

**THE STRUCTURE OF MAMMALIAN TELOMERES ASSOCIATED WITH  
THE SHELTERIN SUBCOMPLEXES**

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## **ABSTRACT**

### **Ceylan Cakit: The Structure of Mammalian Telomeres Associated with the Shelterin Subcomplexes (Under the direction of Dr. Jack D. Griffith)**

Telomeres are the specialized DNA-protein complexes at eukaryotic chromosome ends. They are essential to protect linear chromosome ends from nucleolytic attack and chromosomal end-to-end fusions, which may lead to chromosomal abnormalities. Genomic instability, a hallmark for both cancer and aging, is majorly induced by chromosomal abnormalities. Therefore, telomere length and structure are equally important for the maintenance of genomic stability. Mammalian telomeres associate with a six-subunit, telomere-specific shelterin complex, as well as shelterin subcomplexes. In this study, I will visualize the structure of mammalian telomeres associated with the shelterin subcomplexes using electron microscopy (EM). In order to achieve this goal, I will examine the association of model telomeres with single-stranded and double-stranded telomeric DNA repeat binding proteins separately. This study will be extremely valuable for understanding the mechanistic details of the regulation of mammalian telomere structure and function by the shelterin complex subunits and subcomplexes.

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## LIST OF ABBREVIATIONS

A	Adenine
ATP	Adenosine 5'-TriPhosphate
ALT	Alternative lengthening of telomeres
bp	Base pair
C	Cytosine
°C	Celsius
cPot1	Chicken protection of telomeres 1
C-terminus	Carboxyl-terminus
ds	Double stranded
DNA	Deoxyribose nucleic acid
EM	Electron microscopy
FBS	Fetal bovine serum
G	Guanine
hPot1	Human protection of telomeres 1
hRap1	Human repressor/activator protein 1
hTpp1	Human tripeptidyl peptidase 1
hTin2	Human Trf1-interacting nuclear protein 1
hTrf1	Human telomere repeat binding protein 1
hTrf2	Human telomere repeat binding protein 2
kb	Kilobase

min	Minute
ml	Milliliter
MOI	Multiplicity of infection
N-terminus	Amino-terminus
nt	Nucleotide
<sup>32</sup> P	Radioactive phosphorus isotope
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
pH	Parts hydrogen
Pip1	Pot1-interacting protein 1
Ptop	Pot1 and Tin2 organizing protein 1
RNA	Ribonucleic acid
ss	Single stranded
SDSPAGE	Sodium dodecyl sulfate
T	Thymine
t-circle	Telomere circle
t-loop	Telomere loop
Tint1	Tin2-interacting protein 1
TRF	Telomere Repeat Fragment
TRFH	Telomere Repeat Fragment Homology

## CHAPTER 1

### MAMMALIAN TELOMERES AND THE SHELTERIN COMPLEX

#### 1.1. Telomeres and Genomic Stability

Throughout the lifetime of every cell and organism, proper maintenance of DNA represents the major challenge. Genomic instability is one of the primary reasons for both cancer and ageing. Protection of genetic material in eukaryotes becomes much more complex due the linear nature of chromosomes. Telomeres are the specialized DNA-protein structures at the ends of linear eukaryotic chromosomes. These structures enable cells to recognize natural and broken chromosome ends as distinct structures (1). Therefore, telomeres serve an essential function by protecting chromosome ends from degradation, terminal fusions, and inappropriate repair activities. Functional telomeres are not recognized and processed as double strand breaks (2, 3), whereas telomere dysfunction caused by either the loss of telomeric DNA or telomere protection activates DNA damage response pathways (4, 5, 6). The characteristics of telomere dysfunction in mammalian cells can be outlined as telomeric DNA degradation and cell cycle arrest accompanied by chromosomal fusions and genomic instability (7, 8). As a consequence, genomic stability relies on the proper maintenance of telomere structure and function. **Therefore, the long-term goal of this study is to elucidate**



## **telomere structure and its regulation by telomere-specific proteins.**

In mammalian telomeres, which are the focus of this study, the DNA component is made up of tandem repeats of double stranded TTAGGG that terminate in a 150-200-nucleotide long single-stranded G-rich strand 3' overhang (7, 8, 9). Telomeric DNA folds into a “telomere loop (t-loop)” structure, where the G-rich 3' single-stranded overhang invades the duplex telomeric tract (Figure 1.1). T-loops were first discovered via visualization of purified mammalian telomeric DNA using electron microscopy (9). To date, telomeres of many organisms including humans, mice, chickens, plants, yeast and ciliates have also been shown to form t-loops (9, 10, 11, 12). It is not known whether the t-loop is the most common structure of natural chromosome ends, or if it is the structures of all chromosome ends *in vivo* (8, 16). However, many studies revealed that the proper protection and regulation of telomeric structures are central to the suppression of the cell cycle checkpoint activation at natural chromosome ends (4, 13, 14, 15). Therefore, structural folding of telomeric DNA into t-loops may be a required conformational change for the inhibition of an inappropriate DNA damage response. Alternatively, t-loop structure may be regulatory for limiting the accessibility of chromosome ends to the telomere-interacting factors such as telomerase (8, 16).

