed mTR FISH

and telomere IF on the coilin KO MEF cells (Figure 5). We found that mTR localized to the

telomere in the absence of coilin and normal coilin-containing Cajal bodies (Figure 5). The

frequency of mTR-telomere associations was the same (approximately 12% of cells) in the wild-

type and coilin KO MEFs.
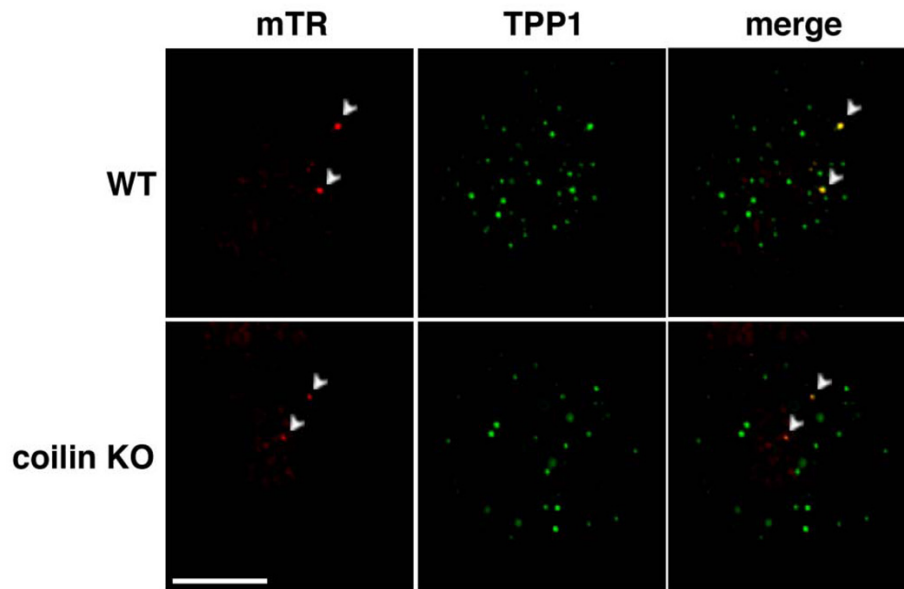
Figure 5. mTR localizes to the telomere in coilin KO MEFs. *mTR FISH (red) and TPP1 IF*

(green, marker for telomeres) were performed on MEF cells derived from wild type (WT) and

coilin -/- (coilin KO) MEFs. Merge panels display an overlay of mTR and TPP1 signals.

Arrowheads denote foci where both mTR and TPP1 signals overlap. Scale bar, 10 microns.

