Insight into the Regulation of Telomerase Access to Telomeres by Shelterin Proteins

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Abstract

Recruitment to telomeres is a pivotal step in the function and regulation of human telomerase. Impaired telomerase function can lead to premature organismal aging, development of cancer and multisystem disorders such as dyskeratosis congenita. Telomerase access to telomeres may be regulated by a telomere-binding complex termed shelterin, which is composed of TRF1, TRF2, RAP1, TIN2, TPP1 and POT1 proteins. However the molecular basis for human telomerase recruitment to telomeres is not known.

Here, we have directly investigated the process of telomerase recruitment via chromatin immunoprecipitation (ChIP) and fluorescence *in situ* hybridization (FISH). We find that depletion of two components of the shelterin complex - TPP1 and the protein that tethers TPP1 to the complex, TIN2 - results in a loss of telomerase recruitment. On the other hand, we find that the majority of the observed telomerase association with telomeres does not require POT1, the shelterin protein that links TPP1 to the single-stranded region of the telomere. Furthermore, we find that the double-stranded telomere binding protein, TRF2 is dispensable for telomerase association with telomeres. Deletion of the oligonucleotide/oligosaccharide-binding fold (OB-fold) of TPP1 further disrupts telomerase recruitment. In addition, while loss of TPP1 results in the appearance of DNA damage factors at telomeres, the DNA damage response *per se* does not account for the telomerase recruitment defect observed in the absence of TPP1. Our findings indicate that TIN2-anchored TPP1 plays a major role in the recruitment of telomerase to telomeres in human cells and that the recruitment does not depend on POT1 or interaction of the shelterin complex with the single-stranded region of the telomere. We propose that the loss of TRF2 can be compensated by the second double-stranded telomere binding protein, TRF1 that anchors TIN2/TPP1-recruited telomerase onto the telomeres.

Dyskeratosis congenita (DC) is a multisystem disorder characterized with bone marrow failure, cancer predisposition and defective telomere maintenance. The *TINF2* gene that encodes for the TIN2 shelterin protein is one of seven mutated genes identified in DC patients. Here, we have examined the effects of TIN2 DC mutants on telomere protection and shelterin protein stability. We find that exogenous expression of TIN2 DC mutants can rescue the TIN2-depleted phenotype, restoring TPP1 protein levels and telomere protection. Our findings indicate that TIN2 DC mutants preserve the intact telomere structure and protection. We propose that defective telomere elongation may be the underlying cause of impaired telomere maintenance in TIN2 DC patients.

Keywords: Telomerase, telomeres, shelterin, TPP1, telomerase recruitment, TIN2, dyskeratosis congenita.

Résumé

Le recrutement de la télomérase est une étape clef dans le fonctionnement et la régulation de la télomérase humaine. La perturbation de ce processus peut conduire à un vieillissement prématuré de l'organisme, au développement de cancers et à des désordres systémiques tels que la dyskératose congénitale. Il se peut que l'accès de la télomérase aux télomères soit régulé par le 'shelterin', un complexe qui se lie aussi aux télomères et qui est composé des protéines TRF1, TRF2, RAP1, TIN2, TPP1 and POT1. Cependant, les bases moléculaires du recrutement de la télomérase humaine n'en sont pas encore connues.

Dans ce travail, nous avons étudié directement le mécanisme de recrutement de la télomérase par immonuprécipitation de la chromatine (ChIP) et par hybridation fluorescente *in situ* (FISH). Nous avons trouvé que la déplétion de TPP1 et celle de TIN2, deux constituants du complexe 'shelterin', empêchent le recrutement de la télomérase. Nous avons également trouvé que la majorité de l'association de la télomérase avec les télomères ne dépend pas de POT1, une protéine qui fait partie des 'shelterin' et qui lie TPP1 à la partie simple brin des télomères. TRF2, une protéine qui se lie à la partie double brin des télomères, n'est pas non plus indispensable pour l'association de la télomérase avec les télomères. La délétion du domaine de liaison oligonucleotide/oligosaccharide (oligonucleotide/oligosaccharide-binding fold, abrégé 'OB-fold', en anglais) de TPP1 interrompt le recrutement de la télomérase. Bien que la perte de TPP1 ait pour conséquence l'apparition de facteurs de dommage à l'ADN aux télomères, la réponse de dommage à l'ADN n'est pas, en soi, la cause du défaut de recrutement de la télomérase. Ainsi, nos résultats indiquent que la protéine TPP1, lorsqu'elle est liée à TIN2, joue un rôle majeur dans le recrutement de la télomérase aux télomères dans les cellules humaines et que ledit recrutement ne dépend ni de POT1, ni d'une interaction du complexe 'shelterin' avec la portion simple brin des

télomères. Nous émettons également l'hypothèse que la perte de TRF2 peut être compensée par l'autre protéine liant la portion double brin des télomères, TRF1, qui ancre la télomérase aux télomères lorsqu'elle est recrutée par TPP1 et TIN2.

La dyskératose congénitale (DC) est une maladie caractérisée par une incapacité de la moelle osseuse à créer les cellules sanguines, une prédisposition au cancer et un mécanisme de régulation des télomères défectueux (les télomères ne sont pas protégés et/ou ne sont pas allongés). Le gène *TINF2*, qui code pour la protéine TIN2, est l'un des sept gènes impliqués dans la DC. Dans ce travail, nous avons examiné les effets des mutants de TIN2 sur la protection des télomères et sur la stabilité des protéines 'shelterin'. Nous avons trouvé que l'expression exogène des mutants de TIN2 permet de faire disparaître les phénotypes associés à une déplétion de TIN2, restaurant les niveaux de TPP1 et la protection des télomères. Nos résultats indiquent que les mutants de TIN2 impliqués dans la DC préservent la structure des télomères et suggèrent que la cause principale du défaut de régulation des télomères serait l'impossibilité de les allonger.

Mots clefs: Télomérase, télomères, 'shelterin', TPP1, recrutement de la télomérase, TIN2, dyskératose congénitale.

Абстракт

Регрутирањето на теломеразата врз теломерите е клучен чекор во функционирањето и регулацијата на хуманата теломераза. Нарушена функција на теломеразата може да предизвика предвремено стареење на организмот, развој на рак и мултисистемски заболувања како во случајот на dyskeratosis congenita. Пристапот на теломеразата кон теломерите зависи од комплексот шелтерин (англ. shelterin) кој ја врзува и заштитува ДНК на теломерите, а е составен од протеините TRF1, TRF2, RAP1, TIN2, TPP1 и POT1. Сепак молекуларните основи на процесот на хуманата теломеразна регрутација врз теломерите се непознати.

Во овој труд, ние го проучувавме процесот на теломеразна регрутација преку техниките на имунопреципитација на хроматин (ChIP) и флуоресцентна *in situ* хибридизација (FISH). Пронајдовме дека намалување на нивото на шелтерин протеинот TPP1 и протеинот TIN2, кој го врзува TPP1 за комплексот шелтерин, го попречува процесот на теломеразна регрутација врз теломерите. Од друга страна, пронајдовме дека поголемиот дел од теломеразната асоцијација со теломерите не зависи од протеинот POT1, компонента на шелтерин комплексот кој го поврзува протеинот TPP1 со едноверижната ДНК на теломерите. Исто така пронајдовме дека намаленото ниво на протеинот TRF2, кој ја врзува двојноверижната ДНК на теломерите, не ја нарушува теломеразната асоцијација со теломерите. Одстранувањето на олигонуклеотидниот/олигосахаридниот врзувачки домен (OB-fold) на протеинот TPP1, резултира со дополнително намалување на теломеразната регрутација врз теломерите. Понатаму, додека намаленото ниво на протеинот TPP1 предизвикува појава на ДНК оштетувачки фактори на теломерите, оштетувањето на теломерите само по себе не придонесува за дефектот во теломеразната регрутација предизвикан од

недостаток на протеинот TPP1. Нашите резултати индицираат дека протеинот TPP1 поврзан со протеинот TIN2, има главна улога во теломеразната регрутација врз теломерите во хумани клетки. Дополнително, теломеразната регрутација не зависи од протеинот POT1 ниту од интеракцијата на комплексот шелтерин со едноверижната ДНК на теломерите. Предложуваме дека дефицитот на протеинот TRF2 може да биде надополнет со протеинот TRF1, кој исто така ја врзува двојноверижната ДНК на теломерите и со тоа го доведува комплексот TIN2-TPP1-теломераза врз теломерите.

Dyskeratosis congenita (DC) е мултисистемско заболување кое се карактеризира со нарушена функција на коскената срж, предиспозиција кон рак и дефекти во одржувањето на теломерите. Генот *TINF2*, кој го кодира протеинот TIN2, е еден од седумте мутирани гени кои се пронајдени во пациенти со DC. Во овој труд, ние ги истражувавме ефектите на DC мутирани TIN2 протеини врз заштитата на теломерите и стабилноста на протеинскиот комплекс шелтерин. Пронајдовме дека надворешната експресија на TIN2 протеини со DC мутации може да го нормализира фенотипот предизвикан од недостаток на протеинот TIN2 и да ги воспостави нормалните нивоа на протеинот TPP1, а со тоа и заштитата на теломерите. Нашите резултати покажуваат дека TIN2 протеини со DC мутации ја задржуваат нормалната физиолошка структура на теломерите и нивната заштита. Последователно, предложуваме дека можна главна причина за нарушеното одржување на теломерите во пациенти со TIN2 DC мутации е дефект во теломеразното издолжување на теломерите.

Клучни зборови: Теломераза, теломери, шелтерин, TPP1, теломеразна регрутација, TIN2, dyskeratosis congenita.

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Chapter I: Introduction

Our chromosome ends are specialized nucleoprotein structures termed telomeres. They are composed of simple tandem DNA repeats which are bound by telomere-binding proteins (de Lange, 2005). Some telomeres are also coated with telomeric repeat-containing RNA (TERRA) (Azzalin et al., 2007; Schoeftner and Blasco, 2008). Telomeres are essential in all organisms with linear chromosomes due to their protective function at the chromosome termini. Compromising human telomere structure causes chromosome instability, eventually leading to aging and cancer. Thus, telomere maintenance is indispensable and is mediated through a combined action of telomere-associated proteins, telomerase and possibly TERRA. Telomerase is a reverse transcriptase that uses its RNA moiety to elongate and maintain functional telomeres. In this chapter I will introduce the telomere and the basic features of human telomere maintenance by telomerase and telomere-bound proteins. TERRA and transiently telomere- and telomerase-associated proteins will not be covered. The consequences of telomere dysfunction in humans will be discussed as well.

The discovery of telomeres

The first description of telomeres and their function came from the studies of Barbara McClintock and Hermann Muller. McClintock and Muller experimented with X-ray irradiation to induce chromosome alterations in maize (McClintock, 1939; McClintock, 1941) and fruit flies (Muller, 1938). They described the deleterious outcome of the irradiation in form of chromosome translocations, inversions, deficiencies and ring chromosomes. A plausible explanation for such events was that broken chromosome ends fuse with each other. However, they observed that natural ends of linear chromosomes were not susceptible to such events. Thus, the ends of chromosomes must be protected by special structures. It was Muller who coined the

term telomere. At that time, he referred to telomeres as essential genes that cells could not lose, rather than capping structures at the chromosome end.

Human telomeric DNA

First insights into the sequence of telomeric DNA came from studies in the holotrichous ciliate *Tetrahymena thermophila* (Blackburn and Gall, 1978) and the human telomere repeat sequence 5'-TTAGGG-3' was first identified from a human recombinant repetitive DNA library (Moyzis et al., 1988). This sequence is characteristic for all vertebrates but it is also found in several fungi, protozoa and plants. The human telomere DNA is organized in a double stranded part and a 3' G-rich overhang (Makarov et al., 1997). The length of telomere DNA is an inherited trait and in humans is around 10 kb at birth with gradual decrease with each cell division cycle (Allshire et al., 1988; Cooke and Smith, 1986; Harley et al., 1990). Telomerase-positive human cells have highly variable telomere length ranging from 2 to 30 kb. In addition, each telomere is heterogeneous in size. For comparison, in other organisms the double stranded telomere part ranges from less than 30 bp in ciliates, to 200-300 bp in budding yeast and up to 100 kb in mice. The G rich-overhang length varies from few nucleotides in ciliates, up to 100 nucleotides in human (Chai et al., 2005; Makarov et al., 1997). The G-overhang length in humans is reported longer on lagging strand telomeres (telomeres that are synthesized by lagging strand DNA replication) than on leading strand telomeres. However, unlike yeast overhangs that become very short soon after DNA synthesis, human G-rich overhangs are longer at most of the telomeres throughout the cell cycle (Chai et al., 2006a; Makarov et al., 1997). The DNA damage sensor complex Mre11-Rad50-Nbs1 is implicated in the G-overhang length regulation in humans, as its reduction produces transient shortening of the G-overhang in telomerase-positive but not in telomerase-negative cells (Chai et al., 2006b). Another study that determined the terminal ends of both C-rich and G-rich strands, reveals a highly specific processing events at the C-strand with a preference for the sequence 3'-

CCAATC-5' at the 5' end (Sfeir et al., 2005). On the other hand, the G-rich overhangs display more variable sequence terminations GGTTAG-3', GGGTTA-3' and AGGGTT-3' with a strong preference for the first sequence termination in cells that express telomerase.

Higher-order telomere structures

Telomere DNA is proposed to form higher order structures. G-quadruplex structures arise in physiological salt conditions when four guanines form G-quartets, which stack on top of each other while being stabilized with potassium and sodium ions. In *Stylonichia lemnae*, these structures are observed *in vivo*, by immunofluorescence with a G-quadruplex-specific antibody (Schaffitzel et al., 2001). Their formation is regulated in a cell-cycle dependent manner by the telomere end binding proteins TEBPα and TEBPβ (Paeschke et al., 2005) In *Oxytricia nova*, telomerase activity *in vitro* is inhibited on substrates forming G-quartet structures (Zahler et al., 1991). However in *Stylonichia lemnae* the unfolding of G-quadruplex structure in S-phase is dependent on telomerase which is recruited by phosphorylated TEBPβ (Paeschke et al., 2008). The *in vivo* existence of human telomere G-quadruplex structure remains unknown.

Isolated and protein-free human and mouse telomeric DNA, which was *in vivo* cross-linked with psoralen/UV and analyzed by electron microscopy, revealed formation of lasso-like structures referred to as telomeric loops or t-loops (Griffith et al., 1999). T-loop formation is accomplished through strand invasion of the 3' overhang into the duplex part of the telomere. It was shown that a TTAGGG-3' overhang of at least 6 nucleotides is sufficient for t-loop formation (Stansel et al., 2001). T-loop formation is assumed to be important in sequestration of the chromosome ends. Closed terminal loops were also observed under electron microscopy in isolated telomeric chromatin fibers covered by nucleosomes, in both chicken and mice (Nikitina and Woodcock, 2004). The t-loops may be conserved structures, as they have been

detected on chromosomes from a wide range of organisms such as humans, mice, ciliates, trypanosomes and peas (Wei and Price, 2003).

The end replication problem

Telomeres are replicated by the conventional DNA replication machinery. The ability of DNA polymerase to replicate only in 5' to 3' direction, and the additional nucleolytic processing on the 5' end of the leading telomere, results in the so-called end-replication problem (Lingner et al., 1995). During the replication fork progression at telomeres, one daughter telomere is replicated by the leading strand synthesis that creates a blunt ended telomere. To produce a 3' overhang this telomere is additionally eroded by an unknown nuclease. The other daughter telomere is replicated by the lagging strand synthesis, where - after the removal of the last RNA primer - and additional processing, a lagging telomere with a 3' overhang is created. These replication and processing events cause telomere loss with each cell division and are the molecular basis of the end replication problem. When telomeres reach a critical length they lose their capping function and trigger a DNA damage response at chromosome ends (d'Adda di Fagagna et al., 2003). The end replication problem can be buffered by the action of telomerase (see below).

Discovery of telomerase

In 1985, Carol Greider and Elizabeth Blackburn first detected telomerase activity in cell free extracts of the holotrichous ciliate *Tetrahymena thermophila* (Greider and Blackburn, 1985). Later, it was demonstrated that the telomerase activity co-purifies with an RNA moiety (Greider and Blackburn, 1987) which specifies the telomeric repeat sequence (Greider and Blackburn, 1989; Yu et al., 1990). Telomerase genes were first identified through genetic analysis in yeast (Lendvay et al., 1996) and later telomerase was purified from the hypotrichus ciliate *Euplotes aediculatus* (Lingner and Cech, 1996). The telomerase reverse transcriptase (TERT) protein is

phylogenically closely related to reverse transcriptases encoded by non-LTR retrotransposons and group II introns (Lingner et al., 1997b). TERT orthologs are found in most organisms as telomerase belongs among the most ancient eukaryotic reverse transcriptases (Eickbush, 1997; Nakamura and Cech, 1998). The human telomere terminal transferase activity was identified in crude HeLa cell extracts (Morin, 1989) and the RNA component (Feng et al., 1995) and the catalytic subunit (Kilian et al., 1997; Nakamura et al., 1997) were cloned.

Telomerase structure and mechanism of action

The catalytic core of human telomerase is composed of two subunits: the human telomerase reverse transcriptase (hTERT) and the human telomerase RNA component (hTR or TERC). The telomerase RNA contains the single-stranded RNA template that is flanked 5' by a long-range base-pairing boundary element and 3' by a conserved pseudoknot domain required for stable association with TERT (Chen et al., 2000). Outside of these two conserved regions TR sequence and structure is highly divergent probably due to the different telomerase-associating factors that bind mostly towards its 3' end. The 3' end of hTR contains a Cajal body localization sequence and an H/ACA motif characteristic for small nucleolar RNAs (snoRNAs) (Mitchell et al., 1999a). TERT protein has seven defined reverse transcriptase domains in its carboxy-terminal half. In the amino-terminal half there are two TR binding domains RID1 and RID2. RID2 interacts with the CR4-CR5 domain of TR whereas RID1 binds the pseudoknot-template region of TR (Mitchell and Collins, 2000; Moriarty et al., 2004).

Telomerase action begins with alignment of its RNA template with the 3' end of telomeres. The RNA template is approximately 1.5-2 times of telomeric repeat length and allows annealing of the 3' overhang and its subsequent elongation. One nucleotide is added at a time until the end of the RNA template is reached. Nucleotide addition processivity refers to non-dissociation of telomerase from the substrate during addition of a single telomeric repeat. Once the end of the RNA template is reached, the 3'

telomere end needs to translocate again and reanneal with the RNA moiety, for further elongation. For most telomerases the 3' telomere end remains bound to telomerase during this translocation step, and the probability of such event defines the repeat addition processivity of telomerase. The alignment, elongation and translocation events during telomerase action give rise to the repetitive nature of telomere DNA.

Human telomere maintenance by telomerase

The proper function of telomeres relies on their intact structure and their telomere length reservoir. Telomerase activity is crucial for telomere length homeostasis, which in turn provides a tumour suppression mechanism and at the same time prevents premature cellular senescence. In the absence of telomerase, telomeres of cultured human cells shorten with approximately 50-150 bp per cell division cycle (Harley et al., 1990). Ectopic expression of TERT induces telomere elongation in normal cells and at the same time extends their normal life span (Bodnar et al., 1998). In normal human cells, hTERT is ubiquitously expressed only during the first weeks of embryogenesis and subsequently downregulated in most cell types after 12 to 21 weeks (Ulaner and Giudice, 1997). Besides in germ cells, telomerase activity is detectable in both embryonic and adult stem cell compartments such as the intestine, hair follicle, skin and bone marrow (Forsyth et al., 2002; Wright et al., 1996). The hTR subunit and other telomerase-associated proteins are ubiquitously expressed and are often considered as housekeeping genes (Feng et al., 1995). Telomerase becomes reactivated in 90% of tumors (Kim et al., 1994), where it maintains short but stable telomeres. hTERT expression is regulated by multiple tumour suppressor pathways including Mad/c-Myc and TGFβ (Lin and Elledge, 2003). However, the mechanisms that lead to activation of hTERT in tumors have not been elucidated. The essential role of telomerase in telomere length maintenance and long-term survival of highly proliferative tissues is demonstrated by studies in the telomerase knockout mice (Blasco et al., 1997; Lee et al., 1998; Liu et al., 2000).

Telomerase concentration limits telomere length in human cells (Cristofari and Lingner, 2006). Increasing human telomerase activity in primary and cancer human cells increases its association to telomeres causing telomere elongation in a length-independent manner far beyond the physiological size of telomeres. Thus, even long telomeres are extendible. Another study in human cells showed that upon TERT expression short telomeres are gradually elongated during initial population doublings, whereas longer telomeres displayed limited elongation at the same time (Britt-Compton et al., 2009). On the contrary, in cancer cells, human telomerase extended most chromosome ends during each S-phase and was not preferentially recruited to the shortest telomeres (Zhao et al., 2009). The current model of telomere length homeostasis, based on studies in yeast, proposes that the transition between non-extendible and extendible telomeres is telomere length dependent (Teixeira et al., 2004). In addition, studies in human cells expand this model proposing that the transition between extendible and extending telomeres is depended on telomerase concentration (Cristofari and Lingner, 2006). In conclusion, a low cellular concentration of telomerase is critical to achieve preferential elongation of short telomeres and telomere length homeostasis.

Cell-cycle regulation of human telomerase

In humans, semi-conservative DNA replication of telomeres occurs throughout S-phase, rather than in late S-phase as in budding yeast (Wright et al., 1999). The *in vitro* activity of telomerase was suggested to increase in S-phase as well (Zhu et al., 1996). In another study, measurements of telomerase *in vitro* activity did not reveal cell cycle dependent changes in the amount of extracted telomerase activity (Holt et al., 1997). *In vivo*, it was shown that telomeres become more accessible for telomerase during S-phase, when they loss part of their protective structure and are recognized as DNA damage sites (Verdun and Karlseder, 2006). Cytological analyses of human telomerase recruitment have revealed S-phase-specific assembly of telomerase to

telomeres (Cristofari et al., 2007; Jady et al., 2006; Tomlinson et al., 2006). Throughout interphase, hTR localizes to Cajal bodies, dynamic structures involved in the biogenesis of small RNPs (Jady et al., 2006). hTR localization to Cajal bodies requires a short sequence motif called CAB box. hTR CAB box mutants are fully functional in forming catalytically active telomerase *in vivo*, but they impair the recruitmet of telomerase to telomeres. Another telomerase subunit, telomerase Cajal body protein 1, TCAB1 was identified to facilitate hTR association with Cajal bodies (Venteicher et al., 2009). TCAB1 was found enriched in Cajal bodies, where it associates with active telomerase to further promote telomere synthesis by telomerase. hTERT on the other hand was found to localize in nucleoplasmic foci of unknown composition (Zhu et al., 2004). In addition, trafficking of hTR to Cajal bodies and telomeres depends on hTERT (Tomlinson et al., 2008). Early in S-phase, Cajal bodies containing hTR are present at the periphery of nucleoli while hTERT becomes nucleolar. During S-phase, hTERT and hTR both colocalize to foci adjacent to Cajal bodies. Some Cajal bodies, hTERT and hTR are also found to associate with telomeres during S-phase. These experiments suggest the cell cycle-dependent assembly of active telomerase and its association with telomeres.

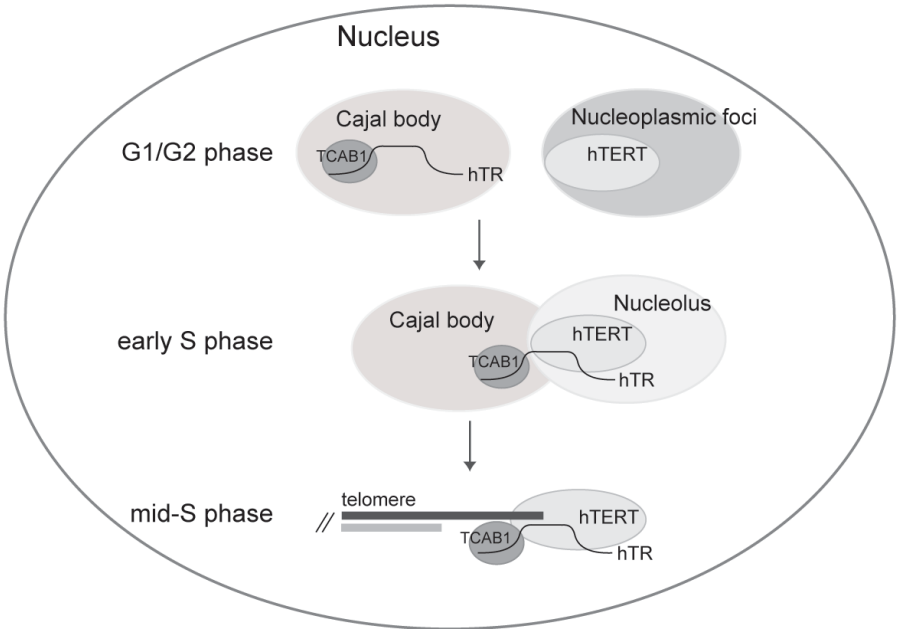


Figure 1. Current model for cell cycle-regulated trafficking of human telomerase (Jady et al., 2006; Tomlinson et al., 2008; Yang et al., 2002)

Human telomere maintenance by telomeric proteins

Telomeric DNA is coated with six abundant proteins which form the so-called shelterin complex: TRF1, TRF2, TIN2, TPP1, POT1 and RAP1. Shelterin polypeptides are present as high molecular weight complexes in fractionated nuclear extracts (Liu et al., 2004a; Ye et al., 2004a). Shelterin is essential for telomere capping and it regulates telomere length. In the next section, I will introduce the function of each of the shelterin components.