### **1.2. Maintenance of Telomere Length**

The average telomere length in human cells is within a range of 5-15 kb and becomes shorter by 150-200 base pair with every successful DNA replication, since the very end of

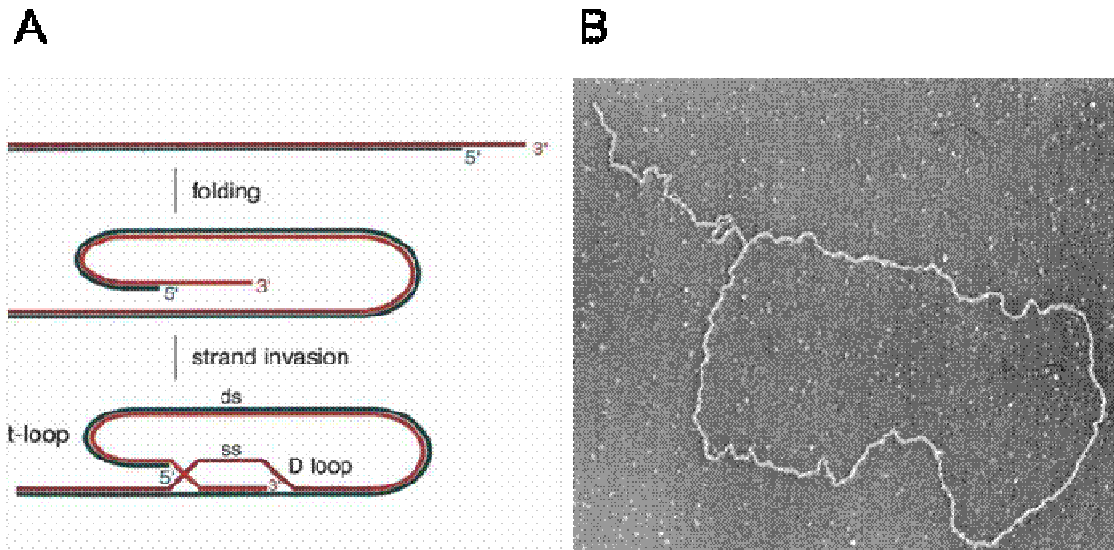


Figure 1.1: Structure of DNA in a telomere loop (t-loop). A) Schematic of DNA structure in a telomere loop (t-loop) (16). Red line indicates the G-rich telomeric repeat sequence, while black line indicates C-rich telomeric repeat sequence. B) Electron micrograph of telomeric DNA isolated from HeLa subclone 1.2.11 in a t-loop conformation (9).

the lagging strand cannot be replicated by DNA polymerases, referred to as the “end-replication problem” (17, 18, 19). As a consequence, telomeres reach a critical length after about 50 cell divisions (Hayflick limit), where normal somatic cells stop dividing and permanently arrest, a phenomenon called “replicative senescence” (20). This gradual decline in telomere length sets a limit to the lifespan of normal somatic cells. Therefore, telomeres act as the biological clock of a cell (19).

In contrast to normal somatic cells, the majority of cancer cells circumvents “replicative senescence”, and become immortal by expressing telomerase. Telomerase is a unique eukaryotic reverse transcriptase and is responsible for adding telomeric repeats to the 3' G-rich overhang of DNA at telomeres using its own RNA subunit as a template (21, 22). Telomerase expression is not a general feature of normal somatic cells. Most cancer cells turn on telomerase expression, whereas some telomerase deficient cancer cells use “Alternative Lengthening of Telomeres” (ALT) (23, 24) for immortalization. ALT cells depend on homologous recombination for telomere length maintenance (23, 24, 25, 26); the exact mechanistic details of this pathway are still being investigated. However, highly heterogeneous telomeres in length ranging in between 2-20 kb in humans and free telomeric circles (t-circles) are markers for ALT cells (25, 26, 27). Alternatively, both telomerase expression and ALT pathway activation may be operational simultaneously for some cancer types or under certain circumstances in order to maintain telomere length (28).