Human telomerase RNA localizes to Cajal bodies (and telomeres) in mouse cells

The lack of localization of mTR to Cajal bodies in mouse cells could be attributable to a difference in the mouse TR or in some aspect of the mouse cell environment (such as the nature of the Cajal bodies). To investigate the molecular basis for the difference in the localization patterns of mouse and human telomerase RNA, we examined the localization of hTR expressed in mouse cells. hTR and telomere localization were visualized by FISH, and Cajal bodies by coilin IF in both n2a and MEF26 cells (Figure 6). Unlike mTR, human telomerase RNA localized to Cajal bodies in mouse cells (Figure 6). hTR was also found at telomeres in synchronized S phase cells (Figure 6); co-analysis of PCNA staining patterns in the n2a mouse cells indicated that hTR-telomere co-localization was restricted to S phase. We did not observe any significant occurrence of hTR at foci outside of Cajal bodies and telomeres in the mouse cells. The results indicate that inherent features of mouse telomerase RNA dictate the distinct trafficking observed for TR in mouse.
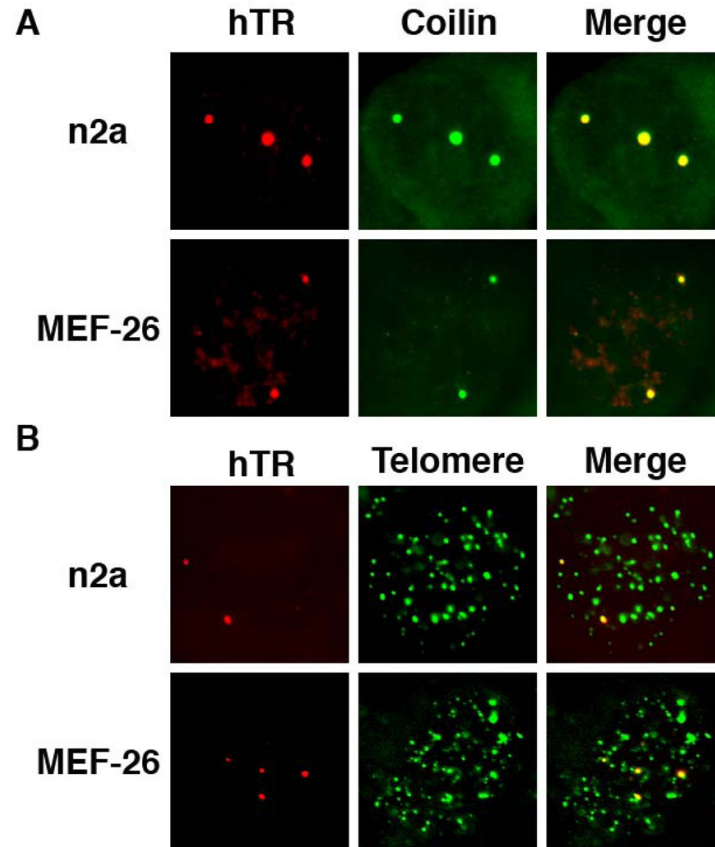
Figure 6. Human telomerase RNA localizes to Cajal bodies and telomeres in mouse cells. *A.*

hTR co-localizes with Cajal bodies in mouse cells. Human telomerase RNA (hTR) was expressed

in n2a and MEF-26 mouse cell lines and co-analyzed for hTR (detected by FISH, red panels)

and coilin (detected by IF, green panels). Merge panels display an overlay of hTR and coilin

signals. B. hTR co-localizes with telomeres in mouse cells. hTR-transfected n2a and MEF-26

mouse cells were examined for hTR (detected by FISH, red panels) and telomere (detected by

FISH, green panels) signals. Merge panels display an overlay of hTR and telomere signals.

A novel localization pattern is associated with TR-telomere association in mouse cells

Given that telomerase does not require Cajal bodies for delivery to telomeres in mouse

cells, we wondered how mTR arrives at its functional site. During the course of our analysis

with synchronized cells we noticed an interesting pattern of mTR localization: mTR foci linked