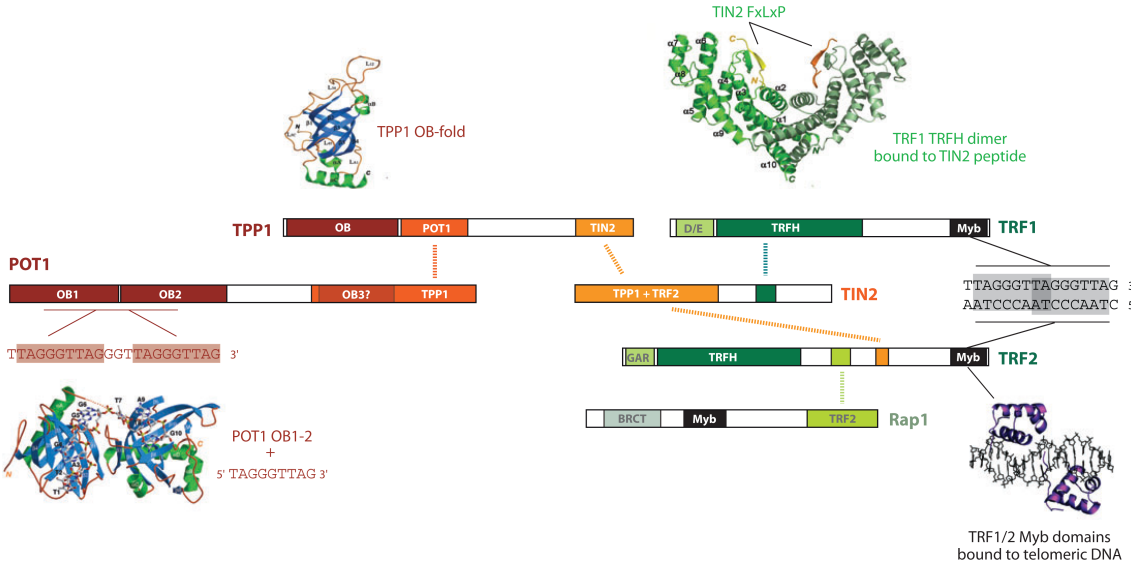


Figure 2. Shelterin complex: domain structures and interactions among the six proteins of human shelterin complex. Domains whose structures have been solved are presented. (Adapted from Palm and de Lange, 2008)

TRF1 (TTAGGG-repeat-binding factor 1)

The TRF1 protein was first identified in HeLa cell nuclear extracts due to its DNA-binding affinity specifically towards duplex TTAGGG repeats (Zhong et al., 1992). This 439-amino-acid protein has an amino-terminal acidic domain, a TRF homology domain (TRFH), and a carboxy-terminal SANT/Myb domain (Bianchi et al., 1997; Chong et al., 1995). TRF1 was found to dimerize via its TRFH domain, and the dimer was the preferable form for its DNA-binding activity (Bianchi et al., 1997). The SANT/Myb domain is the DNA-binding domain. Immunofluorescence and ChIP data

identified TRF1 as telomere binding protein throughout the cell cycle (Chong et al., 1995; Scherthan et al., 2000; van Steensel and de Lange, 1997). Alternative splicing generates two forms of functionally identical, closely migrating TRF1 proteins that differ by 20 amino acids in their linker region (van Steensel and de Lange, 1997). Initial functional analysis in telomerase-positive human cells characterized TRF1 as a negative regulator of telomere length. Long-term ectopic overexpression of TRF1 resulted in gradual and progressive telomere shortening. Expression of a dominant negative TRF1 mutant, that inhibited binding of endogenous TRF1 to telomeres, induced telomere elongation (Smogorzewska et al., 2000; van Steensel and de Lange, 1997). In mice, targeted deletion of *Trf1* gave an embryonic lethal phenotype and no evidence of telomere deprotection (Karlseder et al., 2003), whereas conditional *Trf1*-null mice exhibited telomere uncapping together with reduced TRF2 and TIN2 levels at telomeres (Iwano et al., 2004). Recent studies implicate TRF1 in mediating sister telomere association in human cells, via a novel connection between the cohesin and telomeric chromatin (Canudas et al., 2007). TRF1 has also a role in telomere replication, promoting efficient replication of TTAGGG repeats and preventing replication fork stalling (Sfeir et al., 2009).

TRF2 (TTAGGG-repeat-binding factor 2)

Cloning of TRF2 revealed homology to the Myb-related DNA-binding domain of TRF1 (Broccoli et al., 1997). Like TRF1, TRF2 as a homodimer, binds specifically duplex TTAGGG repeats via its carboxy-terminal SANT/Myb motif, and localizes to telomeres *in vivo*. In addition, TRF2 can bind to interstitial telomeric repeat-related sequences (Smogorzewska et al., 2000). The 500-amino-acid protein was shown to have a similar architecture as TRF1 in that it has a large TRFH domain close to its amino-terminus. However, unlike TRF1, the amino terminus in TRF2 is rather basic and rich in glycine and arginine residues (GAR domain). Furthermore, the TRFH domains of TRF1 and TRF2 do not interact suggesting that these proteins exist

predominantly as homodimers or oligomers formed through homotypic interactions in the TRFH domain (Broccoli et al., 1997). TRF2 has tendency to remodel telomeric DNA into t-loop structures *in vitro*, as shown by electron microscopy (Griffith et al., 1999). Within the t-loop structure, TRF2 preferentially localizes to the junction between the duplex repeats and the single-stranded overhang (Stansel et al., 2001). In human cells, RNAi mediated reduction of TRF2 triggers gradual and progressive telomere elongation (Takai et al., 2010). *Trf2*-null mice are embryonic lethal, and conditional *Trf2* deletion in p53 null mouse embryonic fibroblasts gave rise to telomere DNA damage (Celli and de Lange, 2005). Thus TRF2, as TRF1, is an essential gene with a protective function at telomeres.

TIN2 (TRF1-interacting nuclear protein)

TIN2 was discovered by interaction cloning using TRF1 as a bait in a yeast-two hybrid screen (Kim et al., 1999). This 354-amino-acid protein binds the TRFH domain of TRF1 with its central region (amino acids 195-284) (Kim et al., 1999). Later studies revealed TIN2 interaction with TRF2 and TPP1 (Houghtaling et al., 2004; Liu et al., 2004a; Liu et al., 2004b; Ye et al., 2004a; Ye et al., 2004b). However, TIN2 does not bind TRF2 within its TRFH domain, but within a more central region of TRF2 (Chen et al., 2008). The amino-terminal half of TIN2 (amino acids 1-195) is a TRF2 and TPP1 binding domain (Houghtaling et al., 2004). It was shown that TIN2 binds TRF1 and TRF2 simultaneously and stabilizes TRF2 at telomeres. Consequently, disrupting the TRF1-TIN2-TRF2 interaction led to telomere deprotection (Kim et al., 2004). Expressing TIN2 mutant, lacking the amino-terminal part, elongates telomeres in a telomerase-dependent manner (Kim et al., 1999). In addition, TIN2 depletion by RNAi leads to telomere elongation (Ye and de Lange, 2004), defining TIN2 as a negative regulator of telomere length. TIN2, together with TRF1, was implicated to play a role in telomere cohesion (Canudas et al., 2007). More precisely, the TIN2 depletion phenotype resembles cohesin (SA1) depletion, and results in loss of sister-telomere

cohesion after DNA replication in S-phase, with dramatic consequences on chromosome morphology and function (Canudas and Smith, 2009). In mice, TIN2 inactivation is also embryonic lethal and not rescued by inactivation of the *Tert* gene (Chiang et al., 2004).

RAP1 (Repressor activator protein 1)

The RAP1 protein emerged as a TRF2-interacting protein in a yeast-two hybrid screen (Li et al., 2000). RAP1 is 399-amino-acid protein with an amino-terminal BRCT domain, a central Myb domain, followed by a predicted coiled domain, a carboxy-terminal RCT domain homologous to the carboxy-terminus of budding yeast Rap1 protein, and a carboxy-terminal nuclear localization signal. The RCT domain mediates TRF2 interaction, and within the heterodimer the two proteins bind in a 1:1 stoichiometry. RAP1 binds to telomeres solely via protein interaction with TRF2, whereas its Myb domain does not have an overall positive charge for DNA binding. RNAi depletion of endogenous RAP1 resulted in longer telomeres (Li et al., 2000; O'Connor et al., 2004). Telomere length phenotypes of RAP1 deletion mutants implicated both the Myb domain and the BRCT domain in telomere length regulation, in particular expression of the BRCT lacking mutant diminished telomere length heterogeneity (Li and de Lange, 2003). RAP1 prevents non-homologous end joining at telomeres in a TRF2-independent manner, despite presence of DNA damage in TRF2-deficient cells (Sarthy et al., 2009). Mice lacking RAP1 are viable and RAP1 is dispensable for TRF2 function in telomere protection, however required to repress telomere homology-directed repair and recombination (Martinez et al., 2010; Sfeir et al., 2010). In mice, RAP1 also binds to telomeric repeats enriched in the subtelomeric regions, mostly in the vicinity of genes and a subset of these genes are deregulated in *Rap1*-null cells, implicating RAP1 in transcriptional regulation (Martinez et al., 2010).

TPP1 (PTOP/PIP1/TINT1)

TPP1 is the last identified protein of the shelterin complex. It emerged as a TIN2-interacting factor in a yeast two hybrid screen and it was also isolated by biochemical purification (TPP1 was previously referred to as TINT1 (Houghtaling et al., 2004), PTOP1 (Liu et al., 2004b)) and PIP1 (Ye et al., 2004b)). TPP1 interacts with TIN2 through its carboxy-terminal, TIN2 interaction domain and with POT1 through its centrally located, POT1 recruitment domain (Houghtaling et al., 2004; Liu et al., 2004b). In between these two domains lies a serine rich domain of unknown function. Sequence analyses and solving the crystal structure of the amino-terminal domain of TPP1 revealed an oligonucleotide/oligosaccharide-binding fold domain. The OB-fold domain of TPP1 is structurally similar to the beta-subunit of the telomere end-binding protein (TEBPβ) of a ciliated protozoan, suggesting that TPP1 may correspond to the missing beta-subunit of the human POT1 protein (Wang et al., 2007; Xin et al., 2007a). Several *in vitro* studies have provided evidence for TPP1 interaction with telomerase. The OB-fold domain of TPP1 pulls down telomerase activity *in vitro* (Xin et al., 2007a) and POT1-TPP1 dimers increase telomerase processivity and translocation efficiency on telomeric substrates (Latrick and Cech, 2010; Zaug et al., 2010). TPP1 is important for recruitment of POT1 to telomeres through its interaction with TIN2. Silencing of TPP1 by RNAi, or overexpression of TPP1 mutants defective in POT1 binding cause telomere elongation and telomere deprotection, phenotypes consistent with POT1 loss (Houghtaling et al., 2004; Liu et al., 2004b; Xin et al., 2007a; Ye et al., 2004b). Furthermore, the TIN2-TPP1 interaction is important for the subcellular localization of POT1 and TPP1. TPP1 contains a nuclear export signal that controls the amount of nuclear and cytoplasmic TPP1 and POT1, and binding of TIN2 to TPP1 promotes nuclear localization of both TPP1 and POT1 (Chen et al., 2007). The splice defects in the *Tpp1* gene from the ACD (adrenocortical dysplasia) mice, give rise to homologous and nonhomologous chromosome fusions, confirming the telomere protection role of

TPP1 *in vivo* (Else et al., 2007; Keegan et al., 2005). Conditional deletion of *Tpp1* from mouse embryo fibroblasts, removes POT1 proteins from telomeres giving rise to similar telomere dysfunction phenotypes as the double knockout *Pot1a/b* mice (Kibe et al., 2010).

POT1 (Protection of telomeres 1)

The identification of human POT1 emerged through its homology to the alpha-subunit of the telomere end-binding protein (TEBPα) in *Oxytricha nova* (Baumann and Cech, 2001). Similarly to the TEBPα, POT1 contains two OB-fold domains in its amino-terminal part with which it can recognize telomeric G-rich overhangs *in vitro* (Baumann and Cech, 2001; Lei et al., 2004). Solving the crystal structure of the N-terminal half of POT1 bound to telomeric single-stranded DNA, and *in vitro* DNA binding assays, indicate that a decamer of 5'-TTAGGGTTAG-3' is sufficient for POT1 binding and that POT1 has a strong preference for telomeric repeats either at the 3' overhang or within a longer single stranded region (Lei et al., 2004; Loayza et al., 2004). The carboxy-terminal half of POT1 binds to TPP1 and this interaction is important for POT1 loading on telomeres, whereas its OB-fold domains are indispensable for POT1 telomere localization (Liu et al., 2004b; Loayza and De Lange, 2003). ChIP against POT1 revealed more POT1 protein on longer telomeres, probably bound to the other shelterin components along the double-stranded part of telomeres. Human POT1 has crucial functions in both telomere length homeostasis and telomere protection. POT1 acts as transducer of telomere length control, and diminished POT1 levels or expression of POT1 mutants lacking the DNA-binding domain induces telomere elongation (Liu et al., 2004b; Loayza and De Lange, 2003; Ye et al., 2004b). Partial depletion of POT1 causes telomere deprotection and alteration of both 3' and 5' ends of telomeres (Hockemeyer et al., 2005; Yang et al., 2005). Mice encode for two POT1 proteins, POT1a and POT1b, which are nearly identical in sequence. Both associate with telomeres, however they differ in their function. Conditional double knockout of *Pot1a/b*

caused telomere DNA damage, endoreduplication and senescence; whereas single knockouts identified POT1a as a suppressor of DNA damage, and POT1b as a regulator of the 3'overhang length (Hockemeyer et al., 2006).

Telomere protection by shelterin

First indications for the requirement of the shelterin complex in telomere protection came from studies in TRF2 depleted human cells and cells expressing $TRF2^{\Delta B \Delta M}$, a dominant negative mutant that can not bind DNA. In these cells telomeres undergo telomeric fusions that correlate with the loss of G-strand overhang, implying that telomeric DNA *per se* is not sufficient for telomere protection (van Steensel et al., 1998). However, telomere degradation is not the trigger for the DNA damage response. Telomeres in TRF2, p53 and DNA ligase IV deficient mice retain their 3' overhangs and are recognized as DNA damage sites (Celli and de Lange, 2005). Dysfunctional telomeres created by TRF2 inhibition accumulate DNA damage response factors, such as 53BP1, gamma-H2AX, Rad17, and Mre11. ATM kinase is the major transducer of the damage signal in TRF2 deficient cells (Denchi and de Lange, 2007) leading to p53 mediated apoptosis or senescence (Karlseder et al., 1999). Telomere domains coated with DNA damage factors are referred to as telomere dysfunction-induced foci (TIFs) (Takai et al., 2003). TRF2 may suppress ATM damage response at telomeres either by inducing t-loop formation that renders telomeres inaccessible or by binding ATM kinase itself and preventing its activation (Karlseder et al., 2004).

TIF formation is also a prominent phenotype of POT1 inhibition, but mostly limited to G1 phase (Hockemeyer et al., 2005). In contrast, TRF2 induced TIFs are seen throughout interphase. POT1 induced TIF formation is diminished when ATR, but not ATM, signalling is impeded, suggesting ATR as the major transducer of the DNA damage pathway (Denchi and de Lange, 2007). In mice, the two POT1 paralogs function differently in repressing the ATR kinase pathway (Hockemeyer et al., 2006; Wu et al., 2006). *Pot1a* conditional deletion elicits telomere sister chromatid exchanges

and formation of telomere circles followed by p53-dependent replicative senescence. POT1b regulates the amount of single-stranded DNA at the telomere terminus, which is the substrate for RPA binding and ATR activation. However, POT1a by itself, its sufficient to prevent ATR signalling at telomeres, whereas POT1b has a relatively minor role in ATR repression (Hockemeyer et al., 2006).

TPP1 is required for the protective function of POT1, as POT1 alleles not able to bind TPP1, are incapable of repressing the DNA damage response (Hockemeyer et al., 2007; Kibe et al., 2010). TPP1 not only recruits POT1 to telomeres but *in vitro* increases POT1 affinity for single-stranded DNA (Wang et al., 2007), two features relevant for ATR repression at telomeres. TPP1 inhibition causes DNA damage at telomeres indistinguishable from the POT1 inhibition phenotype (Denchi and de Lange, 2007; Hockemeyer et al., 2007). Similarly, TIN2 mutants defective in binding to both TRF1 and TRF2 induce TIFs at telomeres and destabilize TRF2 at human telomeres (Kim et al., 2004). TRF1 inhibition also leads to TIF formation and loss of TIN2 association to telomeres (Iwano et al., 2004). In conclusion disrupting the telomere structure, either by depletion or overexpression of dominant negative shelterin components leads to telomere deprotection and TIF formation.

Telomere length control by shelterin

As constitutive part of the telomeres, shelterin components play crucial roles in telomere length maintenance. Telomere length homeostasis is established by a negative feedback loop, where the shelterin complex binds telomeres and inhibits the action of telomerase. As telomeres become elongated, they bind more shelterin complexes and prevent telomerase action. Initial studies in human cell lines confirmed this mechanism, as depletion or dominant-negative overexpression of TRF1 leads to telomere elongation or shortening respectively (van Steensel and de Lange, 1997). The amount of TRF1 increases as telomeres elongate, establishing TRF1 as a telomere-length counting protein, a concept previously reported in yeast (Marcand et

al., 1997). Furthermore, direct tethering of TRF1 to specific telomeres, in telomerase-positive cells, induces telomere shortening, suggesting that TRF1 acts in cis to inhibit telomerase mediated elongation (Ancelin et al., 2002). TRF2 acts similarly to TRF1, since TRF2 levels increase when telomeres are elongating and its overexpression leads to telomere shortening (Smogorzewska et al., 2000). RAP1 depletion results in telomere elongation, a phenotype characteristic for its full-length overexpression or mutant lacking the TRF2 binding domain, both acting as dominant negative mutants (O'Connor et al., 2004). Interestingly, RAP1 alleles lacking the N-terminal BRCT domain result in a more homogenous telomere length (Li and de Lange, 2003). TIN2 and TPP1 behave similarly as negative regulators of telomere length (Houghtaling et al., 2004; Kim et al., 1999; Liu et al., 2004b; Ye et al., 2004b). POT1 being the only shelterin that binds the substrate of telomerase is the crucial negative regulator of telomere length. A POT1 mutant lacking the OB1-fold domain induces rapid telomere lengthening, while the other shelterin components remain intact on telomeres, suggesting that POT1 acts downstream as terminal transducer of telomere length control by shelterin (Loayza and De Lange, 2003). POT1 binds the 3' overhang and inhibits telomerase activity *in vitro* (Kelleher et al., 2005; Lei et al., 2005). However, if POT1 binds one telomeric repeat before the 3' end, telomerase can act on its substrate with improved processivity (Lei et al., 2005). Consistent with this observation, overexpression of POT1 leads to telomerase-dependent elongation (Colgin et al., 2003). The role of POT1 in aiding telomerase action at telomeres is emphasized with more *in vitro* data, where POT1 in a complex together with TPP1 stimulates highly processive extension of telomerase, and increases its translocation efficiency (Latrick and Cech, 2010; Wang et al., 2007). From these studies a positive role of TPP1 in telomerase regulation emerged, supported further by TPP1 pull down of telomerase activity and the sequence specific TPP1-telomerase interaction necessary for telomerase high-processivity *in vitro* (Xin et al., 2007a; Zaug et al., 2010).

Outcome of telomere dysfunction

In 1961, Hayflick demonstrated that normal human cells derived from embryonic tissues have limited replicative life span and senesce after approximately 50 to 70 divisions (Hayflick and Moorhead, 1961). The correlation between the replicative senescence and telomere length was demonstrated in cultured human fibroblasts. Namely, their telomere length decreased as a function of serial passage during aging *in vitro* (Harley et al., 1990). It was proposed that finite doubling capacity of these cells is due to loss of telomeric DNA, since critically short telomeres cease to function as protective units and cause cell death. More evidence that telomeres are rate-limiting for indefinitive cell proliferation, emerged when hTERT was introduced into normal telomerase-negative cells (Bodnar et al., 1998). The cells that expressed *de novo* transfected telomerase continued to grow in normal manner with indefinite life span, however not developing any malignant transformation (Jiang et al., 1999; Morales et al., 1999). Thus, our telomeres direct cellular aging and delay early entry into replicative senescence. In humans, telomerase maintains the telomere length reservoir and the indefinite life span in the totipotent, embryonic stem cells and the germ-line cells (Wright et al., 1996). Rapidly dividing pluripotent cells of highly regenerative tissues, such as those of the immune system, skin, and intestine, express telomerase activity, however they exhibit progressive telomere shortening throughout life (Forsyth et al., 2002). Finally, in most adult tissues telomerase activity is greatly reduced or transcriptionally silenced causing telomere shortening and accumulation of senescent cells with advance aging (Dimri et al., 1995). In addition, cells derived from individuals with premature aging syndromes and short telomeres, have diminished *in vitro* replicative potential (Faragher et al., 1993). The mTR-/- aging mice with shortened telomeres also exhibit reduced life span and a range of aging phenotypes, as compared to age-matched early generation mTR-/- mice (Rudolph et al., 1999). These findings confirm the link between replicative senescence, telomere length and aging.

On the other side, telomere shortening and the onset of replicative senescence present a powerful tumor suppressor mechanism. Cells with critically short telomeres activate the DNA damage response and enter apoptosis and/or senescence. Cells that continue to divide past their normal replicative limit lose the remaining telomeric DNA and undergo chromosome end-to-end fusions, the so-called breakage-fusion-bridge cycle. This triggers the second proliferative block called crisis. Crisis is defined by massive genomic instability and cell death. However, rare transformed clones emerge from crisis and upregulate telomerase activity to maintain short but stable telomeres. Indeed, many precancerous tissues have critically shortened telomeres prior to telomerase detection (Kitada et al., 1995; O'Sullivan et al., 2002; Wu et al., 2003). Telomerase is reactivated in more than 90% of cancers (Kim et al., 1994) and human cancer cells have shorter telomeres than their normal tissue counterparts (Hastie et al., 1990). In addition, a genetic screen identified tumor suppressor/oncogene pathways, such as Mad1/c-Myc, TGFβ and tumor suppressor Menin, to coordinately repress hTERT expression (Lin and Elledge, 2003). Thus, telomerase is a rate-limiting enzyme that overcomes the growth limitation due to telomere loss, which occurs in most cancers.

However telomerase-deficient aged mice, unexpectedly show an increase in age-dependent cancers (Rudolph et al., 1999). At the same, time mice with long and functional telomeres but deficient for either *Ink4a/Arf* or *p53* tumor suppressors are highly cancer prone. When telomerase is removed from *Ink4a/Arf* deficient mice, there is a marked decrease in cancer incidence (Greenberg et al., 1999). On the other hand, telomerase and *p53* deficient mice display dramatic increase in cancer incidence (Chin et al., 1999). These findings suggest that the status of tumor suppressor pathways determines whether telomere dysfunction will suppress or enhance tumorigenesis. Taken together telomerase deficiency and chromosome instability can either repress cancer formation in presence of intact DNA damage response, or they can facilitate the emerging of nascent cancer cells in the absence of functional checkpoint pathways.

There are several human telomerase and telomere deficiency disorders that are characterized with telomere shortening and multisystem abnormalities including bone marrow failure and features of premature aging. These include classical dyskeratosis congenita, aplastic anemia, myelodisplasia, Hoyeraal-Hreidarsson syndrome and idiopathic pulmonary fibrosis (Walne and Dokal, 2009).

The first disease-associated mutation in telomerase was identified in dyskeratosis congenita (DC) patients. Clinical manifestations of classical DC appear during childhood and include mucocutaneous triad of abnormal skin pigmentation, nail dystrophy and mucosal leukoplakia. Bone marrow failure usually develops in the second decade of life but it is the principal cause of mortality, followed by pulmonary complications and malignancy (Dokal, 2000). DC is a genetically heterogeneous disorder with several subtypes: X-linked recessive, autosomal dominant and autosomal recessive inheritance. X-linked DC is caused by mutations in the *DKC1* gene located on Xq28, which encodes dyskerin protein (Heiss et al., 1998; Knight et al., 1999). Dyskerin is a highly conserved nucleolar protein that interacts with GAR1, NHP2 and NOP10 to form the core of H/ACA ribonucleoproteins (Pogacic et al., 2000). This complex also interacts with hTR, which contains an H/ACA motif (Mitchell et al., 1999b). hTR processing and stability depends on dyskerin binding to its H/ACA motif, consequently patients with X-linked DC have reduced hTR levels, but also reduced telomerase activity and accelerated telomere shortening (Mitchell et al., 1999b; Wong and Collins, 2006). Mutations in NHP2 and NOP10 are described in autosomal recessive DC patients with a similar telomere phenotype as X-linked DC, showing significant telomere shortening and low hTR levels (Vulliamy et al., 2008; Walne et al., 2007). TCAB1 mutations that result with mislocalization of the telomerase complex were identified in patients with autosomal recessive DC (Zhong et al., 2011). Autosomal dominant DC is associated with mutations in hTR and hTERT (Armanios et al., 2005; Vulliamy et al., 2001). hTERT mutations lead to haploinsufficiency of telomerase and telomere shortening. hTR mutations cause either impaired hTR

stability or a catalytic defect in telomerase, both resulting in a short telomere phenotype. Experiments reconstituting telomerase with mutant hTR molecules suggest that hTR-DC mutations act via haploinsufficiency rather than by a dominant negative mechanism (Fu and Collins, 2003; Marrone et al., 2004). Multiple heterozygous mutations in the shelterin component TIN2 are implicated in autosomal dominant DC (Savage et al., 2008; Walne et al., 2008). Patients with TIN2 mutations have by far the most sever DC phenotypes, with very short telomeres, but stable hTR levels.