### **1.3. Shelterin Complex**

Telomeres are known to associate with telomere-specific proteins as well as with many repair and recombination proteins. In humans, shelterin is a telomere-specific, multi-

protein complex, which interacts with chromosome ends throughout the cell cycle. It has six subunits: Trf1, Trf2, hRap1, Tin2, Tpp1, and Pot1 (Figure 1.2) (16, 29). The proposed functions of the shelterin complex at the telomeres include the protection of telomeric DNA protection and the regulation of telomere accessibility (16). In any case, the dynamic interactions of shelterin complex with telomeres, as well as the dynamic interactions within the shelterin complex subunits, are central for telomere structure and function. However, the molecular details of these interactions are still unknown. **Therefore, the objective of this study is to elucidate the structure of mammalian telomeres associated with the shelterin complex and to understand telomere structure regulation by the shelterin complex. I hypothesize that shelterin complexes formed by different combinations of shelterin subunits will regulate telomere function by inducing structural alterations in telomere structure.**

The shelterin complex specifically interacts with telomeric DNA, since the shelterin subunits; Trf1, Trf2 and Pot1 have the ability to recognize and bind to TTAGGG repeats. Trf1 and Trf2 interact with double-stranded TTAGGG repeats through their single myb-like DNA binding domain at their carboxyl-terminus (30). Single base-alterations in TTAGGG repeat sequences are sufficient to diminish the association of Trf1 and Trf2 with telomeres (31, 32). Furthermore, binding of Trf1 and Trf2 to telomeres as homodimers (33), or as higher order oligomers, increases the level of specificity, so that only a tandem array of TTAGGG repeats becomes the DNA target. As a consequence, shelterin can distinguish isolated occurrences of TTAGGG repeats in the rest of the genome. Similarly, Pot1 recognizes and binds specifically to the single-stranded 3'-end and internal 5'-TAGGGTTAG-3' sequences via its N-terminal DNA binding domain (34, 35). Taken as a whole, the highly specific association of Trf1, Trf2, and Pot1 with TTAGGG repeats forms

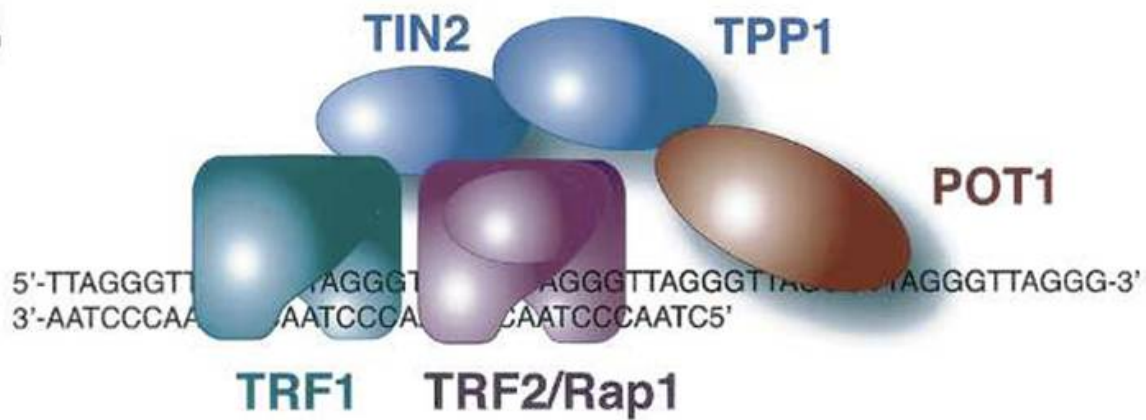


Figure 1.2: Schematic of the shelterin complex on mammalian telomeric DNA. Pot1 binds to the 3'-end single-stranded TTAGGG repeats. Tpp1 is the only Pot1-interacting subunit. The interaction within the shelterin complex is bridged by the interaction between Tpp1 and Tin2. Tin2 binds to both Trf1 and Trf2, which recognize and bind to double-stranded TTAGGG repeats. hRap1 only binds to Trf2 in the complex. As shown in the figure, Tpp1, Tin2, and hRap1 do not directly associate with TTAGGG DNA repeats (16).

the scaffold for the loading the other three subunits of the shelterin complex: Tin2, Tpp1, and hRap1 (16).

Trf1 is a “negative regulator” of telomere length. Trf1 overexpression results in gradual decrease in telomere length, while Trf1 inhibition results in telomere elongation (36). Equally important Trf2 also plays role in telomere length regulation and it is essential for telomere protection. Trf2 overexpression protects even the critically short telomeres, whereas Trf2 inhibition results in total telomere protection loss followed by terminal fusions (6, 37). Recently, Trf2 has been suggested to have a role in telomere replication, where the replication is challenging due to its repetitive nature of telomeric DNA (38, 39).