together in chains of 2-5 foci. We analyzed the localization
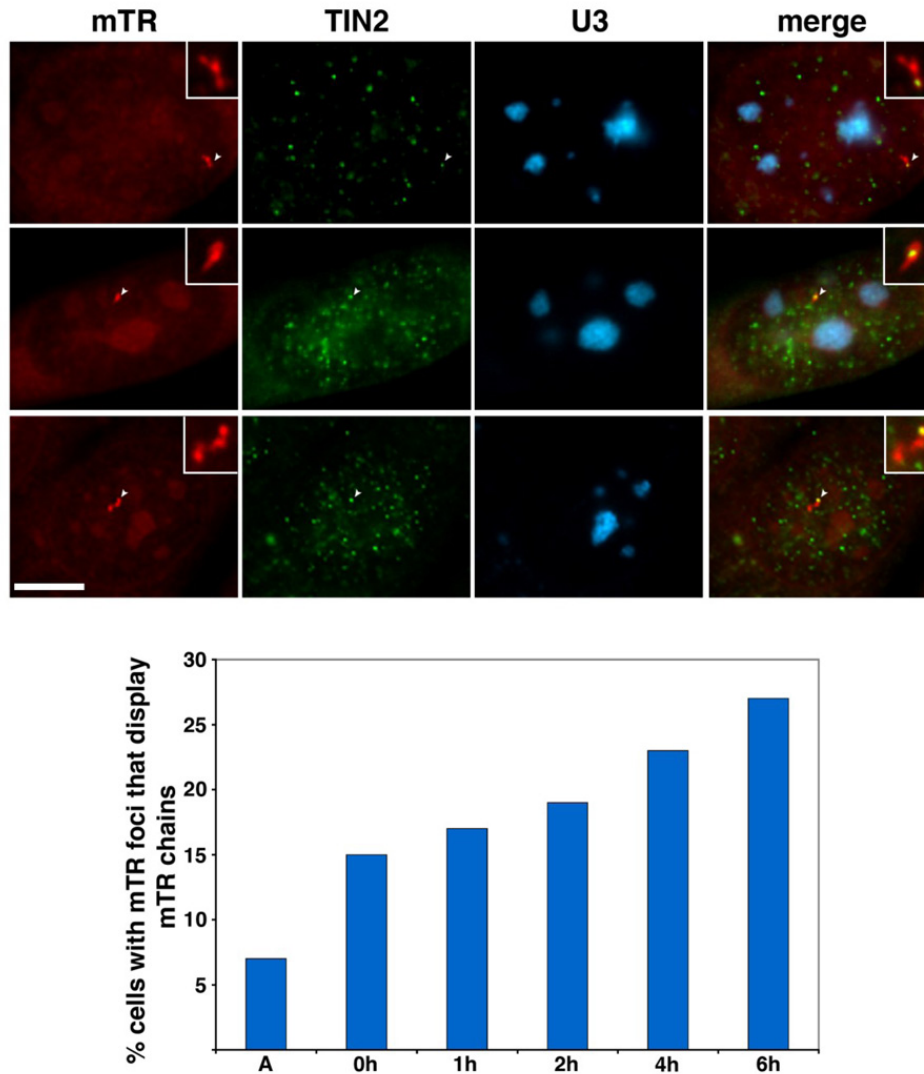
Figure 7. mTR foci coalesce during S phase of the cell cycle. *A. mTR foci link together to*

form chains that are frequently associated with telomeres but not nucleoli. 3T3 cells were

stained for mTR (detected by FISH, red panels), TIN2 (telomere marker detected by IF, green

panels), and U3 snoRNA (nucleolar marker detected by FISH, blue panels). Merge panels

display a superimposition of all 3 panels. Insets show an enhanced magnification of the mTR

chains. Arrowheads point to mTR foci within the chain that overlap with a telomere. Scale bar,

10 microns. B. The frequency of mTR chains gradually increases over S phase of the cell cycle.

The percentage of 3T3 cells with mTR foci that display a mTR chain are plotted relative to time

after release from a double thymidine block. A, asynchronous cells. Data compiled from two

separate experiments.

of the chains of mTR foci relative to U3 snoRNA (FISH) and telomere (TIN2 or TPP1 IF)

localization patterns, and found that in 60% of the chains, at least one of the mTR foci directly

co-localized with a telomere (Figure 7A). Less than 10% of the chains overlapped nucleoli

(visualized via U3 snoRNA) (Figure 7A). The appearance of the mTR chains coincides with the

timing of mTR trafficking to telomeres; both MEF and 3T3 cells displayed a gradual increase in

mTR chains as cells progressed through S phase (Figure 7B, Supplemental Figure 1B and data

not shown). Taken together, our data suggest that the localization of mTR to telomeres during S

phase is associated with the formation of distinct chains of mTR foci in mouse.

Discussion

Studies in human cells have established a remarkable mechanism for the regulation of telomerase activity: regulated trafficking of the enzyme [19, 21-24]. In this work, we have delineated key steps in telomerase RNA trafficking in the genetically tractable mouse system, where we found fundamental similarities as well as interesting differences that expand our understanding of telomerase biology and open up new avenues of investigation.