Hoyeraal-Hreidarsson syndrome (HH) is a severe multisystem disorder with similar features as DC, causing severe growth retardation, bone marrow failure, immunodeficiency and neurological abnormalities. Several mutations in the *DKC1* and *TERT* genes are identified in HH patients, as severe variants of DC, where death from bone marrow failure occurs before appearance of the diagnostic features of DC (Knight et al., 1999; Marrone et al., 2007b). hTR mutations are identified in patients with idiopathic aplastic anemia and myelodysplasia, diseases characterized with bone marrow failure and short telomere phenotype (Keith et al., 2004; Vulliamy et al., 2002; Vulliamy et al., 2004; Xin et al., 2007b; Yamaguchi et al., 2005). Idiopathic pulmonary fibrosis is a chronic, progressive and fatal disease defined by irreversible lung fibrosis and shortened telomeres, harboring heterozygous hTR and hTERT mutations (Alder et al., 2008; Armanios et al., 2007; Marrone et al., 2007a).

Mutations in the telomerase genes and the short telomere phenotype suggest a common molecular mechanism that underlies a range of clinical abnormalities. Thus, understanding the role of telomeres and telomerase in disease has important implication for their diagnosis and treatment. Primarily, some of the crucial aspects of human telomerase regulation need to be elucidated. In particular, it is still not clear how telomerase can bypass the presence of telomeric proteins and reach its substrate, the telomeric DNA, for further elongation. In the following presented work, I have established TPP1 and TIN2 among the shelterin proteins to regulate telomerase

recruitment to telomeres and further investigated their role in a form of dyskeratosis congenita caused by mutations in the *TINF2* gene.

Chapter II: TIN2-Tethered TPP1 Recruits Human Telomerase to Telomeres *In Vivo*

This chapter has been published as presented, except that here Figure 5 was added, in:

E. Abreu, *, E. Aritonovska*, et al. (2010). "TIN2-tethered TPP1 recruits human telomerase to telomeres in vivo." Mol Cell Biol **30**(12): 2971-2982 (2010)

*These authors contributed equally to this work

E. Aritonovska carried out the immunobloting, chromatin immunoprecipitation, coimmunoprecipitation, qRT-PCR, and RQ-TRAP experiments shown in Figure 4A to F, Figure 5, Figure 6, Figure 7A and B, Figure 8, Figure 10A to C, Figure 11 and Figure 12A and B.

Introduction

The physical ends of eukaryotic chromosomes, termed telomeres, are maintained by the cellular reverse transcriptase telomerase. Telomerase uses an internal RNA moiety as a template to add short telomeric repeats to the 3' ends of chromosomes (Greider and Blackburn, 1989; Lingner et al., 1997a). Telomeres protect chromosomes from nucleolytic degradation and inappropriate DNA repair reactions (Palm and de Lange, 2008). In humans, telomerase is developmentally regulated and is expressed primarily during the first weeks of embryogenesis (Cong et al., 2002). Later in life, most normal human somatic cells express only very low levels of telomerase, and telomeres shorten with continuous cell division cycles due to the end-replication problem and nucleolytic processing of chromosome ends. Upon reaching a critical length, short telomeres activate a DNA damage response that leads to a permanent cell cycle arrest or apoptosis (d'Adda di Fagagna et al., 2003). Reactivation of telomerase is a key requisite for human cancer cells to attain unlimited proliferation potential (Bodnar et al., 1998). Telomere shortening suppresses tumor formation, but at the same time, the telomere reserve must be long enough to allow tissue renewal by healthy cells during the entire life span (Lansdorp, 2009). Indeed, accelerated telomere shortening causes dyskeratosis congenita, a bone marrow failure syndrome that leads to premature death due to aplastic anemia (Vulliamy and Dokal, 2008). Telomere dysfunction has also been linked to the pathogenesis of idiopathic pulmonary fibrosis (Armanios et al., 2007), ICF syndrome (Yehezkel et al., 2008) and Werner syndrome (Crabbe et al., 2004).

The maturation and activity of telomerase depends on subcellular trafficking. A minimal, catalytically active telomerase enzyme (that can add telomeric repeats to the ends of DNA oligonucleotide substrates *in vitro*) can be formed by the telomerase reverse transcriptase (TERT) and the telomerase RNA moiety (TR) (Autexier et al., 1996; Weinrich et al., 1997). However, the human TERT (hTERT)-hTR core complex is

not competent for telomere elongation *in vivo*. Within cells, hTR accumulates in Cajal bodies (CBs), subnuclear structures that also contain the subset of box H/ACA pseudouridylation guide RNAs termed small CB-specific RNAs (scaRNAs), which modify snRNAs (Jady et al., 2004; Zhu et al., 2004). hTR accumulation in Cajal bodies is not needed for assembly of the catalytic core of telomerase, but is required to render telomerase competent for telomere association and extension *in vivo* (Cristofari et al., 2007; Venteicher et al., 2009). The telomerase holoenzyme subunit, TCAB1 (telomerase Cajal body factor 1) mediates the essential CB-localization step (Venteicher et al., 2009). Telomere synthesis occurs during S phase, and hTR localizes to telomeres specifically during this phase of the cell cycle (Jady et al., 2006; Tomlinson et al., 2006). However, factors that function in the recruitment of telomerase to telomeres are not known.

The six-component telomere capping complex termed shelterin is important for telomere length control *in vivo* (Palm and de Lange, 2008; Xin et al., 2008), suggesting potential roles for the complex in the regulation of telomerase access to telomeres. Interestingly (and perplexingly), current evidence suggests that shelterin components can both inhibit and stimulate telomere elongation. The six shelterin components are: TRF1, TRF2, RAP1, TIN2, POT1 and TPP1 (de Lange, 2005; Xin et al., 2008) (Figure 3A). The shelterin complex associates with the double-stranded region of the telomere through direct interactions of TRF1 and TRF2 with the DNA (Broccoli et al., 1997; de Lange, 2005). POT1 binds the single-stranded region of the telomere (Baumann and Cech, 2001; Lei et al., 2004). Depletion of TRF1 leads to telomere elongation, and overexpression of TRF1 causes telomere shortening in human telomerase-positive cells without affecting *in vitro*-assayed telomerase activity (Smogorzewska et al., 2000), suggesting that reinforcement of the shelterin complex inhibits telomerase function. Similarly, depletion of TPP1 by RNAi or disruption of the TPP1-POT1 interaction (which are both accompanied by loss of the POT1 signal at telomeres) also results in telomere lengthening (Liu et al., 2004b; Ye et al., 2004b). At the same time,

however, several findings support positive roles of shelterin in telomere length regulation. In particular, TPP1 together with POT1 has been shown to improve telomerase activity and processivity *in vitro* (Wang et al., 2007) by slowing primer dissociation and aiding telomerase translocation (Latrick and Cech). Dissection of the apparently opposing roles of the shelterin complex components in telomerase function awaits further investigation.

TPP1 has been hypothesized to play a role specifically in the recruitment of telomerase to telomeres based on its association with telomerase (Xin et al., 2007a). Xin et al. demonstrated that tandem affinity purification (TAP)-tagged hTERT and glutathione S-transferase (GST)-tagged TPP1 copurify when fractionated from cellular extracts derived from cells coexpressing the tagged proteins (Xin et al., 2007a). In addition, both GST-tagged TPP1 and the oligonucleotide/oligosaccharide binding fold (OB-fold) of TPP1 pull down *in vitro*-translated hemagglutinin (HA)-tagged TERT and telomerase activity (Xin et al., 2007a), indicating that the TPP1 OB-fold is important for association of TPP1 with telomerase. It is not clear whether the interaction between TPP1 and hTERT is direct. These studies did not examine recruitment of telomerase to telomeres. However, based on their findings, the authors speculated that TPP1 together with POT1 could play a role in positively (and negatively) regulating access of telomerase to telomeres (Xin et al., 2007a).

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

Results

TPP1 depletion results in loss of association of telomerase with telomeres

The recruitment of telomerase to telomeres is essential for telomere maintenance; however the mechanism of recruitment is not known. Association of telomerase with telomeres can be observed in cancer cells during S phase by FISH with oligonucleotide probes complementary to hTR or IF with hTERT antibodies (Cristofari et al., 2007; Jady et al., 2004; Jady et al., 2006; Tomlinson et al., 2008; Tomlinson et al., 2006; Venteicher et al., 2009; Zhu et al., 2004). Analysis of telomerase recruitment is facilitated by the use of so-called super-telomerase cells, which concomitantly over-express hTERT and hTR, allowing detection of telomerase association with telomeres by ChIP as well as by microscopy in all phases of the cell cycle (Cristofari et al., 2007; Cristofari and Lingner, 2006). In order to identify proteins that are necessary for accumulation of telomerase to telomeres, we depleted candidate recruitment factors using shRNAs in super-telomerase HeLa cells and examined the localization of telomerase by FISH and ChIP analysis. We found that shRNA-induced depletion of the shelterin component TPP1 (Figure 4A) caused a striking loss of telomerase localization to telomeres as assessed by FISH (Figure 3) and ChIP (Figure 6).

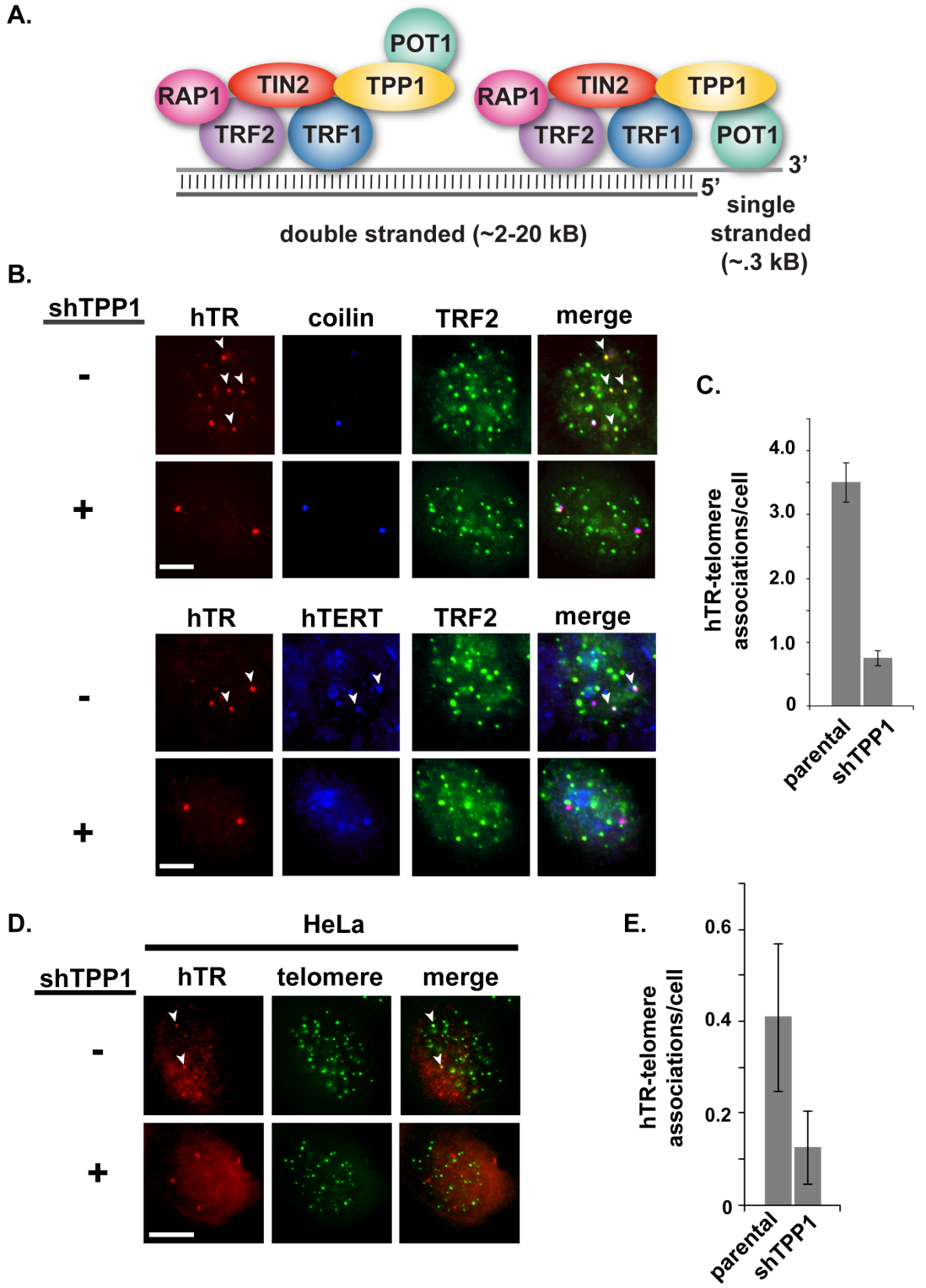


Figure 3. TPP1 depletion results in loss of localization of telomerase to telomeres (assessed by FISH). (***A***) Mammalian chromosome end structure is regulated by a complex of six core telomere-associated proteins (indicated) that make up the shelterin complex (de Lange, 2005; Xin et al., 2008). TPP1 is associated with the double-stranded and single-stranded portion of telomeres via direct interactions with TIN2 and POT1, respectively (de Lange, 2005; Xin et al., 2008). (***B***) Fluorescence *in situ* hybridization (FISH) was used to detect hTR (red), and immunofluorescence (IF) was used to detect TRF2 (telomere marker, green), and hTERT (blue) or coilin (Cajal body marker, blue) in parental (-) and TPP1-depleted (+) super-telomerase Hela

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

In untreated super-telomerase cells, hTR (visualized by FISH) is found at the Cajal bodies (visualized via the Cajal body marker protein coilin) and numerous telomeres (visualized via the telomere-binding protein TRF2) (Figure 3B, -shTPP1, a subset of the hTR-telomere colocalizations is indicated with arrowheads). Following TPP1 depletion, hTR remains at Cajal bodies, but is found only at very few telomeres (Figure 3B and 3C, +shTPP1). TPP1 depletion reduced the number of observed hTR-telomere colocalizations by 77%: from a mean of 3.5 ±0.3 (standard error of the mean (SEM)) colocalizations per cell (one focal plane) in parental super-telomerase cells to 0.8 ±0.1 (SEM) after TPP1 depletion (Figure 3C). hTERT localization to telomeres was also noticeably reduced by depletion of TPP1 (Figure 3B, lower panels; a subset of hTR-hTERT-telomere colocalizations is indicated with arrowheads). At the same time, progression through the cell cycle (assessed by percentage of cells found in S phase), cellular telomerase levels (assayed *in vitro* by RQ-TRAP) and hTERT protein levels (assayed by immunoblot analysis) were not affected by TPP1 depletion (Figure 4 and Figure 5).

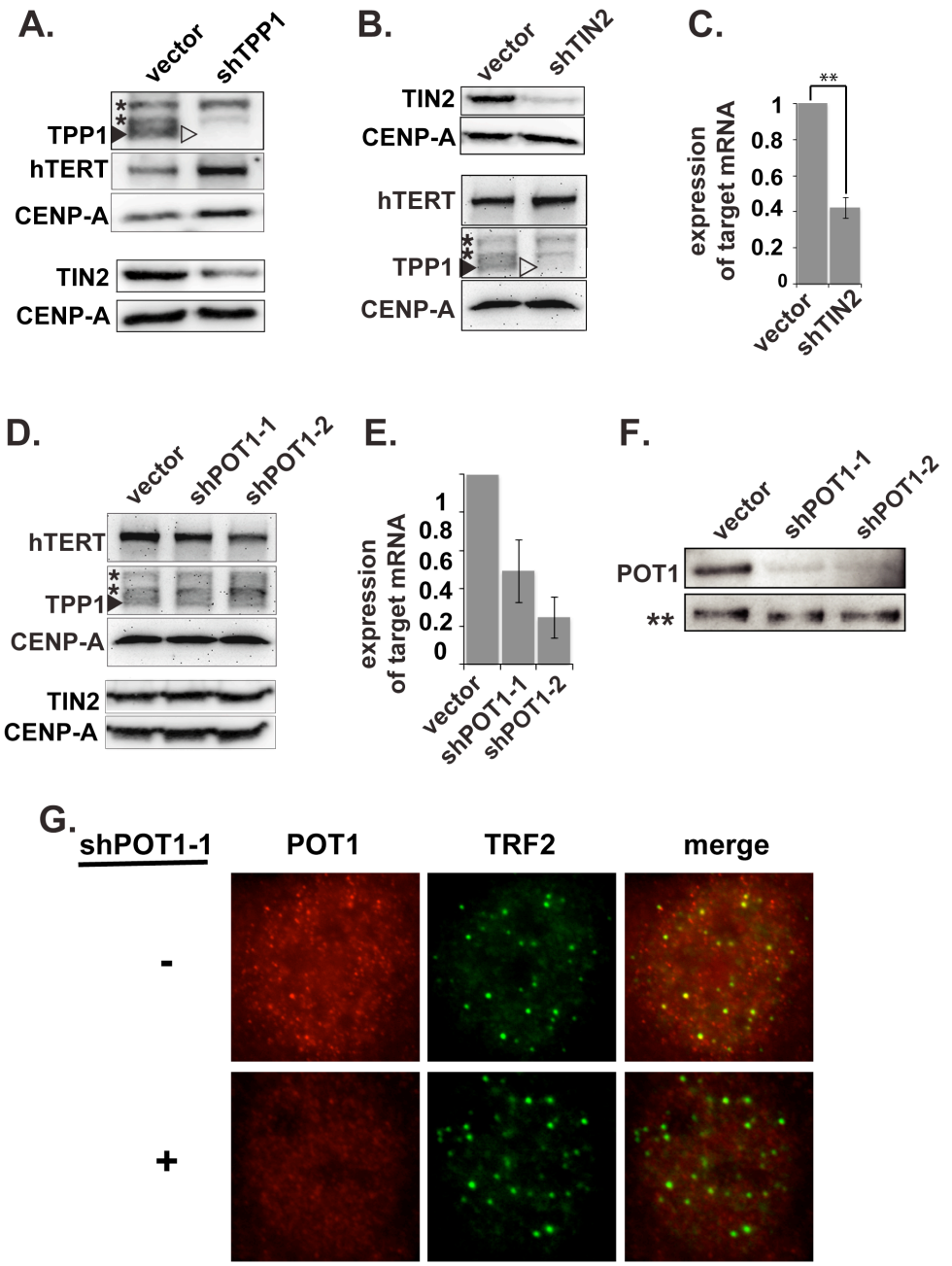


Figure 4. Depletion of TPP1, TIN2 and POT1. (***A***) Immunoblot analysis of TPP1-depleted cells. Super-telomerase HeLa cells were transfected with pSUPER-Puro (vector) or pSUPER-Puro-TPP1 shRNA vector (shTPP1). Four days after transfection TPP1, hTERT, TIN2 and CENP-A expression was analyzed by immunoblotting. The arrowheads indicate the position of endogenous TPP1 protein. Asterisks indicate nonspecific bands. CENP-A was used as loading control. (***B***) Immunoblot analysis of TIN2-depleted cells. Four days after transfection, protein expression was analyzed as described for panel A. (***C***) qRT-PCR analysis of TIN2-depleted cells. qRT-PCR detection of mRNA levels of TIN2 in super-telomerase HeLa cells, 4 days after transfection with TIN2-shRNA, relative to empty-vector control. Error bars correspond to standard deviations of results of three independent experiments. Statistical analyses were done using a two-tailed Student's *t* test (**, $P<0.01$). (***D***) Immunoblot analysis of POT1-depleted cells. Six days after transfection, protein expression was analyzed as described for panel A. (***E***) qRT-PCR analysis of POT1-depleted cells. qRT-PCR showing mRNA levels of POT1 in super-telomerase HeLa cells, 6 days after transfection with two different POT1-shRNAs, relative to empty-vector control. Error bars correspond to standard deviations of results of two independent experiments. (***F***) IP/immunoblot analysis of POT1-depleted cells. Coimmunoprecipitation of endogenous POT1 with TPP1 in POT1-depleted cells. TPP1-immunoprecipitated complexes

from super-telomerase HeLa cells transfected with the indicated plasmids were resolved by 8% SDS-PAGE. Immunoblot antibodies are indicated on the left. No detection of TPP1 was observed in the supernatant fraction after IP (data not shown). A nonspecific band recognized by the TPP1 antibody in the IP fraction (**) served as a loading control. (***G***) Direct IF analysis of POT1-depleted cells. POT1 (red) and TRF2 (green) were detected by IF in parental (-) and POT1-depleted cells.

These results suggest that TPP1 is necessary for the recruitment of telomerase from Cajal bodies to telomeres in super-telomerase cells. Importantly, TPP1 depletion results in a similar loss of hTR localization to telomeres during S phase in standard HeLa cells (not expressing exogenous telomerase) (Figures 3D and 3E; telomeres are detected with a DNA probe; a subset of hTR-telomere colocalizations is indicated with arrowheads). TPP1 depletion reduced the number of hTR-telomere colocalizations by 68%, from a mean of 0.41 ± 0.16 (SEM) per cell to 0.13 ± 0.08 (SEM) after TPP1 depletion (Figure 3E).

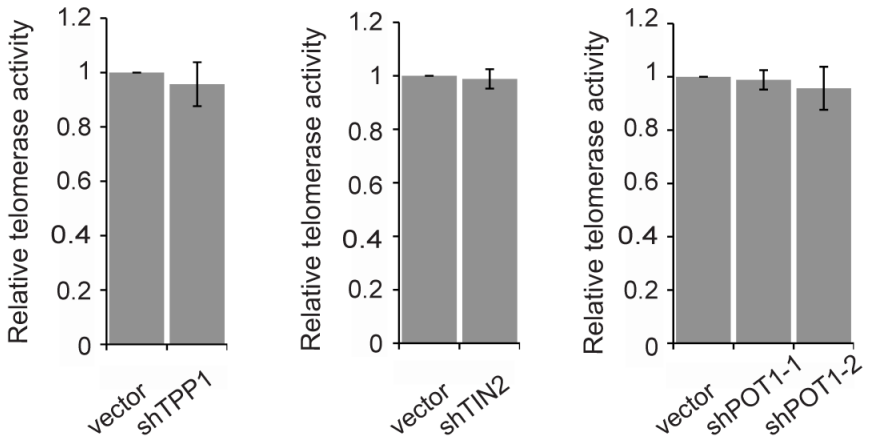


Figure 5. Telomerase activity measured by RQ-TRAP was not changed in super telomerase HeLa cells treated with the indicated shRNAs. Telomerase activity of the empty vector transfected cells was set to 1. Values indicate mean telomerase activity and standard deviation from two independent transfections.

We also examined telomerase recruitment to telomeric DNA by ChIP in super-telomerase HeLa cells (Figure 6). ChIP relies on formaldehyde-mediated covalent linkage of proteins to DNA and therefore reflects close physical as well as spatial associations of proteins and DNA. TPP1 was depleted (Figure 4), and the association of telomerase with telomeres (and Alu repeats) was assessed by IP with hTERT antibodies.

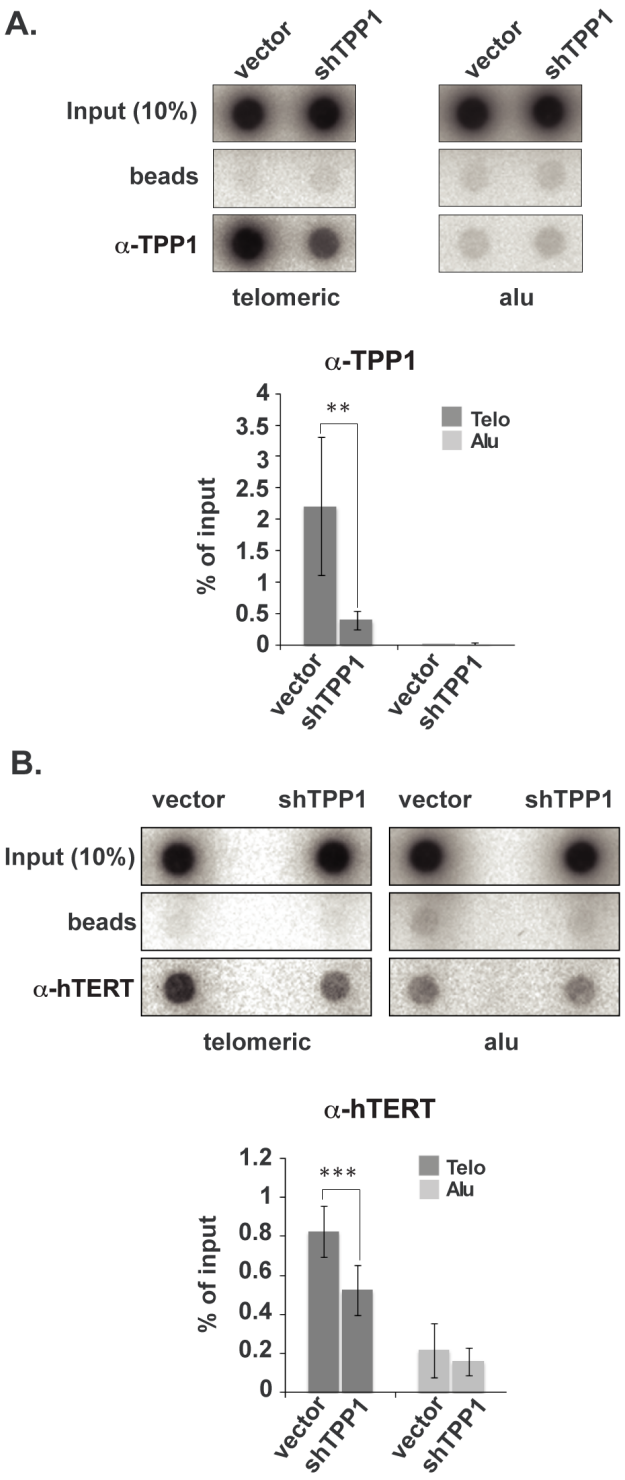


Figure 6. TPP1 depletion results in loss of physical association of hTERT with telomeres (assessed by ChIP). ChIP of telomeric and Alu DNA with TPP1-specific ***(A)*** and hTERT-specific ***(B)*** antibodies in super-telomerase HeLa cells. The percentage of telomeric and Alu DNA recovered in each ChIP is indicated. Error bars correspond to standard deviations of results of three (hTERT ChIP) and five (TPP1 ChIP) independent experiments. Statistical analyses were done using a two-tailed Student's *t* test (***, $P<0.001$; **, $P<0.01$).