Pot1, being the only single-stranded TTAGGG repeat binding protein, is the most conserved subunit of the shelterin complex (16, 35, 40). Pot1 inhibition leads to increased telomere length in telomerase positive cells, indicating that single-strand 3'overhang becomes accessible to telomerase in the absence of Pot1 (41). On the contrary, a recent report suggests a role for Pot1 and its interaction partner Tpp1 in telomerase progression (42). On the other hand, another recent study reveals mammalian Pot1's requirement for Tpp1 for its telomere safeguarding function (43). In agreement with this, Tpp1 [Ptop (44), Pip1 (45) and Tint1 (46)] have already been shown to have an effect on Pot1's localization to telomeres (44, 45).

Within the shelterin complex, the single-stranded 3'overhang region of the telomeric DNA is linked to the duplex telomeric tract by the interaction of Tpp1 to Tin2 (47). Tin2 was first identified as Trf1-interacting protein (48). However, subsequent studies revealed the association of Tin2 with Trf2 and its role in Trf2 function and stabilization on telomeres (49, 50). Tin2 can bind to both Trf1 and Trf2 at the same time (50). The Trf1-Tin2 interaction is

mediated by TRF homology domain (TRFH) of Trf1, while Trf2-Tin2 interaction is outside the TRFH domain of Trf2 (51). Lastly, hRap1 was discovered as a Trf2-interacting factor. It localizes to the telomeres by associating with Trf2 through the C-terminus (52). It has been indicated to have a role in telomere length regulation as well (52, 53, 54).

Throughout these aforementioned studies, shelterin subcomplexes associated with telomeres have also been detected (29, 50). These subcomplexes may have different biological functions. Therefore, characterization of both these subcomplexes and the shelterin complex as a whole, in terms of their localization on telomeres, copy number, composition and biological roles, are equally important for discovering the exact mechanistic details of telomere structure and function regulation, as well as telomere maintenance.

#### **1.4. DNA Remodeling Activities of Shelterin Subunits and Subcomplexes**

Telomeres may be protected and regulated by the shelterin complex subunits through DNA remodeling activities of either individual shelterin subunits, subcomplexes, or the whole shelterin complex. This possibility is partly supported by the induction of senescence with Trf2 overexpression in the absence of telomeric DNA loss (6). Further evidence reveals that Trf2, which is not a helicase, facilitates t-loop formation by itself on telomeric substrates *in vitro* (9). This reaction is not efficient. Only 15-20% of the model telomere substrates form t-loop structures in the presence of Trf2 (55). Therefore, the synergistic action of shelterin subunits altogether, or in different combinations may be required for efficient DNA remodeling and subsequent structural folding into t-loop. **Therefore, in Specific Aim 1 of this study, I will test the binding and DNA remodeling activities of human Pot1 alone, and human Tpp1-Pot1 fusion protein on model telomeres using electron microscopy**

(EM). In order to achieve this goal, I will purify Pot1, Tpp1 and Tpp1-Pot1 proteins and visualize their interaction with telomeric DNA substrates using EM. I will verify these interactions by gel retardation assays. This study will indicate whether single-stranded TTAGGG repeat binding protein, Pot1, has the ability to affect telomere function by inducing structural changes in telomeric DNA structure on its own, or in association with Tpp1.

Alternatively, binding of Trf1, Trf2, hRap1, and Tin2 to double-stranded TTAGGG repeats can remodel telomeric DNA, facilitate, or inhibit t-loop generation. **Therefore, in Specific Aim 2, I will test the binding and DNA remodeling activities of shelterin subcomplexes; (1) Trf2, hRap1 and (2) Trf2, hRap1, Tin2 on model telomeres using electron microscopy.** Since N-terminal-[His]<sub>6</sub>-tagged human Trf2, hRap1, and Tin2 proteins are readily available in our laboratory, I will assemble the aforementioned complexes on model telomere substrates, visualize protein-DNA complexes using EM, and verify interactions by gel retardation assays. This study will reveal whether the DNA remodeling activity of the double-stranded TTAGGG repeat binding protein, Trf2, will be facilitated, or inhibited by its interaction partners: hRap1, and Tin2.

Understanding the function of telomeres and the shelterin complex relies on elucidating the structure of telomeres and telomere bound shelterin complex subunits. Once completed, the structural information of the shelterin subunits and subcomplexes bound to model telomeres will be a powerful tool for further insights into the mechanisms responsible for the telomere structure regulation during aging and oncogenesis. Furthermore, this study will also be of great value for identification of possible therapeutic targets of anti-aging and anti-cancer therapies.