Targeting telomerase to telomeres in mouse

Our results indicate that, in mouse as in human cancer cells, telomerase RNA localizes to subsets of telomeres during S phase (Figure 4A). Retinoblastoma (Rb) proteins that regulate S phase transitions and progression have been shown to play a role in telomere elongation in mouse [55], providing a link between telomerase regulation (perhaps trafficking) and the cell cycle that merits further investigation. But why telomerase is found at subsets of telomeres at any given time during S phase in both human and mouse remains a question of great interest.

One possibility is that telomerase visits all of the telomeres during the course of S phase, but is only found at a subset at any given time. We observe telomerase RNA at telomeres throughout most of S phase in both mouse (Figure 4) and human [23], and recent work provides evidence that human telomerase extends the majority of (or all) telomeres each cell cycle [34].

On the other hand, the observed localization pattern may reflect selective recruitment of telomerase to a fraction of telomeres during each cell cycle. Indeed, considerable evidence indicates that telomerase selectively extends the shortest telomeres in mouse cells [56, 57]. When mice with short telomeres are crossed with those with long telomeres, the shorter telomeres are extended first in the offspring [56]. Likewise, when telomerase levels are limiting (in a mouse heterozygous for TERT expression), the shortest telomeres in the population are

maintained (while the longer ones grow shorter over time) [57]. Preferential elongation of short telomeres in human cells was also recently observed [58]. In yeast, it has been shown that only a small fraction of telomeres are extended by telomerase within any given cell cycle and that telomerase exhibits a preference to act on the shortest telomeres [59]. However, a direct correlation between telomere length and the frequency of telomerase recruitment has not yet been established for mammalian cells.

In mouse, we found that telomerase recruitment to telomeres reaches a maximum in late S phase (Figure 4B), whereas in human cells, the association peaks during mid S phase [23]. The distinct timing of telomerase recruitment in the two species may point to factors important in the recruitment process that differ between the organisms. For example, telomerase recruitment may be coordinated with replication of telomeric DNA, which may occur with distinct kinetics in various mammalian cells. Differences in average telomere length (20-150 kb in mouse cells versus 5-15 kb in human cells [60]) or in core telomerase- or telomere-associated components that regulate recruitment [1, 2, 36, 37] may also be important. Differences in the epigenetic modification of telomeric DNA or telomere-bound proteins, or in expression of telomeric repeat-containing (TERRA) non-coding RNAs could also influence telomerase recruitment dynamics in the two species [61-64]. However, while the precise kinetics may vary, our results suggest that S phase-specific recruitment of telomerase to telomeres is a conserved process in mammals.

Telomerase RNAs possess signals for distinct trafficking pathways

While in human cells it appears that localization of telomerase RNA to telomeres depends on trafficking through (and perhaps delivery by) Cajal bodies, in mouse cells we found that telomerase RNA trafficking does not appear to involve Cajal bodies (Figures 2,3,5,7). This

divergence could reflect a difference in the mouse cell environment (such as the nature of Cajal bodies in mouse cells), however we found that hTR expressed in the mouse cell environment localizes to mouse Cajal bodies and telomeres just as is observed in human cells (Figure 6). The finding that hTR retains its distinct trafficking pattern in mouse cells indicates that telomerase RNAs possess signals that dictate subcellular transport pathways independent of other cellular components.

At the same time, other evidence indicates that TERT and other cellular factors also play important roles in telomerase trafficking that could affect differences observed among species. In human, localization of hTR to both Cajal bodies and telomeres depends on association with hTERT [22, 23]. A number of previous studies have demonstrated that hTR assembles with mTERT to form catalytically active telomerase complexes both in vitro and in mouse cells [14, 15, 65-67]. Interestingly however, while hTR assembles with mTERT [14, 15, 65-67] and is trafficked to telomeres in mouse cells (Figure 6), when hTR and mTERT are co-expressed in human cells (VA13 cells that are naturally devoid of hTR and hTERT) localization of hTR to telomeres is not detected [66]. These findings suggest that interactions between TERT and cellular components also contribute to localization.