As expected, little telomeric DNA was immunoprecipitated with TPP1 antibodies upon depletion of TPP1 (Figure 6A). In addition, however, immunoprecipitation of telomeric DNA with hTERT antibodies was reduced by 36% when cellular TPP1 was depleted (Figure 6B). The small degree of association of hTERT observed with Alu-repeat DNA, which served as a negative control, was not affected by TPP1 depletion. Thus, both FISH and ChIP analyses implicate TPP1 as a telomerase recruitment factor.

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

TPP1 interacts with telomeres via two proteins: TIN2, which binds to the double-stranded telomere-bound TRF1 and TRF2 proteins, and POT1, which mediates interaction with the single-stranded region of the telomere (de Lange, 2005; Xin et al., 2008) (see Figure 3A). We found that TPP1 depletion reduced TIN2 protein levels (Figure 4A), suggesting that the recruitment defect that we observed in TPP1-depleted cells may require TIN2. At the same time, a current model suggests that TPP1 functions with POT1 to recruit telomerase to telomeres (Xin et al., 2007a). To further investigate the mechanism of TPP1-mediated telomerase recruitment, we depleted TIN2 or POT1 using shRNAs (Figures 4B to G). ChIP analysis indicated that association of hTERT with telomeres was reduced 43% upon depletion of TIN2 (Figure 7A and 7B), similar to the reduction observed with TPP1 depletion (Figure 6). However, immunoblot analysis revealed that depletion of TIN2 also resulted in a reduction in TPP1 protein levels (but not telomerase activity or hTERT protein levels) (Figures 4B and Figure 5). On the other hand, POT1 depletion did not detectably change TPP1 levels (Figure 4D). In addition, POT1 depletion did not disrupt hTERT association with telomeres in ChIP analysis (Figures 7A and B).

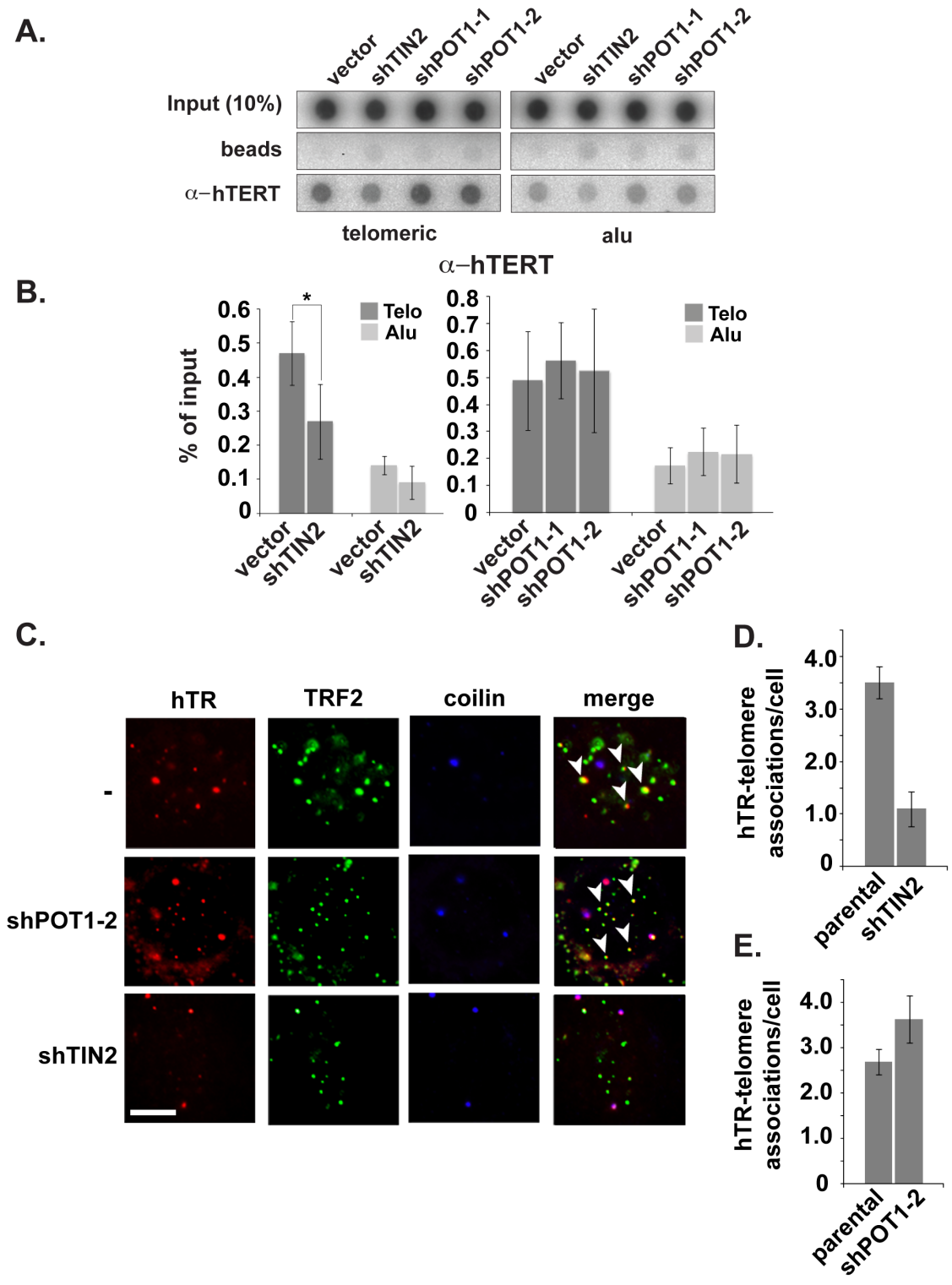


Figure 7. Telomerase recruitment depends on TIN2 but not POT1. (***A***) ChIP of telomeric and Alu DNA with hTERT antibody. (***B***) Quantification of ChIP data shown in panel A. Error bars correspond to standard deviations of results of three (TIN2 data) and two (POT1 data) two independent experiments.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

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

FISH analysis of hTR also showed a marked difference in the effects of TIN2 and POT1 depletion. hTR-telomere colocalization with TIN2 depletion was reduced to an extent similar to that observed with TPP1 depletion (Figure 7C and D). Following TIN2 depletion, hTR-telomere colocalizations decreased by 69%, from a mean of 3.5 ±0.3 (SEM) per cell to 1.1 ±0.3 (SEM) per cell (Figure 7D). However, POT1 depletion did not reduce the colocalization of hTR with telomeres (Figure 7C and E). The results indicate that POT1 and association with the single-stranded region of the telomere (see Figure 3A) are not required for the association of the majority of telomerase with telomeres and suggest a role for TIN2-tethered TPP1 in recruitment.

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

In order to further investigate the function of TPP1 in telomerase recruitment, we rescued the shRNA-mediated depletion of endogenous TPP1 by expression of FLAG epitope-tagged TPP1. Control (empty vector) and rescue (TPP1) plasmids were cotransfected along with the TPP1 shRNA-encoding plasmid. Expression of endogenous and exogenous TPP1 was assessed by immunoblot analysis with TPP1 and FLAG antibodies 4 days following transfection (Figure 8A). The reduction in association of telomerase with telomeres observed with TPP1 depletion by ChIP analysis was not rescued by expression of an shRNA-sensitive TPP1 gene (Figure 8B and C); in both cases, the telomeric DNA precipitated with hTERT antibody was approximately 50% of the shRNA-negative control. However, expression of an shRNA-resistant TPP1 gene (TPP1*, shRNA mRNA recognition site destroyed without altering the protein sequence) encoding full-length FLAG-tagged TPP1 fully restored telomerase association, indicating that the phenotype is related to TPP1 depletion. In order to test the potential role of the N-terminal OB-fold domain of TPP1 (required for coprecipitation of telomerase and TPP1 in pulldown experiments) (Xin et al., 2007a) in

recruitment to telomeres, we introduced an shRNA-resistant truncated version of TPP1 (TPP1ΔOB*). TPP1 lacking the OB-fold did not rescue telomerase recruitment assessed by ChIP (Figures 8B and C).

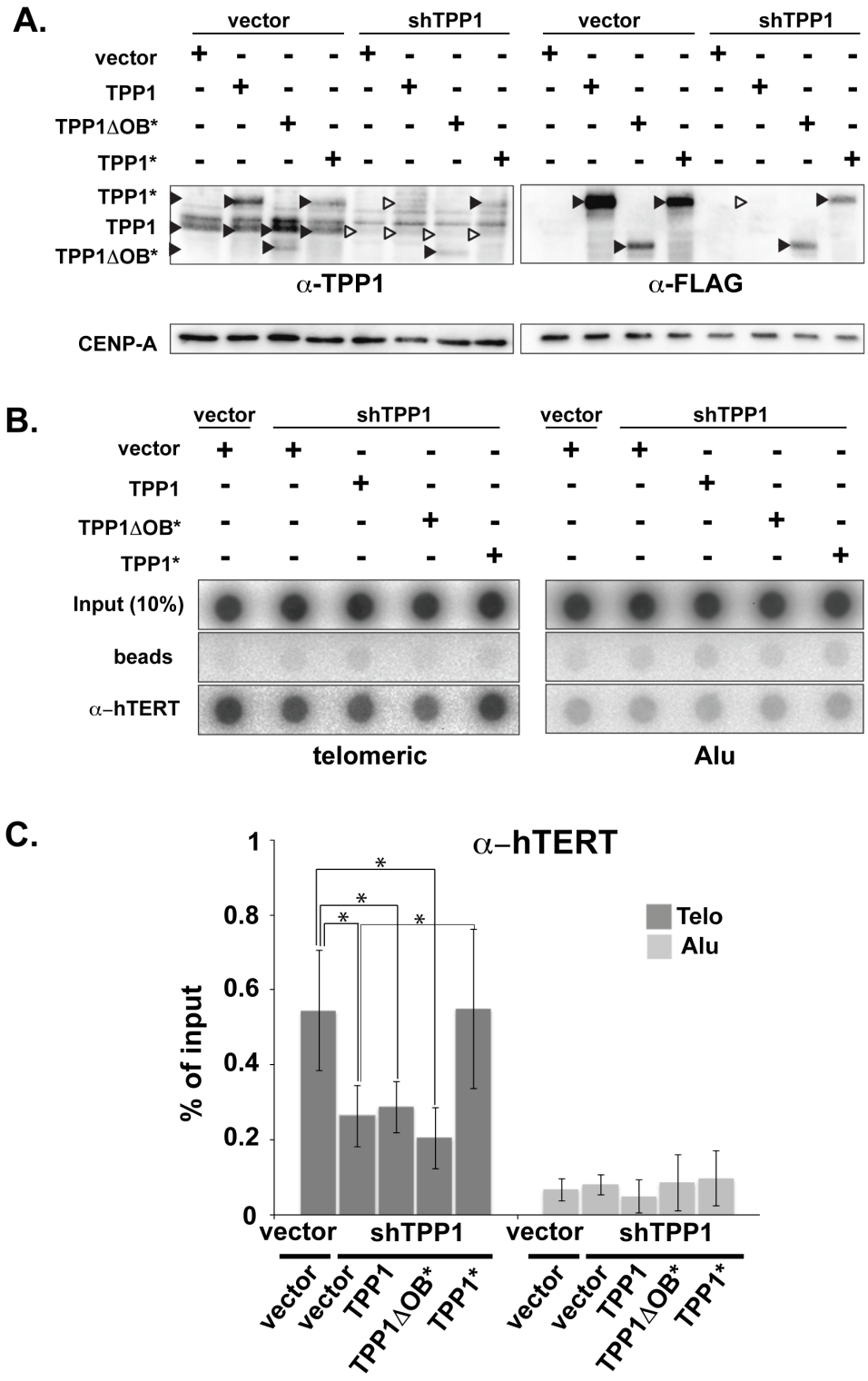


Figure 8. Human telomerase is recruited to telomeres via the OB-fold of TPP1. (***A***) Immunoblot analysis of ectopically expressed FLAG epitope-tagged full-length TPP1 (TPP1), FLAG-tagged,

shRNA resistant full-length TPP1 (TPP1*), and FLAG-tagged, shRNA resistant TPP1 lacking the OB-fold (TPP1ΔOB*). Super-telomerase HeLa cells were cotransfected with the indicated plasmids, and protein expression was analyzed 4 days after transfection. Black arrowheads indicate the presence of the respective TPP1 proteins and white arrowheads indicate the lack of expression. Immunoblots were probed with anti-TPP1, anti-FLAG and CENP-A antibodies as indicated. (***B***) ChIP of telomeric and Alu DNA with hTERT antibody from cells in panel A. (***C***) Quantification of data in panel B representing percent of telomeric and Alu DNA recovered in hTERT ChIP. Error bars correspond to standard deviations of results of four independent experiments. Statistical analyses were done using a two-tailed the Student's *t* test (*, $P<0.05$).

At the cellular level, we also observed that cotransfection of shRNA-resistant full-length TPP1 (TPP1*) restored localization of hTR to telomeres (localized via telomere-binding proteins TRF2 or RAP1) (Figure 9A). The FLAG-tagged TPP1 localized to telomeres, including those where hTR was found (Figure 9A; see arrowheads, TPP1*). Expression of the shRNA-resistant TPP1 increased the number of hTR-telomere colocalizations from a mean of 0.7 ±0.1 (SEM) per cell, observed in TPP1-depleted cells, to 2.4 ±0.4 (SEM) per cell, similar to the 2.1 ±0.3 (SEM) colocalizations per cell observed in parental super-telomerase cells in this experiment (Figure 9B). On the other hand, TPP1 lacking the OB-fold domain was unable to rescue telomerase recruitment (0.3 ±0.1 (SEM) hTR-telomere colocalizations per cell) (Figure 9B), despite the fact that the TPP1ΔOB protein localized to telomeres (see FLAG and RAP1 Figure 9A). These results suggest that the association of TPP1 and telomerase, which is mediated by the OB-fold of TPP1 (Xin et al., 2007a), functions in recruitment of telomerase to telomeres.

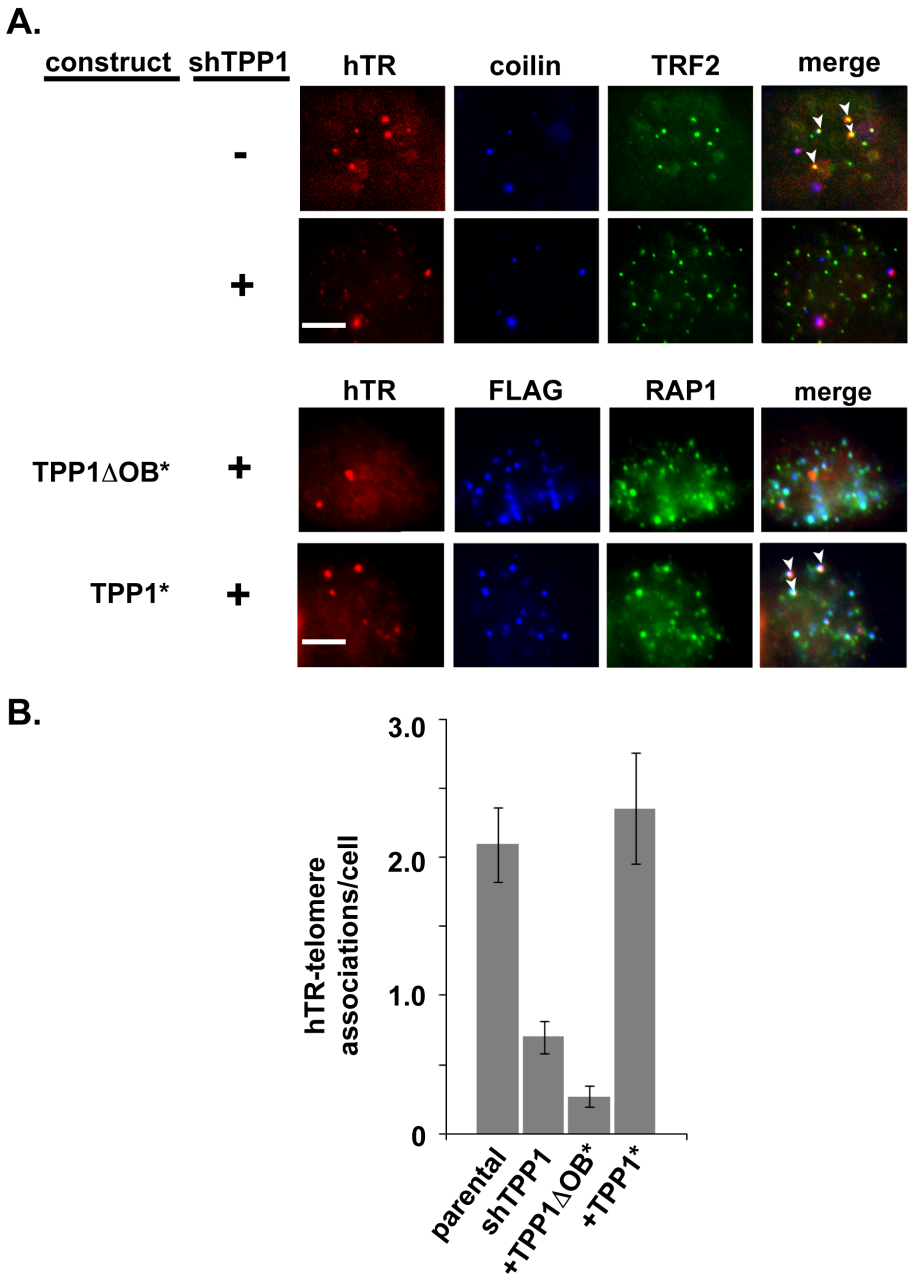


Figure 9. The TPP1 OB-fold is required to rescue telomerase recruitment to telomeres. An shRNA-resistant form of TPP1 is able to restore hTR localization to telomeres in TPP1-depleted cells. However, an shRNA resistant form of TPP1, lacking the OB-fold cannot restore localization. (***A***) Parental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

TIF formation does not impair association of telomerase with telomeres

Previous studies demonstrated that depletion of TPP1 activates a DNA damage response marked by the formation of telomere dysfunction-induced foci (TIFs) at telomeres (Guo et al., 2007; Hockemeyer et al., 2007; Xin et al., 2007a). TIF formation can be detected via the association of DNA damage proteins such as γ-H2AX and 53BP1, with telomeres (Takai et al., 2003). Accordingly, our ChIP analysis of telomeric DNA with antibodies against the DNA damage marker γ-H2AX revealed a 7-fold increase in γ-H2AX at telomeres when TPP1 levels are reduced (Figure 10A and B) (without a detectable change in cellular γ-H2AX levels; Figure 10C). In addition, we found the DNA damage marker 53BP1 at telomeres in 29% ±8% (SEM) of cells by IF when TPP1 was depleted, relative to 0.4% ±0.4% (SEM) of untreated cells in our experiments (Figure 10D) (scoring threshold = 7 or more 53BP1-telomere colocalizations per cell, similar to that used by others (Denchi and de Lange, 2007; Kibe et al., 2010)). These findings support the previous observations of TIF formation in response to TPP1 depletion and suggest general effects of TPP1 knockdown on telomere composition that could lead to the observed loss of telomerase recruitment. However, additional observations indicate that TIF formation *per se* does not account for the loss of telomerase recruitment observed in the absence of TPP1.

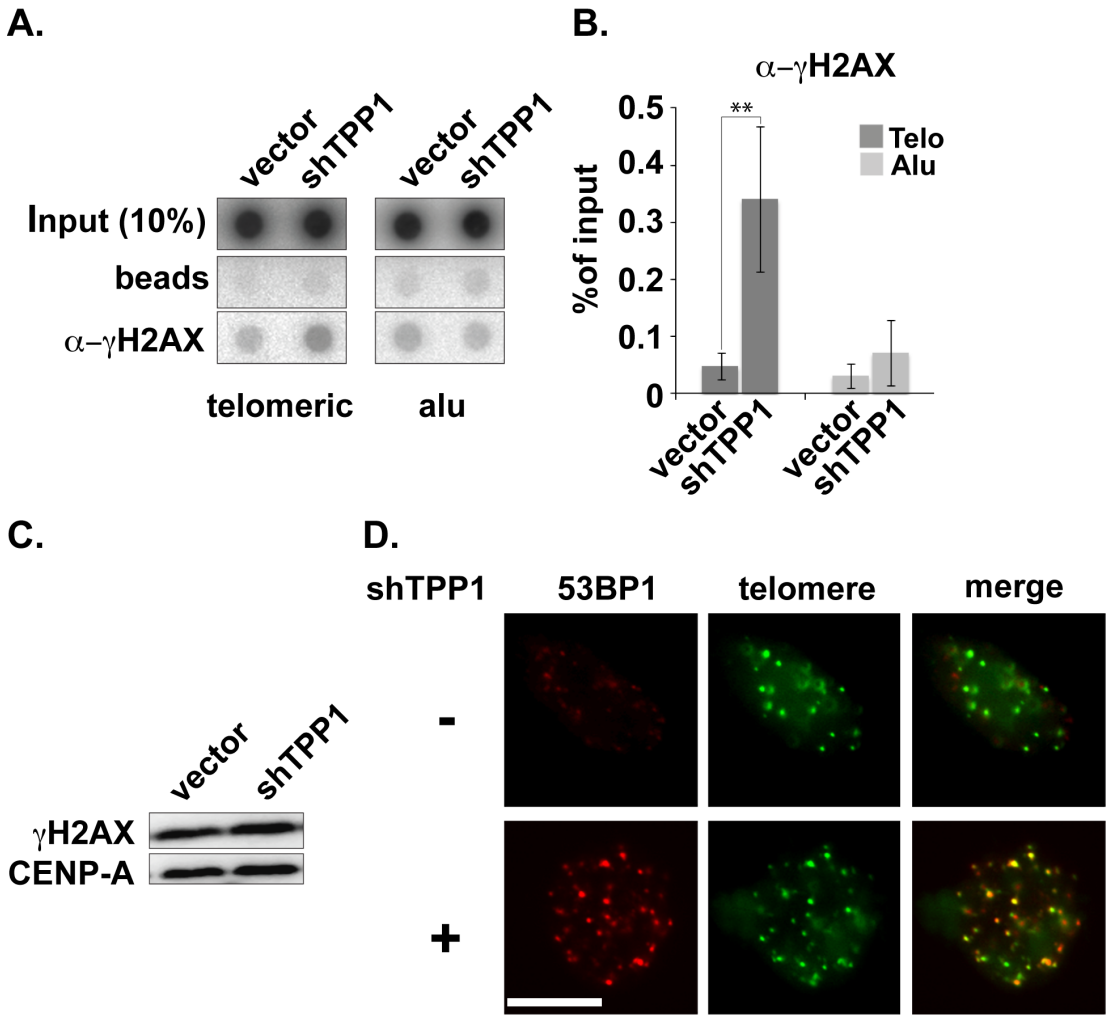


Figure 10. DNA damage response at telomeres following TPP1 depletion. (***A***) ChIP of telomeric and Alu DNA with γ-H2AX antibodies. Transfected plasmids are indicated. (***B***) The graph represents the quantification of the dot blot indicating the percentage of telomeric and Alu DNA recovered with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

Depletion of POT1 (and TIN2) also leads to a DNA damage response at telomeres (Hockemeyer et al., 2005; Kim et al., 2004) evidenced by an increase in γ-H2AX association with telomeric DNA by ChIP analysis (6-fold increase in TIN2-depleted cells and 8- to 10-fold increase in POT1-depleted cells) (Figure 11).

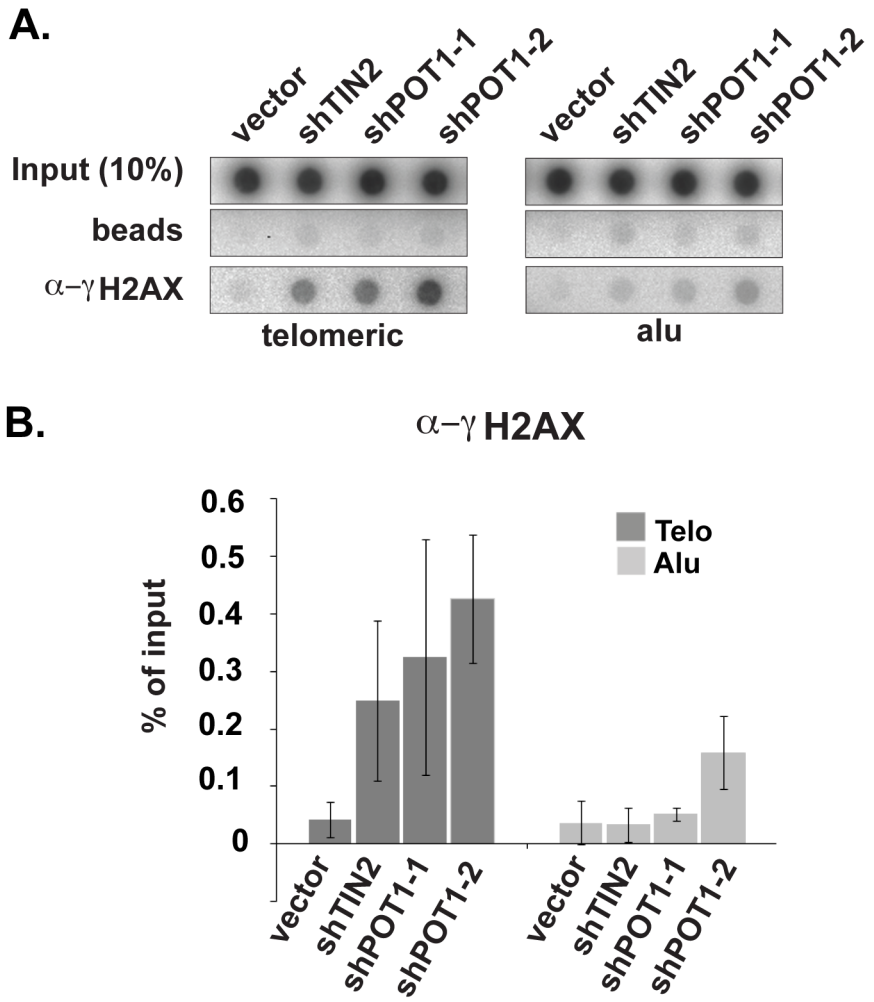


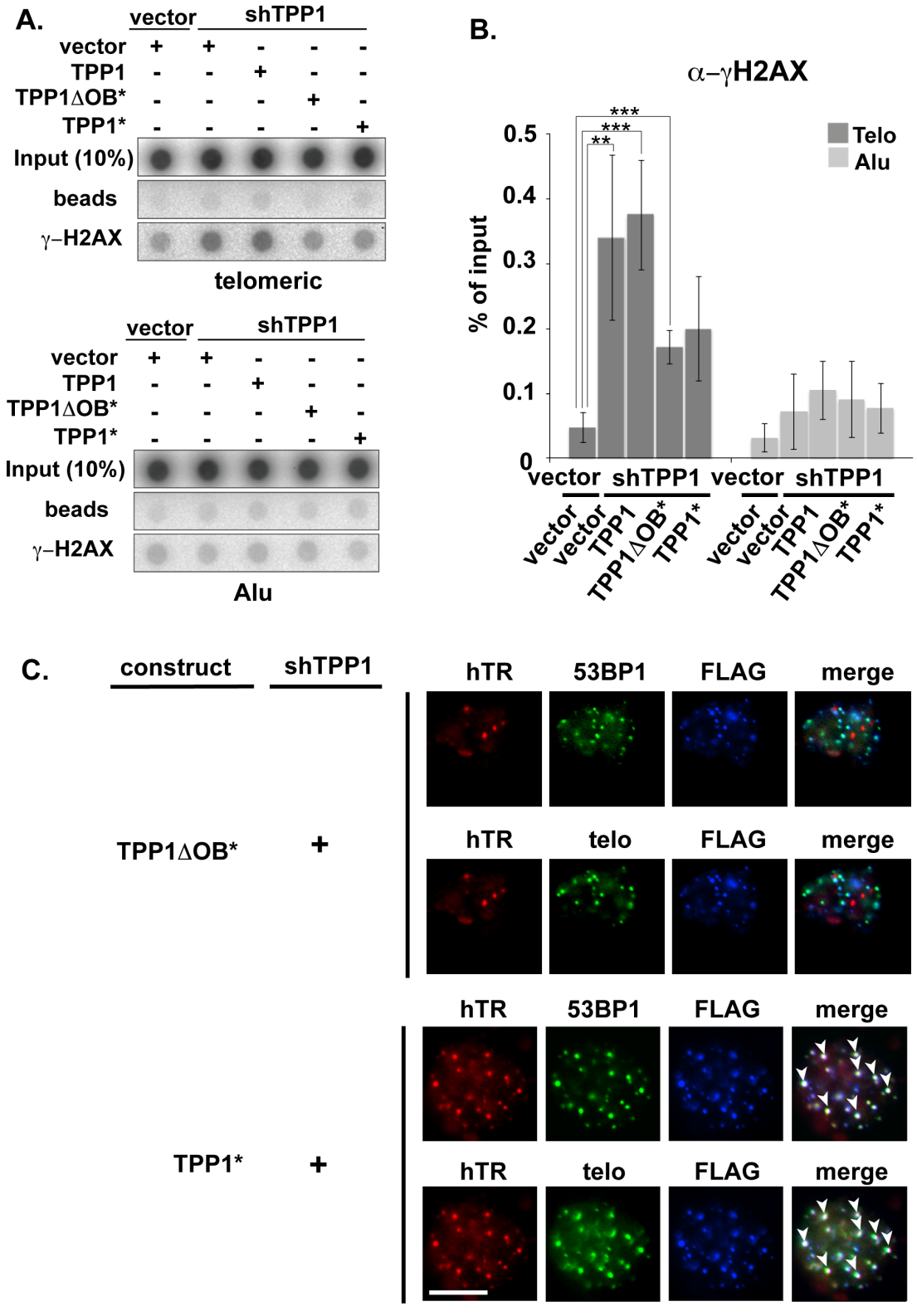
Figure 11. Increased DNA damage response at telomeres upon TIN2 or POT1 depletion. (***A***) ChIP of telomeric and Alu DNA with γ-H2AX antibodies. (***B***) The graph represents the quantification of the dot blot. Error bars correspond to standard deviations of results of two independent experiments.

However, the DNA damage response associated with POT1 depletion does not disrupt recruitment of telomerase to telomeres (Figure 7). In addition, we found that TIFs remained in our cells rescued with the wild-type TPP1 construct. ChIP analysis of γ-H2AX suggests that the TIF formation that occurred with TPP1 depletion was only partially rescued by the full-length TPP1 (or by the TPP1ΔOB protein) under the conditions of the experiment (TPP1*; Figure 12A and B). (The incomplete suppression of TIFs may reflect lower expression levels of transgenic TPP1 than of endogenous TPP1 (Figure 8A lanes 1 and 8)). Moreover, in microscopy experiments, 53BP1 was found at telomeres in 19% ±6% (SEM) of cells rescued with full-length TPP1 (Figure

12C) where telomerase recruitment was restored (Figures 8 and 9) (compared to 0.4% ±0.4% (SEM) of untreated cells), suggesting that the loss of telomerase recruitment is not a result of secondary effects of TPP1 knockdown on telomere structure. In fact, telomerase was observed at the same telomeres as the TIF marker protein 53BP1 in the rescued cells (Figure 12C), clearly indicating that TIF formation does not prevent recruitment of telomerase. At the same time, TIFs (indicated by the presence of γ-H2AX or 53BP1 at telomeres) were present in cells depleted of TPP1 (Figure 10) or expressing the TPP1ΔOB protein (Figure 12), indicating that the DNA damage response at telomeres also does not stimulate telomerase recruitment in the absence of intact TPP1. The results indicate that the association of telomerase with telomeres depends on TPP1 and, in particular, on the OB-fold domain of TPP1.

Figure 12. The presence of TIFs (telomere dysfunction-induced foci) does not impact the ability of TPP1 to rescue telomerase associations with telomeres. (***A***) ChIP of telomeric and Alu DNA with γ-H2AX antibodies. Transfected plasmids are indicated. Expression of TPP1* and TPP1ΔOB* partially rescues TIF formation observed in TPP1-depleted cells. (***B***) The graph represents the quantification of the dot blot indicating the percentages of telomeric and Alu DNA recovered with γ-H2AX antibodies. Error bars correspond to standard deviations of results of four independent experiments. Statistical analyses were done using a two-tailed Student's *t* test (***, $P<0.001$; **, $P<0.01$). (***C***) Although TIFs were detected in TPP1-depleted cells

coexpressing TPP1* or TPP1ΔOB*, TIFs did not inhibit rescue of hTR recruitment to telomeres by TPP1*. Super-telomerase HeLa cells were cotransfected with shTPP1 and either TPP1* or TPP1ΔOB*. Treated cells were subjected to FISH and IF to label for hTR (red), FLAG (blue), 53BP1 (green), and telomeres (green). Merge panels show superimposition of hTR, 53BP1, and FLAG; or hTR, telomeres, and FLAG.



Discussion

It is now clear that one primary mechanism for the regulation of telomerase activity is through regulated intracellular trafficking of the enzyme (Jady et al., 2006; Tomlinson et al., 2008; Tomlinson et al., 2006). Accumulation of hTR in Cajal bodies is mediated by TCAB1 and is required to render telomerase competent for association with telomeres in S phase of the cell cycle (Cristofari et al., 2007; Venteicher et al., 2009). The factors responsible for the recruitment of telomerase to telomeres have remained unidentified. In this study, using combined ChIP and FISH analyses, we have determined that depletion of shelterin proteins TPP1 and TIN2 (but not POT1) prevents association of telomerase with telomeres (Figures 6 and 3). These findings indicate that the majority of telomerase is recruited to telomeres by TPP1 proteins, bound to telomeres via TIN2 in humans and likely other vertebrates (Figure 3A).

TPP1 could theoretically function in telomerase recruitment specifically when bound to the single-stranded 3' overhang of the telomere via POT1 (de Lange, 2005; Palm and de Lange, 2008; Xin et al., 2008) (Figure 3A). Indeed, TPP1 has been speculated to function with POT1 to recruit telomerase (Xin et al., 2007a). However, we did not detect significant changes in telomerase recruitment upon depletion of POT1 (Figure 7), indicating that interaction with POT1 and the single-stranded end of the telomere is not required for recruitment of telomerase to telomeres by TPP1. At the same time, our results do not exclude the possibility that POT1 also plays an important role (positive or negative) in telomerase recruitment. The single-stranded 3' overhang of the telomere is generally much less extensive (typically 0.1 to 0.3 kb) than the double-stranded tract (typically 2 to 20 kb), and thus the fraction of telomerase that may be present at the 3' overhang would be expected to be small relative to that bound to the double-stranded part of the telomere. A specific change in telomerase levels at the single-stranded end may be difficult to detect above the background of telomerase associated with the rest of the telomere. Logically, the association of telomerase with

the single-stranded region of the telomere is important for telomere elongation. Recruitment of telomerase to the double-stranded part of the telomere may anticipate its catalytic action at the 3' end, perhaps by increasing the local concentration of telomerase. Importantly, the previously described S phase-specific trafficking of telomerase to telomeres (Jady et al., 2006; Tomlinson et al., 2008; Tomlinson et al., 2006) is TPP1-dependent (Figures 3D and E), indicating that TPP1-mediated recruitment is regulated by the cell cycle. While we cannot formally exclude an independent role for TPP1 and/or TIN2 in telomerase recruitment (since depletion of one is accompanied by reduction of the other (Figures 4A and 4B)), current knowledge about the organization of the shelterin complex supports co-function in the form of TIN2-anchored TPP1 (de Lange, 2005; Xin et al., 2008). Importantly, TIN2 protein levels remained reduced upon exogenous TPP1 expression and rescue of the TPP1-depletion-induced telomerase recruitment defect (Figure 16). On the other side, current evidence clearly suggest that TPP1 loading onto telomeres is TIN2-dependent (Chen et al., 2007). Thus, we hypothesize that the exogenously introduced TPP1 following TPP1-depletion, binds to the remaining telomere-bound TIN2 and recruits telomerase to telomeres. The significance of telomerase association with telomeres via TIN2-TPP1 is indicated by the recent identification of TIN2 mutations in patients suffering from the short telomere disease dyskeratosis congenita (Savage et al., 2008). Our findings suggest that inefficient telomerase recruitment might contribute to the pathogenesis of dyskeratosis congenita in these patients. It is not yet known whether additional factors are involved in TIN2-TPP1-mediated recruitment of telomerase to telomeres. It was previously demonstrated that TPP1 associates with telomerase in cell extracts (Xin et al., 2007a); however, it is not clear whether TPP1 interacts directly with telomerase.

The results presented here support the emerging view that certain shelterin components act as both negative and positive regulators of telomerase function (Smogorzewska and De Lange, 2004; Wang et al., 2007; Xin et al., 2007a). While collectively the shelterin proteins inhibit telomerase-telomere interactions, evidence

indicates that particular telomere-associated proteins can also interact with and recruit telomerase. These proteins include Cdc13 (*Saccharomyces cerevisiae*), TEBP-beta (ciliates), and TPP1 (humans and other vertebrates (this study)) (Chandra et al., 2001; Lin and Zakian, 1996; Nugent et al., 1996; Paeschke et al., 2008; Paeschke et al., 2005; Wang et al., 2007; Xin et al., 2007a). Recent studies indicate that dynamic phosphorylation of these proteins switches them between negative and positive regulation of telomerase recruitment by modulating the ability of the protein to interact with specific partner proteins. For example, in yeast, phosphorylation of Cdc13 by CDK1 favors an interaction with the Est1 subunit of telomerase (telomerase recruitment) over interaction with Stn1/Ten1 proteins (end protection) (Li et al., 2009; Tseng et al., 2009). Likewise, in ciliates, phosphorylation of TEBP-beta stimulates the function of the protein in telomerase recruitment and interferes with formation of a heterodimer with TEBP-alpha, which functions in telomere protection (Paeschke et al., 2008). TPP1 is a structural homolog of the ciliate TEBP-beta protein (Wang et al., 2007; Xin et al., 2007a) and contains a conserved serine-rich domain with several predicted Cdk2 phosphorylation sites (Wang et al., 2007; Xin et al., 2007a). Our findings establish TPP1 as a central factor in telomerase recruitment in humans. While depletion of TPP1 reduces telomerase recruitment (this study), it can also lead to telomerase-mediated telomere extension (Liu et al., 2004b; Ye et al., 2004b), suggesting that TPP1 is also poised to function as part of telomerase repressing and activating complexes in humans. To fully understand the mechanisms that underlie telomerase recruitment and understand the positive and negative roles of TPP1, POT1 and other shelterin components in telomerase regulation, it will be important to delineate the various telomeric states, identify the components of telomerase that associate with TPP1 during recruitment, and investigate whether phosphorylation of TPP1 plays a role in the regulated recruitment of telomerase to telomeres.

Addendum

TPP1, TIN2 and POT1 depletion does not cause substantial decrease in the protein levels of other shelterin components

Dysfunctional, uncapped telomeres associate with DNA repair and checkpoint markers into domains referred to as telomere-dysfunction induced foci (TIFs) (d'Adda di Fagagna et al., 2003; Takai et al., 2003). TIFs were induced by inhibition of TRF2 binding to telomeres and were characterized by an increase of phosphorylated H2AX (γ-H2AX) (d'Adda di Fagagna et al., 2003). We tested the cellular levels of γ-H2AX in TPP1-, TIN2- and POT1- depleted cells from Figure 4A, B and D respectively. Unexpectedly, in our experiments we did not observe an overall increase of γ-H2AX levels in total cell extracts, from cells depleted for TPP1 (Figure 10C), TIN2 or POT1 (Figure 13). We confirmed the specificity of the anti-γ-H2AX antibody in total cell extracts following replication protein A (RPA) depletion where we detected increased protein levels of γ-H2AX (data not shown) (Araya et al., 2005; Zou and Elledge, 2003). However, further investigation of TPP1-, POT1- and TIN2-depleted cells did reveal telomere-specific DNA damage, manifested as increased association of γ-H2AX with telomeres assayed by ChIP (see Figures 10 and 11).

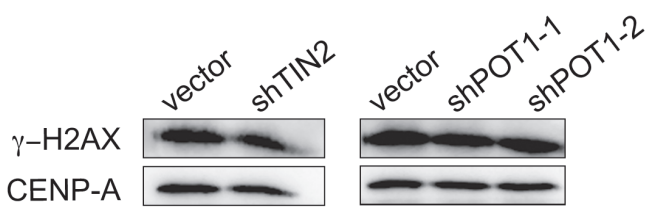


Figure 13. Total levels of γ-H2AX following TIN2 and POT1 depletion. Immunoblots were probed with anti-γ-H2AX and CENP-A antibodies following transfection of super-telomerase HeLa cells with empty vector, TIN2 or POT1 shRNA constructs as indicated. CENP-A was used as a loading control.

Telomere uncapping triggers senescence in human fibroblasts where the observed γ-H2AX extends hundreds of kilobases inwards from the telomeres (d'Adda di Fagagna et al., 2003). It is possible that in the super-telomerase HeLa cells partial uncapping of the telomeres is induced by telomerase overexpression and a slight increase of total γ-H2AX upon depletion of shelterin components was not detectable.

In parallel to the telomere uncapping phenotype, TPP1 depletion affected TIN2 protein levels and vice versa (see Figure 4A and B). On the other side, POT1-depleted cells had unaffected TPP1 and TIN2 protein levels (Figure 4C). We sought to investigate the effects of TPP1, TIN2 and POT1 protein depletion on the stability of other shelterin components. Total cell extracts from TPP1-, TIN2- and POT1- depleted cells from Figure 4A, B and D respectively, were analyzed for TRF1, TRF2 and RAP1 protein expression. We did not observe any substantial changes for TRF1 and TRF2 proteins, nor for the TRF2-binding RAP1 protein, following TPP1, TIN2 or POT1 depletion (Figure 14).

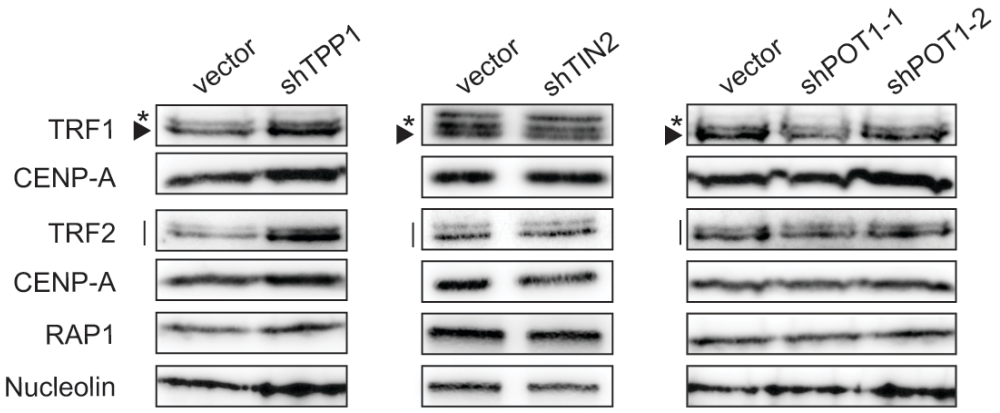


Figure 14. Effects of TPP1, TIN2 and POT1 depletion on the protein levels of other shelterin components. Immunoblot analysis of TPP1-, TIN2- and POT1- depleted cells in Figure 4A, B and D. Super-telomerase HeLa cells were transfected with empty vector or TPP1, TIN2 and POT1 shRNA vectors. Four days after TPP1 and TIN2 shRNA vector transfection and six days after POT1 shRNA transfection, TRF1, TRF2 and RAP1 protein expression was analyzed. CENP-A and nucleolin were used as loading controls. The arrowheads indicate the position of endogenous TRF1. The vertical line indicates endogenous TRF2, which is detected by two specific bands. Asterisks indicate nonspecific bands.

Previous studies have reported the importance of TIN2 for the stability of TRF1 and TRF2 proteins. RNAi-mediated depletion of TIN2 in transiently transfected HT1080 cells led to TRF1 and TRF2 protein reduction (Kim et al., 2008). In another study, however, siRNA-mediated TIN2-depletion affected only TRF1 protein levels, whereas TRF2 and RAP1 proteins were unaffected in transiently transfected HeLa 1.2.11 and HTC75 (Ye and de Lange, 2004). In the super-telomerase HeLa cells used here, TIN2-depletion is accompanied by TPP1 protein reduction and it had no impact on the

abundance of other shelterin proteins (Figure14 and Figure 4B). Likewise, TPP1 depletion reduced TIN2 protein levels and it did not alter the cellular levels of TRF1, TRF2 and RAP1 (Figure 4A and Figure 14). Consistently, stable knock down of TPP1 by shRNA in HTC75 cells did not perturb TRF2 and RAP1 protein levels (Chen et al., 2007). POT1 is the link of the shelterin complex to the single-stranded part of the telomere, however it seems dispensable for shelterin protein stability (Figure 14).

Our immunoblot analyses cannot discriminate whether shelterin proteins are still telomere-associated upon TPP1, TIN2 or POT1 depletion; they are only informative of their depletion-effects on the expression and stability of shelterin proteins. Current studies clearly indicate that TPP1 is responsible for POT1 recruitment to telomeres and TPP1 depletion results in POT1 dissociation from telomeres (Chen et al., 2007; Xin et al., 2007a). Both TPP1 and TIN2 were demonstrated as key components in mediating the shelterin complex assembly. In particular, TPP1 helps to stabilize the TRF1-TIN2-TRF2 interaction and knocking down TPP1 reduced the ability of TRF1 to associate with the TRF2 complex (O'Connor et al., 2006). Furthermore, TIN2 stabilizes TRF2 at telomeres via simultaneous binding of both TRF1 and TRF2; consequently TIN2 RNAi results in dissociation of both TRF2 and RAP1 from telomeres (Ye et al., 2004a). TIN2 binding to TPP1 promotes nuclear localization of TPP1-POT1 complexes and their loading onto the telomeres (Chen et al., 2007). In the scope of these findings it would be interesting to investigate on what level is the protein expression of TPP1 and TIN2 regulated. It may be that TIN2 protein regulates transcription or stability of TPP1 mRNA and vice versa. Alternatively, TIN2-TPP1 heterodimer may simply stabilize the two proteins and once this interaction is disrupted at telomeres, they become degraded. Notably, a recent study has also shown that TIN2 is 10-fold more abundant than TPP1 at telomeres, indicating that not all TIN2 molecules at telomeres form heterodimers with TPP1 (Takai et al., 2010). Accordingly, our immunoblot analyses of TPP1-depleted super-telomerase HeLa cells show that a nearly complete knockdown of

TPP1 triggers partial reduction in TIN2 protein expression (Figure 4A and Figure 16A and B).

Residual TIFs upon rescue of TPP1 depletion may be due to failure in restoring TIN2 protein levels

TIF formation that occurred with TPP1 depletion was only partially rescued when expressing the full-length TPP1 (or by the TPP1ΔOB protein) (TPP1*; Figure 12A and B), whereas the telomerase recruitment defect was restored back to control levels (Figure 8B and C). We hypothesized that the incomplete suppression of TIFs might have reflected lower expression levels of transgenic TPP1 than of endogenous TPP1 (Figure 8A compare lanes 1 and 8 in immunoblots probed with anti-TPP1 and FLAG antibodies). Interestingly, evidence from previous studies suggested that exogenous expression of full-length TPP1 *per se* increases the percentage of TIFs. 10-15% of TIF positive cells were observed following wild-type TPP1 overexpression (Chen et al., 2007; Xin et al., 2007a). To address the DNA damage response to exogenous TPP1 expression, we transfected super-telomerase HeLa cells with vectors expressing the FLAG epitope-tagged full-length TPP1 and TPP1 lacking the OB-fold domain. Expression of exogenous TPP1 was assayed with FLAG antibody four days following transfection (Figure 15A). We confirmed that the exogenous TPP1 proteins associate with telomeres by ChIP (Figure 15B and C).

Next, we examined whether the expression of transgenic full-length TPP1 or TPP1ΔOB increased γ-H2AX levels at telomeres. We could not detect marked increase of γ-H2AX association with telomeres above control levels (Figure 15B). The percentage of telomeric and Alu DNA recovered in γ-H2AX ChIP was the same (Figure 15B and quantification not shown). We conclude that, in super-telomerase HeLa cells, ectopically expressed TPP1 *per se* does not account for increase of basal levels of γ-H2AX, thus it does not contribute to the residual levels of DNA damage following rescue of TPP1 depletion.