Mouse telomerase RNA resides in novel nuclear foci distinct from Cajal bodies

In contrast to human cancer cells [20, 25] and *Xenopus* (frog) oocytes [68, 69], telomerase RNA does not accumulate in Cajal bodies in cultured mouse cells and instead is found in distinct, nucleoplasmic foci during most of the cell cycle (Figures 2 and 3). Our results further suggest that the distinct localization patterns of hTR and mTR derive from inherent properties of the RNAs (Figure 6). It is interesting that mTR is not found in Cajal bodies despite

the presence of an intact CAB box motif with a sequence (UGAG) identical to that shown to be required to target hTR and small Cajal body RNAs (scaRNAs) to Cajal bodies [19, 20, 50, 70]. Furthermore, the protein that recognizes the CAB box motif (TCAB1 or WDR79) and is required for localization of TR to both Cajal bodies and telomeres (as well as for telomerase activity) in human cells is highly conserved in mouse [24, 35]. The most obvious difference between hTR and mTR is in the 5' terminal sequences [70, 71]. hTR includes 45 nts upstream of the template region, and a portion of this sequence participates in intramolecular basepairing within the 5' pseudoknot domain of the RNA to form the P1 stem [70]. In contrast, mTR contains just 2 nts upstream of the template and no P1 stem is formed. Additional work will be required to determine if differences in the 5' regions or other more subtle variations in sequence and structure account for the ability or inability of the RNAs to associate with Cajal bodies. We certainly do not exclude the possibility of limited association of mTR with Cajal bodies in the mouse cells, however telomerase RNA clearly accumulates in distinct foci in mouse cells.

The relationship between mTR, the novel mTR foci identified here, and Cajal bodies may warrant further investigation. Interesting associations have been found between Cajal bodies and other related foci, and molecules found in Cajal bodies under some conditions can be found in distinct foci under other cellular conditions. During S phase in human cancer cells, hTR is found in distinct foci attached to Cajal bodies prior to localization to telomeres [23]. In addition, the Cajal body constituent SMN can be found in foci known as gems (gemini or twins of Cajal bodies [47]) in cells deficient in coilin methylation [72] and at certain times in development [73, 74]. The cell lines examined in this work (Figure 2) are derived from embryonic tissue (MEF, 3T3) or reflect an undifferentiated state (n2a, c2c12), and thus it is possible, for example, that mTR accumulates in Cajal bodies in mouse cells in other developmental states.

The novel mouse telomerase RNA foci identified in this work appear to play a role (akin to that of Cajal bodies in human cells [21, 23]) in the delivery of TR to telomeres. In mouse cells we found that TR localizes to telomeres in the absence of obvious accumulation within Cajal bodies (Figure 4A, and see also Figures 2 and 3) and in the absence of coilin (Figure 5). Instead, chains of mTR foci form and co-localize with telomeres specifically during S phase (Figure 7). The significance of the formation of groups of attached TR foci in both human (Cajal body-associated foci) and mouse (chains of mTR foci) cells at the time of delivery to telomeres is intriguing but currently unclear.

While there may be differences in the pathways in mouse versus man, the paradigm that has emerged from examining telomerase RNA localization in mammalian cells is that the RNA subunit of telomerase resides in subnuclear structures physically separate from telomeres and is mobilized to telomeres specifically during S phase. Moreover, the structures appear to play roles in the delivery of TR to the telomeres. The development of an effective mouse telomerase RNA detection procedure opens the door to a wealth of genetic approaches that should lead to a deeper understanding of how telomerase trafficking is regulated.

Acknowledgements

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