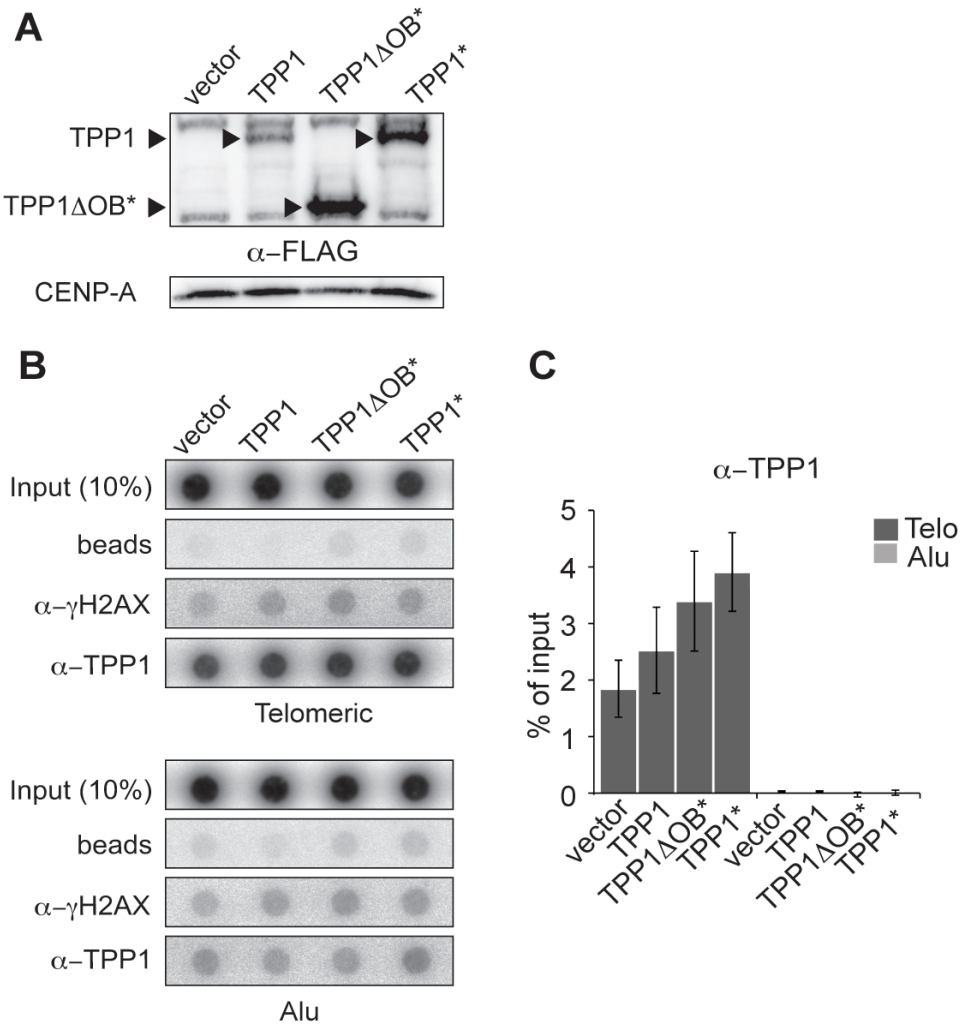


Figure 15. Exogenous expression of TPP1 does not cause a DNA damage response at telomeres. (***A***) Immunoblot analysis of ectopically expressed FLAG epitope-tagged full-length TPP1 (TPP1), FLAG-tagged, shRNA resistant TPP1 lacking the OB-fold (TPP1ΔOB*) and FLAG-tagged, shRNA resistant full-length TPP1 (TPP1*). Super-telomerase HeLa cells were transfected with the indicated plasmids, and protein expression was analyzed 4 days after transfection. Black arrowheads indicate the respective TPP1 proteins. Immunoblots were probed with anti-FLAG and anti-CENP-A antibodies; CENP-A was used as a loading control. (***B***) ChIP of telomeric and Alu DNA with γ-H2AX and TPP1 antibodies from cells in panel A. (***C***) Quantification of data in panel B representing percentage of telomeric and Alu DNA recovered in γ-H2AX and TPP1 ChIP. Error bars correspond to standard deviations of results of two independent experiments.

We showed that TPP1 depletion causes telomere uncapping and TIN2 protein reduction and it does not alter the protein levels of TRF1, TRF2 and RAP1 shelterin components. Furthermore, exogenous TPP1 expression alone does not trigger telomere-specific DNA damage (Figure 15), yet it cannot fully restore TIF formation following endogenous TPP1 depletion, despite restoring telomerase-telomere association (Figure 8 and Figure 12). The rescued telomerase recruitment phenotype

indicates functional TPP1 binding to telomeres. Thus, we set out to examine the levels of TIN2 protein following TPP1 depletion and rescue to determine the source of the remaining telomere-specific DNA damage response. Interestingly, cells depleted for TPP1 and expressing an shRNA-resistant TPP1 gene that encodes full-length FLAG-tagged TPP1, did not restore TIN2 protein levels (Figure 16A and B). As shown before, we observed 60 percent reduction of TIN2 protein following TPP1 depletion (Figure 4A, Figure 16A and B). However, TIN2 expression was still reduced by 50 percent compared to wild-type levels upon introduction of transgenic TPP1, which fully rescued the telomerase recruitment defect (Figure 16A and B and Figure 8). As expected, there was no obvious change in the overall levels of γ-H2AX and hTERT following TPP1 depletion and rescue, assayed by immunoblotting (Figure 16A).

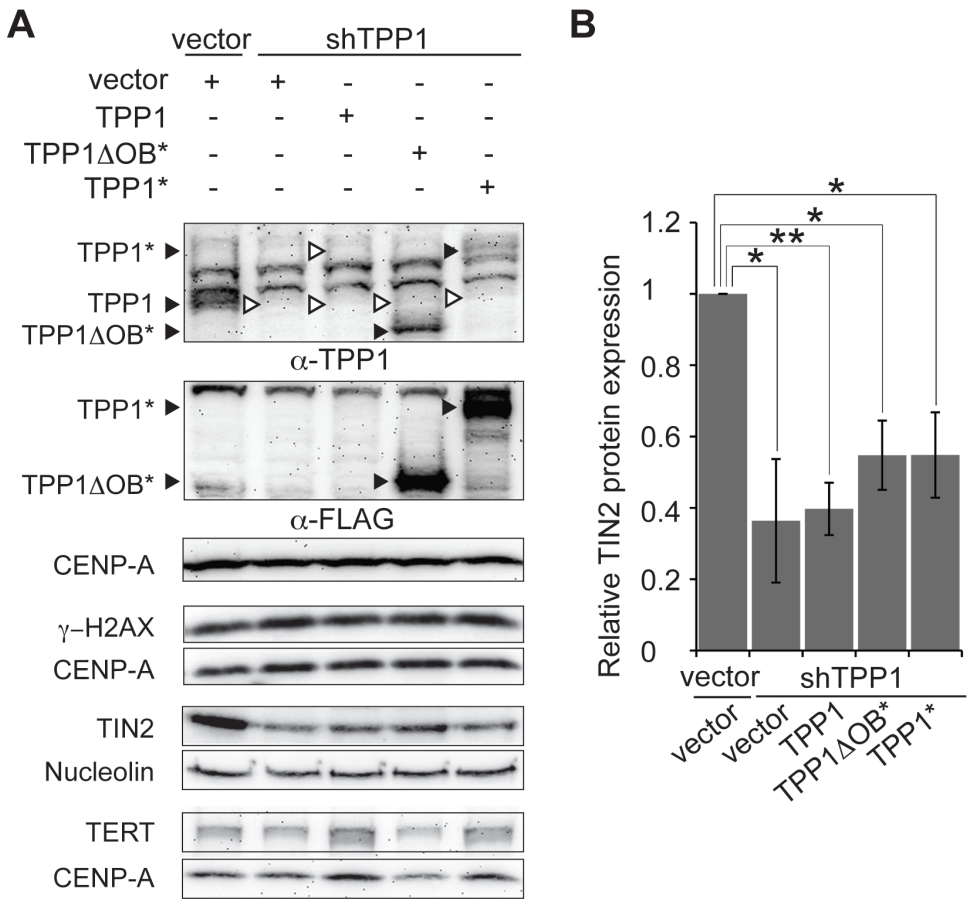


Figure 16. TIN2 protein levels are not fully restored following TPP1 depletion and rescue. (***A***) Immunoblot analysis of ectopically expressed FLAG epitope-tagged full-length TPP1 (TPP1), FLAG-tagged, shRNA resistant TPP1 lacking the OB-fold (TPP1ΔOB*) and FLAG-tagged, shRNA resistant full-length TPP1 (TPP1*). Super-telomerase HeLa cells were cotransfected with the indicated plasmids, and protein expression was analyzed 4 days after transfection. Immunoblots were probed with anti-TPP1, FLAG, CENP-A, γ-H2AX, TIN2, nucleolin, and

hTERT antibodies. CENP-A and nucleolin were used as loading controls. Black arrowheads indicate the presence of the respective TPP1 proteins and white arrowheads indicate the lack of expression. (***B***) Quantification of endogenous TIN2 expression in panel A. TIN2 protein levels were normalized to loading control and expressed relative to TIN2 protein levels in empty vector transfected cells, which were set to 1. Error bars correspond to standard deviations of results of three independent experiments. Statistical analyses were done using a two-tailed the Student's *t* test (*, $P<0.05$; **, $P<0.01$).

Recent studies that aimed at elucidating the stoichiometry of shelterin components *in vivo* provided quantitative analysis of the abundance of each shelterin subunit in human cells (Takai et al., 2010). It was reported that TIN2 is a very abundant protein such that each TIN2 copy is sufficient to bind to each copy of TRF1 and TRF2 dimers. On the other side, TPP1 and POT1 were approximately 10-fold less abundant than TIN2 and present at equimolar amounts. Moreover, the TPP1-POT1 heterodimer was present in excess over the amount that could be bound at the single-stranded DNA of each telomere; suggesting that a fraction of telomere bound TPP1-POT1 is loaded along the double-stranded part of the telomere. The finding that TIN2 is 10-fold more abundant than TPP1 could explain the rescue of telomerase recruitment in the super-telomerase HeLa cells, following ectopic expression of TPP1, despite 50% lower levels of endogenous TIN2 protein remaining. This result suggests that newly formed TPP1-telomerase complexes following transgenic TPP1 expression, can bind to the remaining TIN2 proteins at telomeres and restore telomerase recruitment. However, two-fold reduced TIN2 protein levels still cause telomere uncapping, evidenced by the remaining telomere-associated γ-H2AX following exogenous TPP1 expression and rescue of telomerase recruitment.

TRF2 depletion does not affect association of telomerase with telomeres

We have determined that depletion of each TPP1 and TIN2, but not POT1 significantly reduces the presence of telomerase at telomeres. Thus, telomerase recruitment primarily occurs via the double-stranded part of the telomere. To further support our results, we addressed the role of TRF2, a telomeric double stranded-binding protein, in telomerase association to telomeres. Determining the role of TRF1,

the second telomeric double stranded-binding protein, in telomerase recruitment was hampered by the lack of shRNAs that could efficiently target TRF1 expression. We found that shRNA-induced depletion of TRF2 did not change telomerase association to telomeres as compared to cells harboring endogenous levels of TRF2 (Figure 17).

Human TRF2 is represented in immunoblots by two closely migrating bands. Both bands are derived from the TRF2 mRNA since both disappear upon TRF2 knockdown with shRNA (Figure 17A). The difference between these two forms of TRF2 is not known. Further immunoblot analysis revealed no change of hTERT and γ-H2AX protein levels, similarly as shown for TPP1, TIN2 and POT1 depletion (Figure 17A, Figure 4A, B and D, Figure 10C and Figure 13). As previously demonstrated, TRF2 depletion resulted in telomere deprotection evidenced by the increase of γ-H2AX association to telomeres (Figure 17B and C). In contrast, hTERT association with telomeres did not change significantly following TRF2 depletion (Figure 17B and C).

TRF2 is a double-stranded telomere binding protein exerting a protective function at telomeres (Celli and de Lange, 2005; Karlseder et al., 1999; Takai et al., 2003; van Steensel et al., 1998). Targeting of TRF2 to specific telomeres in telomerase-positive cell lines leads to telomere shortening, and expression of full-length TRF2 in primary cells also leads to increased telomere shortening rate (Ancelin et al., 2002). In addition, TRF2 reduction causes telomere elongation (Takai et al., 2010). Thus, TRF2 is a negative regulator of both telomerase-dependent and -independent telomere elongation. Furthermore, presence of TRF2 on a short linear telomerase substrate did not inhibit telomerase activity *in vitro* (Smogorzewska et al., 2000). In agreement with the previous findings, in super-telomerase HeLa cells TRF2 seems not to play a role in either the telomerase recruitment step, nor does it affect telomerase (hTERT) expression (Figure 17). We hypothesize that the recruitment step of telomerase is established via a TRF1-TIN2-TPP1 subcomplex in the absence of TRF2 and RAP1. RAP1 binds telomeres solely via TRF2 and removal of TRF2 leads to dissociation of RAP1 from telomeres (Li and de Lange, 2003; Li et al., 2000). It would

be interesting to see whether there is a RAP1-TRF2-TIN2-TPP1-telomerase recruitment subcomplex in the absence of TRF1.

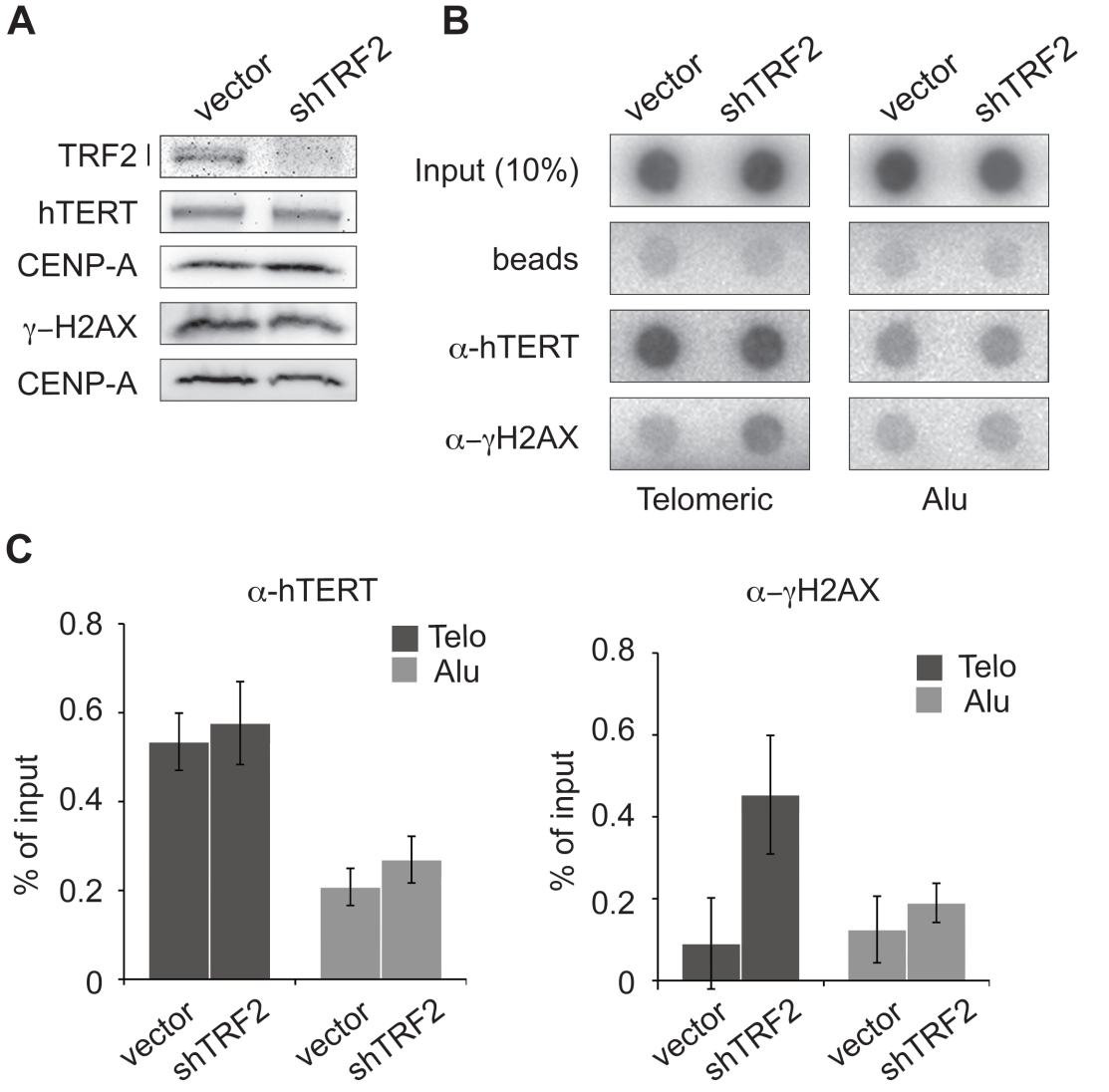


Figure 17. TRF2 is dispensable for telomerase recruitment to telomeres. (***A***) Immunoblot analysis of TRF2-depleted cells. Super-telomerase HeLa cells were transfected with empty vector and TRF2 shRNA vector. Seven days after transfection, TRF2, hTERT, γ-H2AX and CENP-A expression was analyzed. Two closely migrating bands present endogenous TRF2. CENP-A was used as a loading control. (***B***) ChIP of telomeric and Alu DNA with hTERT and γ-H2AX antibodies from cells analyzed in panel A. (***C***) Quantification of data in panel B representing percentage of telomeric and Alu DNA recovered in hTERT ChIP and γ-H2AX ChIP. Error bars correspond to standard deviations of results of two (hTERT ChIP) and four (γ-H2AX ChIP) independent experiments.

Upon TRF1 inhibition, TIN2 remains at telomeres via an increased association with TRF2 (Houghtaling et al., 2004). Likewise, TRF1 remains associated with telomeres in human cells that overexpress a dominant negative allele of TRF2 or in mouse embryonic fibroblasts lacking TRF2 (Hockemeyer et al., 2007; Loayza and De Lange,

2003; van Steensel et al., 1998). Therefore, it is plausible that both TRF1 and TRF2 regulate TIN2-TPP1 binding to the double-stranded telomeric tract promoting further telomerase recruitment. Consequently, in the absence of one TRF protein, the other can compensate to prevent a defect in recruitment of TPP1-telomerase complexes to telomeres.

Chapter III: Dyskeratosis congenita *TINF2* mutations do not affect telomere structure

Introduction

Dyskeratosis congenita (DC) is a rare, heterogeneous bone marrow failure syndrome with cancer predisposition and abnormalities in telomere maintenance. Classical DC is associated with the diagnostic triad of nail dystrophy, abnormal skin pigmentation and oral leukoplakia (Walne et al., 2005). However, this triad is not always observed in a clinical setting. Patients with DC are at high risk of developing various malignancies such as aplastic anemia, myelodysplastic syndrome and leukemia (Alter et al., 2009). Other diseases in the DC spectrum include Hoyeraal-Hreidarsson (HH) syndrome and Revesz syndrome (Walne et al., 2008). Despite the clinical heterogeneity, a unifying feature of all DC patients is the abnormally short telomere length (Alter et al., 2007). DC is also a genetically heterogeneous disease represented in X-linked recessive, autosomal dominant and autosomal recessive forms (Mason et al., 2005). X-linked mutations are linked to the first identified DC causative gene, the *DKC1* gene encoding for the nucleolar protein dyskerin (Heiss et al., 1998) (Knight et al., 1999). The link with telomerase was suggested when dyskerin was found to stabilize hTR and maintain telomere length through catalytically active telomerase (Mitchell et al., 1999b). Autosomal-dominant DC mutations are found in the catalytic core telomerase subunits: hTR and hTERT (Armanios et al., 2005; Vulliamy et al., 2001). Autosomal recessive DC is caused by mutations in the *NOP10* and *NHP2* genes, encoding for nucleolar proteins and integral components of the H/ACA class of snoRNP complexes, and as well in the *WDR79* gene encoding for TCAB1, a constituent of the active telomerase complex implicated in nuclear telomerase trafficking (Vulliamy et al., 2008; Walne et al., 2007; Zhong et al., 2011). The above-

mentioned mutations are within genes that encode for telomerase components and telomerase accessory factors, and represent approximately 40 percent of the genetically characterized DC cases. Recently, analysis of cohort of DC patients revealed a mutation in the *TINF2* gene that encodes for TIN2 protein, making it the seventh DC mutated gene implicated in telomere maintenance (Sarper et al., 2010; Savage et al., 2008; Walne et al., 2008). Currently, heterozygous coding mutations in *TINF2* account for approximately 10 percent of all DC patients (Walne et al., 2008). The genetic basis of DC remains unclassified in about 50 percent of patients. Establishment of a mouse model for DC has not been straightforward. Deletion of *Dkc1*, *Terc* or *Tert* in mice introduces defects that are not seen in DC patients (Autexier, 2008). However, in double Terc and POT1b knockout mice, a phenotype resembling DC was seen. But since humans lack a *Pot1b* gene the model may not mimic all aspects of the human disease (Hockemeyer et al., 2008).

Savage and colleagues first identified three separate mutations within the *TINF2* gene that were linked to DC: Ex6+234 A to G mutation (causing amino acid change K280E); Ex6+241 G to A (R282H); and Ex6+240 C to A (R282S) (Savage et al., 2008). The R282H mutation was also found in a patient with Revesz syndrome. The heterozygous R282H mutation was also reported in a patient presented with ataxia and pancytopenia, that had short telomeres and reduced telomerase activity (Tsangaris et al., 2008). Walne and colleagues further examined an uncharacterized cohort of DC patients for the exon 6 of the *TINF2* gene, which also encodes the affected amino acids 280 and 282. Heterozygous coding mutations were found in 33 of 175 uncharacterized DC patients. A total of 21 of the mutations affected amino acid 282 and the remaining 12 were insertions or deletions in a single base or missense mutations (Walne et al., 2008). In summary, all identified TINF2-mutations are clustered in an 18-amino acid segment of unknown function. The reported *TINF2* mutations do not affect hTR levels as seen with most of the other DC-causing mutations.

TIN2 provides a critical link between the double-stranded and single-stranded telomere binding proteins within the shelterin complex (Houghtaling et al., 2004; Kim et al., 2004; Kim et al., 1999; Liu et al., 2004a; Liu et al., 2004b; Ye and de Lange, 2004; Ye et al., 2004a; Ye et al., 2004b). TIN2 interaction with TPP1 mediates telomeric loading of the TPP1/POT1 heterodimer (Chen et al., 2007; Liu et al., 2004b; Ye et al., 2004b) and its interaction with TRF2 stabilizes the TRF2/RAP1 complex at telomeres (O'Connor et al., 2006; Ye et al., 2004a). TIN2 is also required for TRF1 protein stability by preventing tankyrase 1-mediated poly(ADP)-ribosylation of TRF1 (Smith and de Lange, 2000; Ye and de Lange, 2004). Inhibition of TIN2 by RNA interference leads to telomere elongation (Ye and de Lange, 2004) and its overexpression causes telomere shortening (Kim et al., 1999). Our findings suggest that TIN2 is partially interdependent with TPP1 for its protein stability, and together they cooperate in telomerase recruitment to telomeres (Chapter 3). TIN2 is an essential protein, as germ line inactivation in the mouse causes early embryonic lethality (Chiang et al., 2004). Pre-mRNA alternative splicing of *TINF2* results in three distinct isoforms termed TIN2S (short), TIN2M (middle) and TIN2L (long) (Chen, 2009; Kaminker et al., 2009). All three isoforms are shown to associate with telomeres but they differ in their role in telomere length regulation; TIN2S is identified as a negative and TIN2L as a positive regulator of telomere length (Chen, 2009). In addition, TIN2L is proposed to mediate interaction of the telomeric complex with the nuclear matrix (Kaminker et al., 2009).

In this chapter, we have directly investigated the effects of several *TINF2* DC-related mutations on telomere structure, shelterin proteins stability and telomerase recruitment. We find that overexpression of TIN2 DC-mutants is able to rescue the TIN2 shRNA-mediated depletion restoring the telomere capping function. In particular, overexpressed TIN2-DC mutants associate with telomeres and rescue the reduced TPP1 protein expression and thus rescue the telomere deprotection phenotype caused by endogenous TIN2-depletion. TIN2-DC mutants have no effect on TERT protein stability nor do they affect the stability of other shelterin proteins.

Results

Exogenously expressed TIN2 isoforms harboring distinct DC-related mutations associate with telomeres

Alternative splicing events in the exon 6 generate three TIN2 isoforms containing distinct 3'-end mRNA sequences. Alternative splicing shifts the translational stop codon upstream of the last 9th exon and produces protein isoforms with a size of 39 kDa (TIN2S), 47 kDa (TIN2M) and 50 kDa (TIN2L) (Chen, 2009). The TIN2S isoform is the first identified TIN2 protein, being the most abundant and ubiquitously expressed form (Chen, 2009; Kim et al., 1999). The identification of TIN2L was only possible when cells were lysed under harsh denaturing conditions, which lead to the assumption that TIN2L associates with and anchors telomeres to the nuclear matrix (Kaminker et al., 2009). Both TIN2M and TIN2L isoforms were identified through cDNA cloning (Chen, 2009). We have investigated the expression of all three TIN2 isoforms in super-telomerase HeLa cells by immunoblotting (Figure 18).

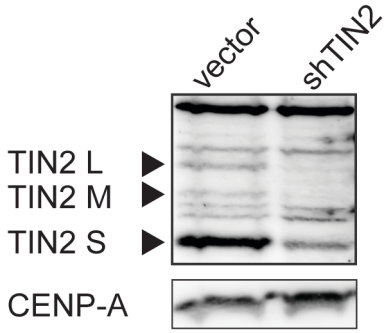


Figure 18. Depletion of TIN2 isoforms. Immunoblot analysis of TIN2-depleted cells. Super-telomerase HeLa cells were transfected with empty vector and TIN2 shRNA vector. Four days following transfection endogenous TIN2 and CENP-A levels were analyzed. Endogenous TIN2 is represented by three isoforms with size of 39 kDa (TIN2S), 47 kDa (TIN2M) and 50 kDa (TIN2L). CENP-A was used as a loading control.

Our TIN2 shRNA was designed to target the expression of all three isoforms and thus resulted in significant reduction of TIN2S, TIN2M and TIN2L proteins (Figure 18). As expected, the expression of TIN2S was the most robust, and TIN2M was the least expressed isoform (Chen, 2009). We have confirmed that super-telomerase HeLa cells regulate TIN2 expression via alternative splicing, resulting in three distinct isoforms.

The finding that *TINF2* is mutated in DC patients prompted us to determine the effects of TIN2 DC mutants on telomere structure. In particular, we decided to examine the effects of few of the most frequent *TINF2* DC mutations causing the following amino acid changes: K280E, R282H, R282C and R282S. For this purpose, following depletion of TIN2, we exogenously expressed FLAG epitope-tagged TIN2-DC mutants rendered inaccessible for the TIN2 shRNA (Figure 19).

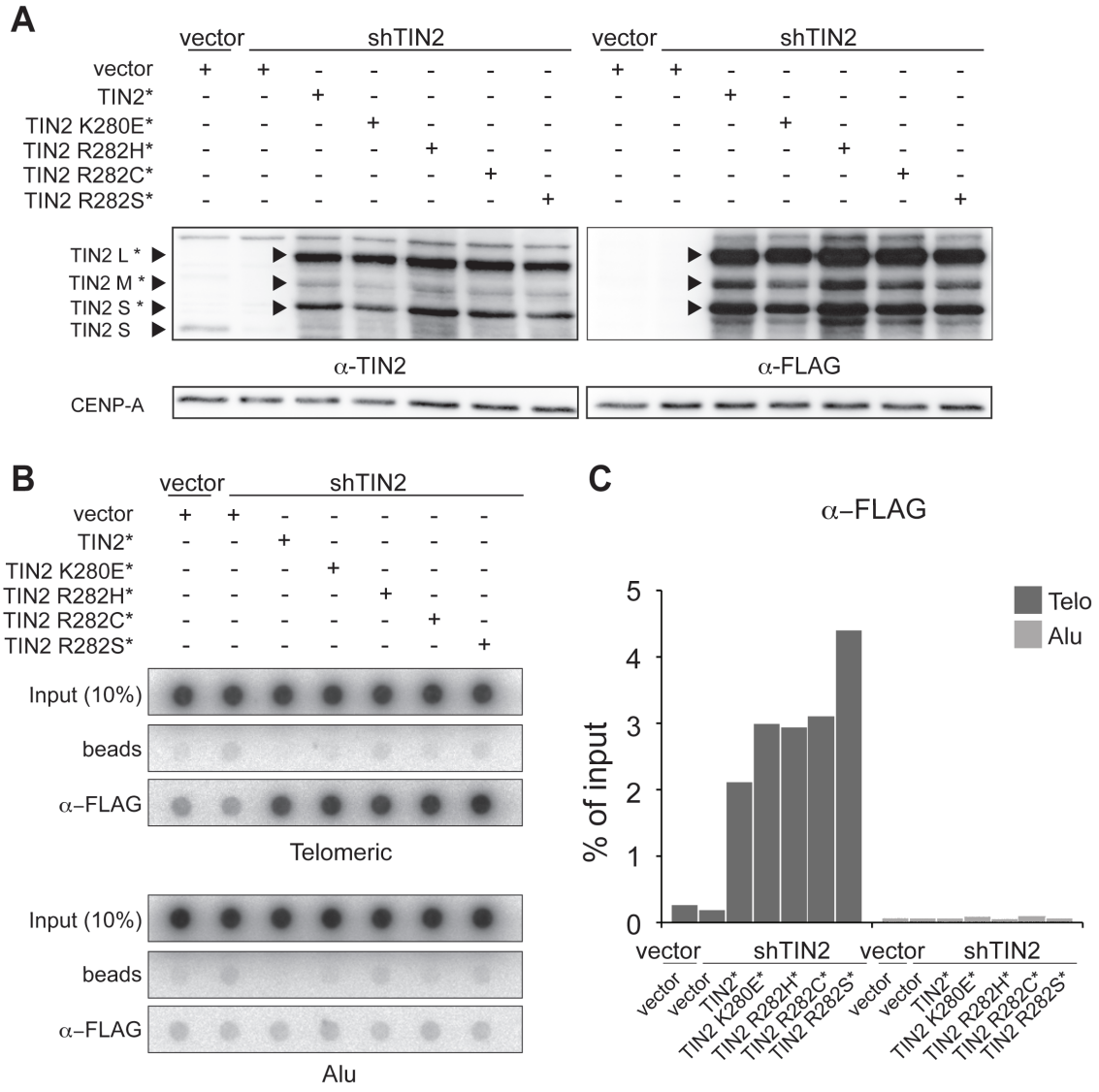


Figure 19. TIN2 DC-isoforms associate with telomeres. (***A***) Immunoblot analysis of ectopically expressed FLAG epitope-tagged, shRNA resistant wild type TIN2 (TIN2*) and FLAG-tagged, shRNA resistant TIN2-DC mutants (TIN2 K280E*; TIN2 R282H*; TIN2 R282C*; TIN2 R282S*). Super-telomerase HeLa cells were cotransfected with the indicated plasmids, and protein expression was analyzed four days after transfection. Arrowheads indicate the presence of endogenous TIN2S and exogenously expressed wild type TIN2* and DC mutant isoforms TIN2S*, TIN2M* and TIN2L*. Immunoblots were probed with anti-TIN2, FLAG and CENP-A antibodies as indicated. CENP-A was used as a loading control. (***B***) ChIP of telomeric and Alu

DNA with FLAG antibody from cells in panel A. (***C***) Quantification of data in panel B representing percentage of telomeric and Alu DNA recovered in FLAG ChIP.

Control (vector) and rescue (TIN2*) plasmids were cotransfected along with the TIN2 shRNA-encoding plasmid. Expression of both the endogenous and exogenous TIN2 was assayed by immunoblot analysis with TIN2 and FLAG antibodies four days following transfection (Figure 19A). The exogenously introduced TIN2 wild type and DC mutants were robustly expressed compared to endogenous TIN2. In particular, the long isoforms (TIN2L) of both TIN2 wild type and DC mutants were more abundantly expressed than their corresponding TIN2S and TIN2M isoforms, an overall expression pattern that differs from the endogenous TIN2 isoforms (Figure 18). Nevertheless, all TIN2 DC mutants were able to associate to telomeres as evidenced by the increase of telomeric DNA recovered in FLAG ChIP, following overexpression of FLAG-tagged TIN2 wild type and DC mutants (Figure 19B and C).

We hypothesize that our system mimics the autosomal dominant expression of *TINF2* mutations in DC patients, due to the robust expression of transgenic TIN2 and incomplete depletion of endogenous TIN2. In addition, we demonstrate that the reported amino acid changes in TIN2 DC mutants do not impair their ability to associate with telomeres.

Overexpression of TIN2 DC proteins does not alter the stability of shelterin proteins

Since TIN2 DC proteins were functional in telomere association, we set out to determine how these telomere-bound TIN2 DC mutants affect the stability of other shelterin proteins. We examined super-telomerase HeLa cells cotransfected with control (vector) or rescue (TIN2*) plasmids along with the TIN2 shRNA-encoding plasmid, for shelterin protein expression by immunoblot (Figure 20). As shown previously, shRNA mediated-depletion of endogenous TIN2 caused destabilization of TPP1 protein and no change in the protein expression of the other shelterin components (Figure 4B, Figure 14 and Figure 20, first and second lane). More

importantly, overexpression of TIN2 DC mutants restored TPP1 protein levels, a phenotype confirmed by the overexpression of the wild type TIN2 (Figure 20 lane 3). The overexpression of wild type TIN2 and TIN2 DC mutants did not trigger changes in TRF1, TRF2 and RAP1 protein levels. POT1 expression could not be assayed due to lack of an antibody for whole cell lysate immunoblot analysis.

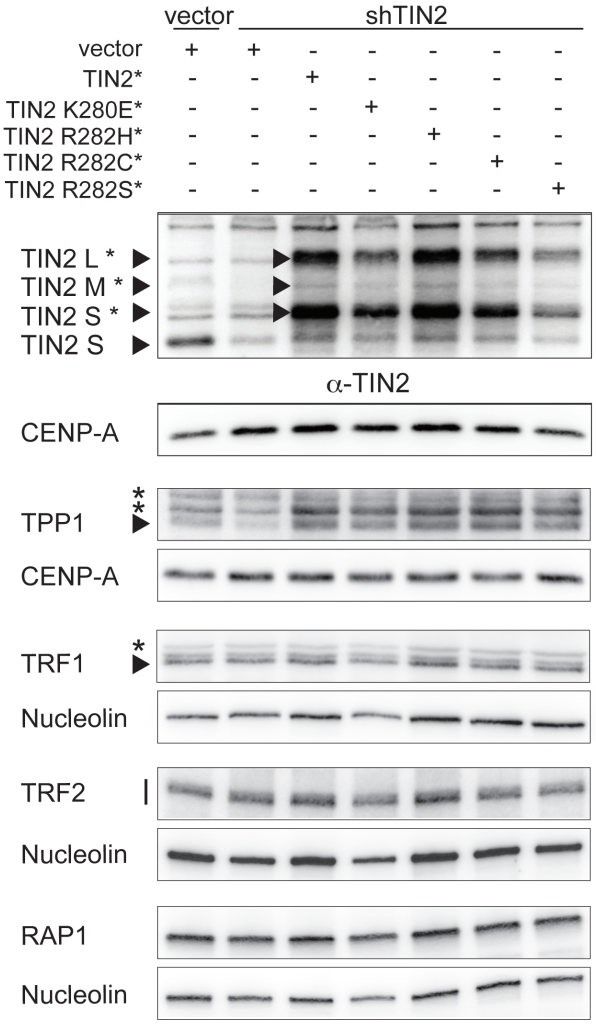


Figure 20. TIN2 DC mutants do not impair protein expression of other shelterin components. Immunoblot analysis of TIN2, TPP1, TRF1, TRF2, RAP1, CENP-A and nucleolin expression. Super-telomerase HeLa cells were cotransfected with the indicated plasmids, and protein expression was analyzed four days after transfection. Arrowheads indicate the presence of endogenous TIN2S, TPP1, TRF1 and exogenously expressed DC mutant isoforms TIN2S*, TIN2M* and TIN1L*. Asterisks indicate unspecific bands. Endogenous TRF2 is detected as two closely migrating bands. CENP-A and nucleolin are used as loading controls.

Our data suggests that TIN2 DC mutants do not compromise the integrity of the shelterin complex; moreover they rescue the reduced TPP1 expression and in this respect they compensate for the loss of endogenous TIN2. However, we did not examine whether shelterin telomere-association is perturbed upon overexpression of TIN2 DC mutants, which would result in a telomere deprotection phenotype, as a proposed cause of defective telomere maintenance in DC.

TIN2 DC proteins do not cause a telomere deprotection phenotype nor do they change hTERT protein levels

In order to test if the telomere protective function is impaired, we set to examine the accumulation of phosphorylated H2AX at telomeres following exogenous expression of TIN2 DC mutants (Figure 21).

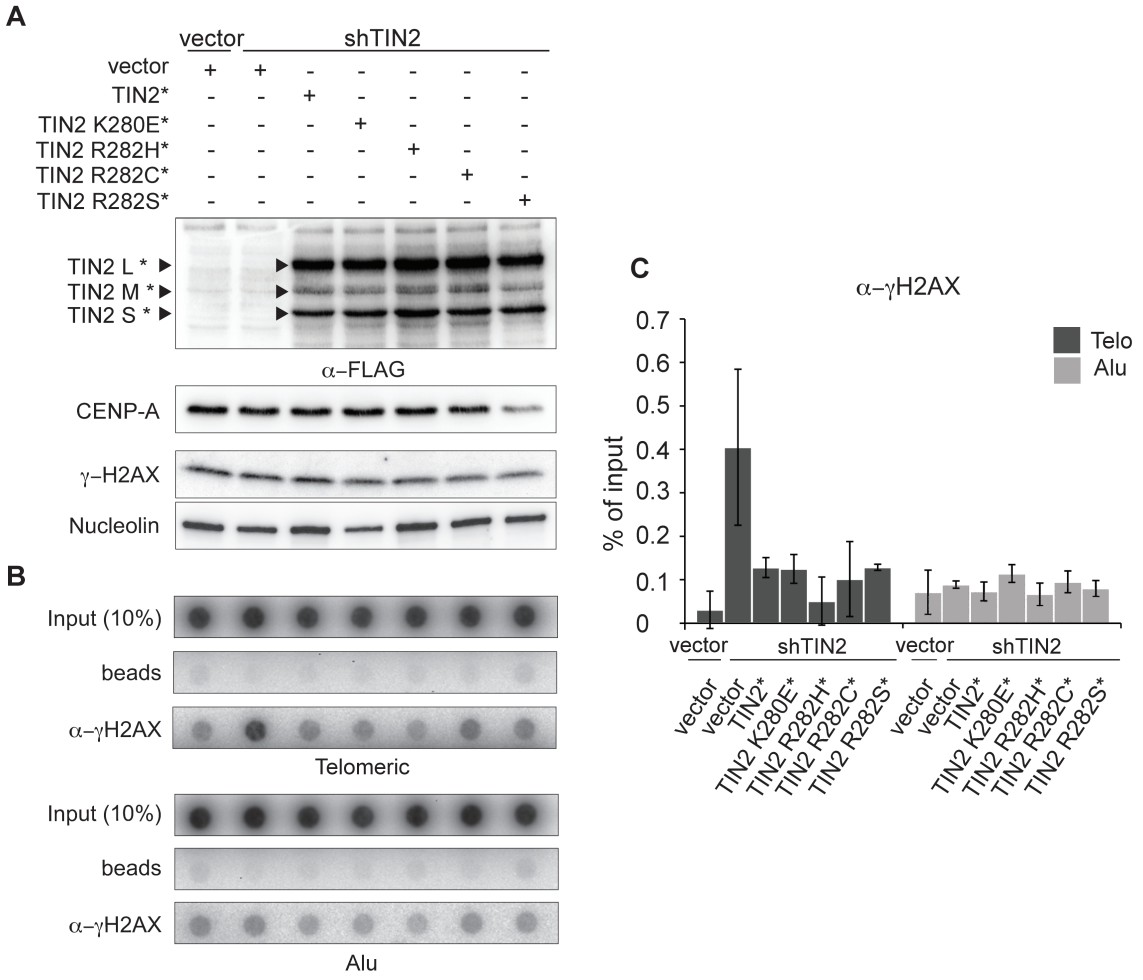


Figure 21. TIN2 DC mutants preserve an intact telomere structure. (***A***) Immunoblot analysis of ectopically expressed FLAG epitope-tagged, shRNA resistant wild type TIN2 (TIN2*), FLAG-tagged, shRNA resistant TIN2-DC mutants (TIN2 K280E*; TIN2 R282H*; TIN2 R282C*; TIN2 R282S*), and cellular levels of γ–H2AX. Super-telomerase HeLa cells were cotransfected with the indicated plasmids, and protein expression was analyzed four days after transfection. Arrowheads indicate the presence of exogenously expressed DC mutant isoforms TIN2S*, TIN2M* and TIN2L*. Immunoblots were probed with anti-FLAG, CENP-A, γ–H2AX and nucleolin antibodies as indicated. CENP-A and nucleolin were used as loading controls. (***B***) ChIP of telomeric and Alu DNA with γ–H2AX antibody from cells in panel A. (***C***) Quantification of data in panel B representing percentage of telomeric and Alu DNA recovered in γ–H2AX ChIP. Error bars correspond to standard deviations of results of three independent experiments.

We observed no change in the overall levels of γ–H2AX upon endogenous TIN2-depletion (Figure 13 and Figure 21A), neither following overexpression of wild type and TIN2 DC mutants (Figure 21A). As expected, TIN2-depletion led to telomere deprotection and accumulation of telomere-associated γ–H2AX, assayed by ChIP (Figure 11A and B; Figure 21B and C). Rescue of TIN2 expression with transgenic wild type TIN2 reduced the accumulation of telomere-specific DNA damage, and similarly the overexpression of TIN2 DC mutants reproduced the rescue effect of wild type TIN2 overexpression (Figure 21 B and C). We note that exogenous expression of wild type and TIN2 DC mutants did not restore the DNA damage response back to endogenous levels and we speculate that this might be an effect from the overexpression *per se*.

The analysis of a large cohort of DC patients with identified *TINF2* mutations, reported no change in hTR levels in whole blood (Walne et al., 2008). However, an identified TIN2 R282H mutation in a patient presented with ataxia-pancytopenia, co-existed with substantially reduced telomerase activity (around 90 percent reduction) compared to control patients (Tsangaris et al., 2008). We reasoned that the reported reduced telomerase activity might be a result of destabilized hTERT protein. Thus, we examined hTERT expression in super-telomerase HeLa cells following overexpression of TIN2 DC mutants (Figure 22).

As shown before, hTERT protein was not affected in TIN2-depleted cells (Figure 4B and Figure 22, lanes 1 and 2). Importantly, the expression pattern of hTERT did not change upon overexpression of TIN2 DC mutants, suggesting that a potential reduction in telomerase activity in super-telomerase HeLa could not be due to impaired hTERT expression. Further analyses on the overall telomerase activity or telomerase activity associated with the TIN2-DC mutants may reveal potential telomerase malfunction at the telomeres harboring *TINF2* mutated proteins.

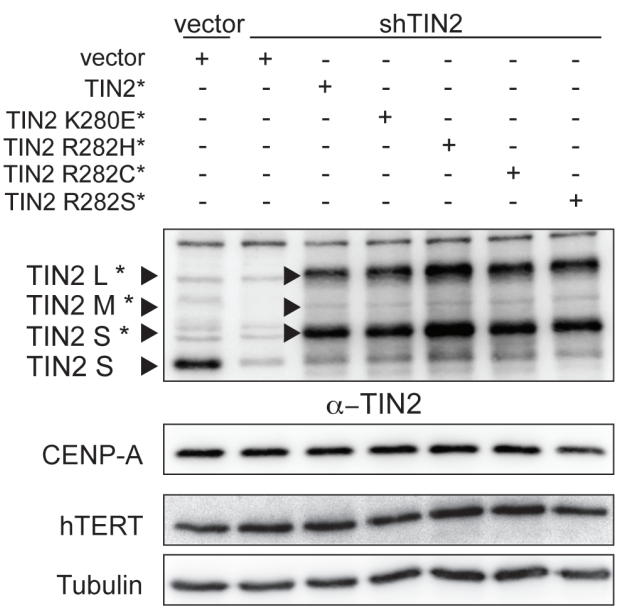


Figure 22. TIN2 DC mutants do not affect hTERT protein levels. Immunoblot analysis of TIN2, CENP-A, hTERT and tubulin expression. Super-telomerase HeLa cells were cotransfected with the indicated plasmids, and protein expression was analyzed four days after transfection. Arrowheads indicate the presence of endogenous TIN2S and exogenously expressed DC mutant isoforms TIN2S*, TIN2M* and TIN1L*. CENP-A and tubulin were used as loading controls.

Taken all together, in super-telomerase HeLa cells the overexpression of TIN2 DC mutants preserved an intact telomere structure and did not affect hTERT stability, therefore an additional defects caused by the DC mutations need to be uncovered in order to explain the short telomere phenotype characteristic for *TINF2*-DC patients.

Discussion

In this chapter we sought to investigate the possible effects of DC-related *TINF2* mutations on telomere protection and telomerase recruitment. *TINF2* is the first reported DC causative gene within the shelterin complex, and *TINF2* mutations account for approximately 10 percent of reported DC cases (Savage et al., 2008; Walne et al., 2008). Another 40 percent of DC patients are reported to have mutations in genes that encode for active telomerase subunits (Bessler et al., 2010). Considering the uniform diagnostic feature of DC, the very short telomere phenotype, it is plausible that the remaining 50 percent of uncharacterized DC patients harbor mutations in the other shelterin genes or other uncharacterized telomerase components.

DC patients with affected *TINF2* gene do not carry mutations in other genes with known telomere functions (Savage et al., 2008). They are characterized with the shortest telomeres compared to other DC subtypes, however it is not clear how the identified TIN2 mutants contribute to this phenotype. The TIN2-DC genomic sequence from which we expressed the mutant proteins in super-telomerase HeLa cells gave rise to all three TIN2 isoforms that were able to associate with telomeres (Figure 19). More importantly, the ectopically expressed TIN2-DC mutants restored the reduced TPP1 expression following TIN2-depletion (Figure 20). We expected that restoring TPP1 protein levels would have reestablished the TIN2/TPP1-tethered telomerase recruitment to telomeres. However, in our preliminary analyses we were not able to properly address this question since our transgenic wild type TIN2 did not rescue the telomerase recruitment defect caused by TIN2 depletion. On the other side, we demonstrated that telomere-bound TIN2-DC mutants do not compromise telomere structure or telomere protective function, moreover they rescue the TIN2-depletion induced DNA damage response at telomeres (Figure 20 and Figure 21). These findings are consistent with previous deletion mutant analyses where TIN2 mutants lacking their carboxy terminal part, downstream of the TRF1-binding domain, did not

induce TIF formation. The carboxy terminal domain of TIN2 also harbors the DC-related amino acid changes, and is dispensable for telomere protection (Kim et al., 2004). Furthermore, we showed that TIN2 DC mutants have no effect on TERT protein expression (Figure 22). *TINF2* mutations did not change hTR levels in samples from analyzed DC patients (Walne et al., 2008), and endogenous TIN2S does not interact with telomerase *in vitro* (Kim et al., 2004). Taken together, the cause of telomere shortening in DC patients with an affected *TINF2* gene could be a defect in telomerase-mediated telomere elongation, rather than impaired telomere protection or catalytically defective telomerase.

Our immunoblot analyses revealed a slightly different expression profile of the transgenic wild type and DC isoforms, from the expression of endogenous TIN2 isoforms (Figure 18 and Figure 19). Namely, transgenic TIN2L was robustly detected and comparable to transgenic TIN2S expression. On the contrary, endogenous TIN2L was less abundant than endogenous TIN2S. Previous expression analyses have shown that TIN2L mRNA levels are elevated and comparable to TIN2S mRNA levels in embryonic stem cells and cancer cells, as opposed to normal somatic cells (Chen, 2009). It is possible that the robust detection of transgenic TIN2L in super-telomerase HeLa cells is due to the strong promoter used in these studies. Alternatively, the nuclear matrix-binding properties of endogenous TIN2L may render it insoluble and difficult to extract for further immunoblot analysis (Kaminker et al., 2009).

Functional analyses of TIN2L and TIN2S have revealed differences in their effect on telomere length regulation. In particular, TIN2L overexpression led to telomere elongation, whereas TIN2S overexpression led to telomere shortening. Taken together, it was suggested that the elevated TIN2L expression, rather than TIN2S, might aid telomerase-mediated telomere elongation in highly proliferative tissues, stem cell compartments and cancer cells (Chen, 2009). Importantly, DC is defined by excessively short telomeres in highly proliferating tissues. We speculate that the elevated expression of TIN2L DC isoforms in super-telomerase HeLa cells resemble

TIN2L expression in highly proliferative tissues and stem cells from DC patients, where they may strongly impair telomerase-mediated telomere elongation. In further support of this notion, patients with *TINF2* mutations tend to have much shorter telomeres at a much earlier age, than patients with DC caused by any other mutated gene. To test this hypothesis we need to examine the telomere length in super-telomerase HeLa cells where TIN2L-DC isoform is selectively expressed in parallel to wild type TIN2L, which is already established as a positive telomere length regulator (Chen, 2009). Additionally, we would need to examine the telomere length phenotype in early generations of super-telomerase HeLa cells that undergo a robust initial telomerase-mediated telomere elongation while expressing TIN2-DC mutants. These data should reconstitute the observed DC-related short telomere phenotype and thus allow us to reflect our conclusions on DC patients with affected *TINF2* gene. Further analyses of the overall telomerase activity, the telomerase activity associated with different TIN2-DC isoforms and the telomerase recruitment will eventually delineate the causative defect of the impaired telomere maintenance observed in DC patients.

Finally, we are constrained in our conclusions by using super-telomerase HeLa cells, as they clearly do not represent a DC environment. It would be interesting to establish cultured cell lines derived from DC patients with affected *TINF2* gene and perform similar analyses. It is possible that these TIN2 mutants have different expression levels in DC patients and/or exhibit different effects on both telomere structure and telomerase-mediated telomere elongation. For now we can only conclude that the proposed amino acid changes within TIN2-DC proteins do not alter their shelterin interactions as they can associate with telomeres and maintain the telomere protective function.

Chapter IV: Conclusions and perspectives

Conclusions

Proper telomere length regulation is crucial for organismal survival as it prevents premature cellular senescence and at the same time suppresses tumor formation. Telomere maintenance is mediated by a joint action of telomerase and telomeric proteins. The molecular mechanisms of human telomerase regulation at telomeres have not yet been elucidated.

Here, we present evidence that shelterin components, regarded both as negative and positive telomere length regulators, recruit telomerase to telomeres *in vivo* and thus, may aid subsequent telomere elongation. In particular, we find that RNAi-mediated reduction of TPP1 reduces telomerase association with telomeres. Furthermore, we find that the recruitment of telomerase by TPP1 depends on its OB-fold domain *in vivo*. Interestingly, TPP1 and TIN2 proteins are partially interdependent for their stability and consequently TIN2 depletion causes the same telomerase recruitment defect seen in TPP1-depleted cells. POT1 binds the telomeric 3' overhang, which is a substrate for the telomerase-telomere elongation. However, in our studies the telomeric 3' overhang seems not responsible for the bulk of telomerase-telomere association. Further, we find that TRF2, a double-stranded telomere binding protein, does not affect telomerase recruitment to telomeres. This indicates that TRF1, another telomeric double-stranded binding protein, is sufficient to recruit telomerase via TIN2 and TPP1 in the absence of TRF2. In conclusion, our study suggests that telomerase is recruited to telomeres primarily via TIN2/TPP1 heterodimer onto the double-stranded part of the telomere, an event that precedes telomere elongation.

TIN2 is the first reported shelterin component to be mutated in dyskeratosis congenita, a disease of defective telomere maintenance. By expressing TIN2-DC mutants in super-telomerase HeLa cells depleted of TIN2, we find that TIN2-DC

mutants are functional in telomere association and they further rescue the reduced TPP1 protein stability and telomere protection. Thus, TIN2 mutants in DC patients most likely impair telomere elongation rather than telomere protection.

Taken together our work has yielded novel insight into the molecular mechanism of telomerase regulation at telomeres. In addition, we hinted the cause of defective telomere maintenance in dyskeratosis congenita patients with mutations in the *TINF2* gene.

Dual role of TPP1 in telomere maintenance

A major concern in our study emerges from previous findings where TPP1 RNAi-mediated depletion, or expression of a TPP1 mutant lacking the POT1 binding domain leads to telomere elongation. These studies indicate that TPP1 is required for POT1 loading onto telomeres (Liu et al., 2004b; Xin et al., 2007a; Ye et al., 2004b) where POT1 acts as a negative regulator of telomere length control. Our data, which implicates TPP1 in telomerase recruitment to telomeres, does not contradict these findings; rather it suggests an additional role of TPP1 fulfilled by its OB-fold domain *in vivo*. The telomerase recruitment role of TPP1 is independent of POT1. We have showed that telomerease is still recruited to telomeres in the absence of POT1 and TPP1 mutant lacking its POT1-binding domain is still able to pull down telomerase activity *in vitro* (Xin et al., 2007a). Due to time constrain, we were not able to conduct and complete telomere length analysis in super-telomerase HeLa cells depleted of TPP1 or expressing a TPP1 OB-fold lacking mutant. Preliminary data, however, did indicate slight telomere shortening both in TPP1-depleted cells and cells expressing a TPP1 OB-fold lacking mutant. Nevertheless, further analyses are required to confirm these telomere length phenotypes. We propose that TPP1 has a double function in telomere maintenance. On one side it mediates POT1 protective function at telomeres and on the other side it recruits telomerase to telomeres independently of POT1.

TPP1-dependent telomerase recruitment to telomeres was recently reported in TPP1-deficient mouse embryonic fibroblasts (MEFs) (Tejera et al., 2010). However, surprisingly in this study the mTERT-chromatin association occurred even in MEFs that carried a homozygous deletion of the telomerase RNA gene. This result contradicts studies in human cells that demonstrate requirement of telomerase RNA expression and hTR binding by TCAB1 for telomerase recruitment to telomeres (Cristofari et al., 2007; Venteicher et al., 2009). Possibly, the discrepancies between these studies reflect differences in telomere biology between mouse and humans or they may arise due to experimental differences. In this same study from the Blasco group, the authors also report that TPP1 null cells fail to elongate their telomeres when reprogrammed into induced pluripotent stem cells (iPS). Previously, telomerase was shown to be upregulated during induction of pluripotent stem cells to levels that can even bypass DC-related dyskerin mutations and restore telomere maintenance (Agarwal et al., 2010). However, this increase in telomerase expression is apparently not sufficient to bypass the need of functional TPP1.

Interdependence of TIN2 and TPP1 in telomerase recruitment to telomeres

It is of a great interest to determine how telomerase interacts TPP1. This interaction could be protein-protein (via hTERT) or protein-RNA (via hTR) dependent or it could be indirect (via other factors/complexes at telomeres). It is also plausible that telomerase in part associates with TIN2. Our ChIP data indicated that not all telomerase is disassociated from telomeres in the absence of TPP1. Furthermore, even though TIN2 protein levels are reduced following TPP1 depletion, there is still about 40 percent residual TIN2 protein detected by our immunoblot analysis. Additionally, rescue of TPP1-depletion by exogenous expression of TPP1 restores completely the telomerase recruitment defect while TIN2 remains reduced by two-fold. In this respect, it would be informative to perform a single and double knock down of

TPP1 and TIN2, analyze telomerase recruitment as well as the telomere length kinetics and TIN2- and TPP1-bound telomerase activity. These results might eventually distinguish independent or interdependent roles of TIN2 and TPP1 in telomerase recruitment. In the same line of analyses, it would be interesting to examine which TIN2 isoform represents the TPP1-binding fraction at telomeres or how much of this TPP1-binding isoform remains at telomeres following TPP1-depletion. This will as well provide additional information on the functional diversity of the TIN2 isoforms.

On the other hand, a recent study on the stoichiometry of telomere-bound shelterin proteins, has suggested that the interpretation of ChIP data on changes in protein occupancy at telomeres may not be straightforward (Takai et al., 2010). For example, expression of dominant negative TRF2 results in an extensive DNA damage response involving most telomeres (Takai et al., 2003). This robust DNA damage response requires loss of more than 90 percent of telomere-bound TRF2, yet TRF2 ChIP efficiency was reduced by only 2-fold (Loayza and De Lange, 2003). The same interpretation could be applied to explain the remaining 60 percent of telomere-associated telomerase following TPP1 depletion. Taken all together, we propose that TPP1 is the predominant telomerase recruitment factor at telomeres.

Telomerase recruitment onto the double-stranded telomere precedes telomere elongation

Our data and previous findings demonstrate that the recruitment step of telomerase is distinct from its telomere-elongation step. TPP1 and POT1 are found in a 1:1 stoichiometry at telomeres, consistent with them forming a heterodimer that is stabilized at telomeres via TIN2 (Chen et al., 2007; Xin et al., 2007a). However, the TPP1/POT1 heterodimer is present in excess over its binding sites onto the 3' telomeric overhang. This indicates that a fraction of telomere bound TPP1/POT1 may not be associated with telomeric single-stranded DNA (Takai et al., 2010). It is tempting to speculate that the fraction of TPP1/POT1 binding the double-stranded part of

telomeres is the dominant fraction that recruits telomerase to telomeres. However, this hypothesis is very difficult to address *in vivo*.

A following question is how telomerase associated to the double stranded telomere part reaches its substrate for telomere elongation. POT1 is the major single-stranded telomere binding protein with high sequence specificity for telomeric substrates (Baumann and Cech, 2001; Lei et al., 2004). Overexpression of POT1 leads to telomere elongation in telomerase-positive cells (Colgin et al., 2003). In addition, POT1 RNAi mediated depletion or expression of POT1 mutant lacking the DNA binding domain also leads to telomere elongation (Loayza and De Lange, 2003; Ye et al., 2004b). *In vitro* studies have already provided evidence of POT1 dual role in regulating telomerase action at telomeres. Namely, POT1 can change its binding site relative to the 3'end and enable telomerase activity and improve its processivity (Lei et al., 2005). Thus, there may be an additional factor that would catalyze interconversion between TPP1/POT1 heterodimers binding the double- and single- stranded telomerc tracts *in vivo*. This will provide telomerase recruited onto the double-stranded telomere to be subsequently positioned on the 3'end for telomere elongation. It is very likely that these factors act in a cell cycle-dependent manner, since telomerase-mediated telomere elongation is restricted to S-phase. The telomerase recruitment step is also restricted to S-phase, evidenced by the S-phase specific hTR recruitment to telomeres in parental HeLa cells. However, we hypothesize that in super-telomerase HeLa cells, telomerase is recruited onto the double-stranded telomere part throughout the cell cycle and only during S-phase is repositioned onto the 3'overhang. Nevertheless, in both scenarios, protein modifications on shelterin components may trigger cell cycle regulated changes in the shelterin conformation and subsequent telomerase repositioning onto the telomeric overhang. Discovery and functional characterization of such transient posttranslational modifications in telomere length regulation awaits further investigation.

Dissecting the shelterin complex: subcomplexes and their role in telomerase recruitment

Meanwhile, our study raises more questions on the different aspects of telomere length maintenance by shelterin proteins. The notion that TRF2 protein is not required for telomerase recruitment to telomeres clearly evokes existence of different shelterin subcomplexes. In particular, we propose a TIN2/TPP1/POT1 ternary complex that binds to TRF1 and thus remains anchored to telomeres independently of TRF2. It would be interesting to investigate the TRF1 effects in this respect and whether its loss can be compensated by TRF2.

Initially, TIN2 was discovered to interact with both TRF1 and TRF2 simultaneously, and disrupting this interaction destabilized both TRF1 and TRF2 at telomeres (Kim et al., 2004). Similarly, RNAi-mediated depletion of TRF1 or TIN2 decreased the presence of both TRF2 and RAP1 at telomeres. On the contrary, gel filtration analysis identified a TRF2 complex containing TIN2 but not TRF1; indicating that TRF1 is not required for this interaction (Ye et al., 2004a). Furthermore, after removal of TRF1 from telomeres by overexpressing tankyrase1, TIN2/TPP1 complex remained on telomeres via an increased association with TRF2 (Houghtaling et al., 2004). Interestingly, another study provided again contrary data where stripping off TRF1 from telomeres, by tankyrase1 overexpression, reduced telomere recruitment of both TIN2 and TRF2 (Liu et al., 2004a). Gel filtration analysis and co-immunoprecipitation experiments in the same study, found all six shelterin components to associate in one high molecular weight complex (Liu et al., 2004a). TPP1 along with TIN2 was identified as a key component in mediating the six-protein complex assembly. In particular, TPP1 reduction also reduced the ability of TRF1 to associate with TRF2 (O'Connor et al., 2006). These findings are supported by the fact that TPP1 loading onto telomeres requires interaction with TIN2 (Chen et al., 2007; Houghtaling et al., 2004). Lastly, dissection of shelterin via immunoprecipitation and gel filtration

identified two major TIN2 complexes, TIN2/TRF1 and RAP/TRF2/TIN2/TPP1/POT1, which functioned differently in cellular senescence and cell survival (Kim et al., 2008).

Taken together, while providing invaluable information these studies lack a systematic investigation of the shelterin subcomplexes at telomeres, eventually resulting in various and contradictory data. The interactions between the six shelterin components must undergo dynamic changes in order to maintain telomeres during different cellular states. It has been shown that the telomere structure does change in a cell cycle-dependent manner and becomes more open and accessible for the DNA damage machinery during and after telomere replication (Verdun and Karlseder, 2006). Thus, we need to investigate shelterin subcomplexes and their interactions throughout the cell cycle. Detailed analyses of the protein composition of these subcomplexes could be performed on cell extracts from synchronized cell populations from different cell cycle stages. In addition, coimmunoprecipitation analysis will reveal cell cycle regulation of protein interactions among shelterin components. These studies will determine shelterin-specific structure for telomerase recruitment and/or telomere elongation, and eventually delineate the regulatory mechanism of telomere structure dynamics.

Functionally distinct shelterin isofoms contribute to shelterin complexity

Another level of shelterin complexity emerged with the identification of TIN2 and POT1 isoforms. cDNA cloning revealed three functionally different TIN2S, TIN2M and TIN2L isoforms with tissue specific regulated expression (Chen, 2009). TIN2L is enriched in highly proliferative tissues, embryonic stem cells and cancer cells and its overexpression leads to telomere lengthening in telomerase positive cells. TIN2S is ubiquitously expressed and acts as a negative telomere length regulator. Both TIN2S and TIN2L interact equally with TPP1 and TRF1, however TIN2L is less associated with TRF2 compared to TIN2S (Chen, 2009). TRF2 is proposed to remodel telomeres into t-loop structures, presenting a closed telomere state inaccessible for telomerase

(Griffith et al., 1999). Therefore, it is tempting to speculate that TIN2L is the predominant isoform that mediates telomerase-dependent telomere elongation in cancer cells and ES cells (Chen, 2009). Furthermore, enriched TIN2L/TPP1 telomerase recruiting complexes at telomeres may explain the telomere extension events at most telomeres in cancer cells. On the other hand, primary human fibroblasts that preferentially elongate short telomeres upon ectopic telomerase expression, could have more TIN2S/TPP1 heterodimers loaded onto their telomeres (Britt-Compton et al., 2009; Zhao et al., 2009).

POT1 expression is also regulated by alternative splicing and produces five variants with a poorly understood function. Similar to TIN2 isoforms, POT1 variants have tissue specific expression with four variants being ubiquitously expressed and the fifth one being leukocyte specific. In addition, all five POT1 variants have different telomeric DNA binding affinity *in vitro* (Baumann et al., 2002). Taken together, POT1 and TIN2 isoforms potentially increase the diversity of protein interactions within the shelterin complex at telomeres. Characterization and functional analysis of shelterin complexes containing different TIN2 and POT1 isoforms will further elucidate different mechanisms in human telomere maintenance.

Shelterin and CST protein complexes coexisting at telomeres

Finally, shelterin is not the only protein complex residing at human telomeres. Recently, a novel complex (CST) comprised of OB-fold containing CTC1, human Stn1 and human Ten1 proteins, was found to associate with a fraction of telomeres throughout the cell cycle (Miyake et al., 2009). CST binds single-stranded DNA with high affinity but in a sequence-unspecific manner. The conserved telomere maintenance component 1 (CTC1) was also identified as one subunit of the alpha accessory factor (AAF-132). The second subunit of the alpha accessory factor corresponds to the mammalian ortholog of Stn1 (AAF-44 also known as OBFC1)(Casteel et al., 2009; Surovtseva et al., 2009). Alpha accessory factor (AAF) is

a heterodimer that stimulates Pol α-primase association with single-stranded DNA, allowing the enzyme to prime and elongate with increased processivity (Goulian and Heard, 1990). CTC1 RNAi-mediated depletion impaired chromosome segregation and telomere integrity leading to formation of chromatin bridges and sporadic telomere loss. More detailed analysis on the telomere structure revealed an increase in G-overhang length following CTC1 depletion (Surovtseva et al., 2009). Similarly, RNAi-mediated reduction of Stn1 increased the amount of the single-stranded G-strand (Miyake et al., 2009). Thus, the CST complex appears to be a specialized replication complex that is required for telomere protection and/or telomere replication.

Human CST was shown to bind to telomeres independently of POT1 (Miyake et al., 2009). However, human Stn1 copurified with TPP1, suggesting possible existence of shelterin-CST complexes (Wan et al., 2009). Since the CST complex is lacking sequence-specificity for telomeric ssDNA it could be that it requires interaction with TPP1 in order to bypass the competing sequence-specific ssDNA-binding protein, POT1, and load onto telomeres. The latter indicates that TPP1 might promote a switch between telomerase activity and Pol α-primase activity via CST. Thus, both complexes might cooperate to couple G-strand elongation by telomerase and subsequent C-strand synthesis by Pol α-primase. It would be interesting to temporally separate the events of CST, telomerase and Pol α-primase recruitment to telomeres and which shelterin-CST interactions mediate the transition from one event to another.

In summary, telomeres are an elaborate higher-order DNA architecture supplemented with a suite of proteins mediating complex network of interactions that enables telomere maintenance. Systematic investigation of separate events like the telomerase recruitment described in this work, will eventually clarify the complex nature of telomeres and broaden our understanding in telomere biology.

Chapter V: Materials and methods

Plasmids

ShRNA vectors were prepared by cloning double-stranded DNA oligonucleotides into pSUPER-Puro (Azzalin and Lingner, 2006). The target sequences were as follows:

shRNA plasmid:	Target sequence:	Source:
shTPP1	GACTTAGATGTTCAGAAAA	(Ye and de Lange, 2004)
shTIN2	GTGGAACATTTTCCGCGAGTACTGGAGT	
shPOT1-1	GTACTAGAAGCCTATCTCA	(Ye et al., 2004b)
shPOT1-2	GGGTGGTACAATTGTCAAT	
shTRF2	GCGCATGACAATAAGCAGA	

Full-length TPP1, TPP1 lacking the OB fold (TPP1ΔOB) (Xin et al., 2007a) and full-length TPP1 bearing two silent mutations in the shRNA target site were epitope tagged (N-terminal 1xFLAG and C-terminal 3xFLAG from Sigma) and expressed from pcDNA6/*myc*-His A (Invitrogen). TIN2 genomic sequence (Chen, 2009) was epitope tagged (N-terminal 3xFlag from Sigma) and expressed from pcDNA6/*myc*-His A (Invitrogen). All TIN2 constructs carried three silent mutations in the shRNA target site. Dyskeratosis congentia (Savage et al., 2008; Walne et al., 2008) and shRNA point mutations were generated by QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) using the following mutagenic primers:

mut shTPP1	AACCAAGACTTGGATGTACAGAAAAAGCTCTATG
mut shTIN2	GCGTGGAACACTTTCCGCGTGTACTGGAATTTCTGCGATCTCTGC
mut K280E	CCACTAGGGGAGGCCATGAGGAGCGCCCCACAGTC
mut R282H	GGGAGGCCATAAGGAGCACCCCACAGTCATGCTGTTTC
mut R282C	GGAGGCCATAAGGAGTGCCCCACAGTCATGCTGTTTCC
mut R282S	GGAGGCCATAAGGAGAGCCCCACAGTCATGCTGTTTCC

Cell culture and transfection

Super-telomerase HeLa cells were generated as described previously (Cristofari and Lingner, 2006) and cultured in high glucose Dulbecco's modified Eagle medium (GlutaMAX from GIBCO) supplemented with 10% fetal calf serum (FCS) (Sigma) and 1% penicillin-streptomycin (Invitrogen). All cells were cultured at 37°C with 5% CO_2. Cells were transfected using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Puromycin (1µg/ml) (InvivoGen) was added to the medium 24h after transfection of pSUPER-Puro derivatives. Puromycin selection was maintained until cells were harvested for analyses and mock-transfected cells were dead approximately 4 days posttransfection. For TPP1 shRNA and TIN2 shRNA-treated cells, ChIP, immunoblot, qRT-PCR and RQ-TRAP analyses were performed 4 days posttransfection. For TRF2 shRNA and POT1 shRNA-treated cells, ChIP, immunoblot, coimmunoprecipitation, qRT-PCR, and RQ-TRAP analyses were done 6 days posttransfection.

For FISH and IF, HeLa cells and super-telomerase HeLa (Cristofari and Lingner, 2006) cells were grown on coverslips in D-MEM media (Fisher Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific). Transfections were carried out using Lipofectamine 2000 transfection reagent, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were selected in 1µg/ml puromycin (Sigma-Aldrich) for 48h following transfection. In some cases, cells were synchronized to mid-S phase using double thymidine block as previously described (Tomlinson et al., 2006) except that 18h thymidine treatments were used. (Cells were released for 9h in between thymidine treatments). Bromodeoxyuridine (BrdU) labeling was performed as described previously (Tomlinson et al., 2006) to confirm S-phase synchronization.

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

Three DNA probes (probes 1, 2, and 3), complementary to different regions of telomerase RNA, were used in hTR FISH (Tomlinson et al., 2006). A fourth DNA probe complementary to the G-rich strand of the telomere (CT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*AACCCTAACCCT*A (T* indicates aminoallyl-modified thymidines)) was synthesized by Qiagen (Valencia, CA) and used to detect telomeres. Probes were conjugated with Cy3 or Oregon green mono-functional reactive dye according to the manufacturer's protocol (GE Healthcare, Little Chalfront, Buckinghamshire, United Kingdom, Invitrogen). A 25-ng of each Cy3-labeled hTR FISH probe and/or 0.2 ng of telomere FISH probe were used per coverslip. FISH was performed as described previously (Tomlinson et al., 2006). However, when hTR FISH was performed in combination with BrdU or telomere FISH, the cells were subjected to a 10 minute denaturation at 85°C in 70% formamide, 2xSSC (1xSSC is 0.15 M NaCl plus 0.015 M sodium citrate) prior to FISH.

Following FISH, cells were analyzed by IF as described previously (Tomlinson et al., 2006). Cells were washed three times with 1X PBS and blocked for 1hr in 0.05% Tween-20 in PBS (PBS-T) or 3% BSA in PBS. Next, cells were incubated with either one of several combinations of the following primary antibodies at the indicated dilution for 1h at room temperature: mouse anti-p80 coilin (1:5000, Π)(Almeida et al., 1998), mouse anti-TRF2 (1:1000, Imgenex Corp., San Diego, CA), rabbit anti-hTERT (1:400, Rockland, Gilbertsville, PA), mouse anti-FLAG (1:500, Sigma-Aldrich, St. Louis, MO), rabbit anti-RAP1(1:2000, Novus Biologicals, Littleton, CO) and rabbit anti-53BP1(1:500, Bethyl, Montgomery, Texas). Cells were washed three times in 1X PBS and then incubated with secondary antibody ((1:100 Cy2-conjugated goat anti-rabbit IgG (H+L), 1:100 Cy2-conjugated goat anti-mouse IgG (H+L), 1:100 Cy5-conjugated goat anti-mouse IgGγ, 1:100 Cy5-conjugated goat anti-rabbit IgG (H+L), 1:100 AMCA-conjugated goat anti-mouse IgG (H+L), or 1:100 AMCA-conjugated goat anti-rabbit IgG

(H+L)) (all from Jackson ImmunoResearch laboratories, West Grove, PA) for 1h at room temperature. Primary antibodies were diluted in PBS-T or 3% BSA in PBS, while secondary antibodies were diluted in PBS-T only. Cells were subjected to three final 1X PBS washes and mounted in Prolong Gold (Invitrogen).

Microscopy

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

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously (Cristofari and Lingner, 2006) with the following modifications. For immunoprecipitations, 25 µl of hTERT R484 rabbit serum (Wenz et al., 2001), 2.5 µg mouse monoclonal TPP1 antibody (ACD; Abnova H00065057-M02), 2 µg mouse monoclonal FLAG M2 antibody (Sigma F3165) or 2 µg mouse monoclonal γH2AX antibody (Millipore 05-636) was used, and the

mixtures were incubated for 6h at 4°C with 50 µl of a 50% slurry of protein A/G-Sepharose beads (GE Healthcare). Telomeric DNA was detected as described previously (Azzalin et al., 2007). For detection of Alu sequences, a 5' ^{32}P-labeled oligonucleotide probe (5'-GTGATCCGCCCGCCTCGGCCTCCCAAAGTG-3') was used.

qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). The isolated RNA fraction was treated with RNase-free DNase (Qiagen) and repurified with the TRIzol LS reagent (Invitrogen). For quantitative reverse transcriptase PCR (qRT-PCR), cDNA was prepared from 2 µg total RNA, using random primers and the SuperScript III reverse transcription (Invitrogen) followed by qPCR on a 7900HT fast real-time PCR System (Applied Biosystems), using the PowerSYBR Green PCR master mix (Applied Biosystems). For PCR amplification of TIN2 cDNA, forward and reverse primers were 5'-GTCAGAGGCTCCTGTGGATT-3' and 5'-CAGTGCTTTCTCCAGCTGAC-3', respectively; POT1 cDNA was amplified with previously described primers (Kondo et al., 2004). Serial dilutions of TIN2 and POT1 cDNAs were used to determine amplification efficiencies. TIN2 and POT1 quantities were normalized to the level of β-actin cDNA.

Immunoblots

A total of $5x10^4$ cells (or $1x10^4$ cells for FLAG immunoblots) were boiled for 5 min in Laemmli loading buffer and fractionated on 4 to 20% SDS-polyacrylamide gradient gels (Lonza) except for TIN2 (10% polyacrylamide gel) and POT1 (8% polyacrylamide gel). Standard immunoblot protocols were used with the following antibodies: mouse monoclonal TPP1 antibody, ACD Abnova H00065057-M02 (1:1000); rabbit polyclonal TIN2C 701 antibody, a kind gift from S. Smith (1:1000); rabbit polyclonal hPOT1 978 antibody, a kind gift from T. de Lange (1:1000); rabbit-

polyclonal α-hTERT antibody, Rockland 600-401-252 (1:2500); rabbit polyclonal CENP-A antibody, Upstate 07-240 (1:2000); mouse monoclonal γ-H2AX antibody, Millipore 05-636 (1:2000); mouse monoclonal FLAG M2 antibody, Sigma F3165 (1:5000); mouse monoclonal TRF2 antibody, Upstate 05-521 (1:1000); mouse monoclonal TRF1 antibody, Sigma T1948 (1:1000); rabbit polyclonal RAP1 antibody, Novus biologicals NB100-292 (1:1000); mouse monoclonal nucleolin antibody, MBL M019-3S (1:1000) and mouse monoclonal tubulin antibody, Sigma T9026 (1:2000). For POT1 immunoblots, guanidine renaturation was performed as described previously (Loayza and De Lange, 2003). Secondary horseradish peroxidase-conjugated goat antibodies against rabbit or mouse IgG (1:3000; Promega) were used to reveal the primary antibodies. The AlphaInnotech chemoluminescence substrate and imaging system was used for signal detection and quantification.

Coimmunoprecipitation

Coimmunoprecipitation of endogenous TPP1 and POT1 proteins was performed in super-telomerase HeLa cells transfected with two different shRNAs against POT1. Pre-cleared cell lysates from 10^6 cells were prepared as previously described (Loayza and De Lange, 2003). Lysates were immunoprecipitated with 10 µg mouse monoclonal TPP1 antibody ACD Abnova H00065057-M02 and immune complexes were bound to a 50% slurry of protein G-Sepharose beads (GE Healthcare). After an overnight incubation at 4°C, beads were washed four times with lysis buffer (Loayza and De Lange, 2003) and proteins were eluted with Laemmli loading buffer for analysis on 8% SDS-PAGE.

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

Telomerase activity was measured as previously described (Cristofari and Lingner, 2006) with the following modifications. Reaction mixtures containing the PowerSYBR Green PCR master mix (Applied Biosystems), 1.8 µg undiluted or 3-fold

diluted cell extracts, 1 μM telomerase primer TS, 0.3 μM reverse primer ACX, and 0.5 mM $MgCl_2$ were incubated for 30 min at 30°C and for 10 min at 95°C. Using the 7900HT fast real-time PCR System (Applied Biosystems) samples were amplified in 40 PCR cycles for 15 s at 95°C and 1 min at 60°C. Threefold serial dilutions of the empty-vector-transfected samples were used to obtain a standard curve of the form $\log_{10}$ (protein quantity) = aC(t)+b, where C(t) is the threshold constant, a is the slope of the curve, and b is the y intercept. Telomerase activity was expressed relative to this standard as the quantity of standard sample extract giving the same C(t) value. All samples were serially diluted to verify the linearity of the RQ-TRAP reaction and heat inactivated to verify that the amplification product was attributable to telomerase activity.

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