## **1.5. Preliminary Data and Progress Report**

### **1.5.1. Progress on Specific Aim 1: I will test the binding and DNA remodeling activities of human Pot1 alone, and human Tpp1-Pot1 fusion protein on telomeric DNA using electron microscopy (EM).**

For the accomplishment of this aim, I have successfully constructed N-terminal-[His]<sub>6</sub>-tagged human Pot1 (hPot1) vector by subcloning PCR-amplified hPot1 cDNA into mammalian expression vector pcDNA4B (Invitrogen) using BamHI and EcoRV restriction sites. I have also constructed N-terminally-[His]<sub>6</sub>-tagged human Tpp1-Pot1 (hTpp1-hPot1) vector by inserting PCR-amplified hPot1 cDNA in frame to the C-terminus of human Tpp1 (hTpp1) cDNA using NotI and XbaI restriction sites after subcloning PCR-amplified hTpp1 cDNA into insect vector pFastBacHTA (Invitrogen) using BamHI and EcoRI restriction sites. Each subcloning was tested by restriction enzyme digestion and verified by sequencing constructed recombinant vectors. In addition, I generated and purified bacmid DNA with hTpp1-hPot1 fusion protein coding sequences using Bac-to-Bac system (Invitrogen). I have analyzed bacmid DNA using standard PCR reaction with M13 forward and reverse primers.

## **1.6. Research Design and Methods**

### **1.6.1. Rationale**

*In vitro*, Trf2 can facilitate t-loop formation by itself on telomeric substrates (9) and it localizes specifically to ss-ds junction in the t-loop structure. This reaction does not require any ATP, and is not efficient (55). Therefore, the synergistic action of other shelterin

complex subunits may be required for efficient telomeric DNA remodeling. In order to test this, experimental techniques, which will be used for both Specific Aim 1 and Specific Aim 2 of this study, will be the same. However, the interaction of different subunits and subcomplexes with model telomeres will be tested. For Specific Aim1, I will focus on the single-stranded telomeric repeat binding proteins (hPot1, and hTpp1), while I will focus on the double-stranded telomeric repeat binding proteins (hTrf2, hRap1, and hTin2) for Specific Aim 2. Furthermore, I will pay particular attention to the localization of the aforementioned proteins, and subcomplexes on model telomeres.

## **1.6.2. Materials and Methods**

**1.6.2.1. Generation of hPot1 and hTpp1-hPot1 expression vectors.** Standard PCR reactions were carried out to amplify hPot1 and hTpp1 cDNAs. An N-terminal- [His]<sub>6</sub>-tagged hPot1 vector was constructed by subcloning amplified hPot1 cDNA into pcDNA4B (Invitrogen). N-terminally-[His]<sub>6</sub>-tagged Tpp1-hPot1 was constructed by inserting hPot1 cDNA in frame to the C-terminus of Tpp1 after subcloning of human Tpp1 cDNA into insect vector pFastBacHTA (Invitrogen). Subcloning was tested by restriction enzyme digestion and verified by sequencing constructed recombinant vectors.

**1.6.2.2. Recombinant hPot1 and hTpp1-hPot1 fusion protein expression.** *In vitro* expression of recombinant hPot1 will be tested using TNT Coupled Transcription/Translation System (Promega) according to manufacturer's guide. The expression will be determined by Western blotting with monoclonal mouse His-tag-antibody (Qiagen). Small scale hPot1

expression *in vivo* will be tested and optimized by transfecting human 293A embryonic kidney cells using Fugene (Roche) transfection reagent in a 6-well culture dish according to manufacturer's procedures. For large scale protein expression, 293A embryonic kidney cells grown as adherent cultures in 10% FBS (Sigma) containing Dulbecco's Modified Eagle Medium with high glucose (Gibco) will be transfected with hPot1 mammalian expression vector using calcium phosphate transfection method as described in (56). After 48 hours further incubation at 37°C incubator containing a humidified atmosphere of 5% CO<sub>2</sub>, cells will be harvested by centrifugation at 2000 rpm for 10 min at 4°C. The cell pellet will be washed once with ice cold 1X PBS, frozen and kept at -80°C until further use.

In order to express hTpp1-hPot1 fusion protein, bacmid DNA with hTpp1-hPot1 coding sequences purified will be used to transfect Sf21 (Invitrogen) in a 6-well culture dish using Cellfectin (Invitrogen). P1 viral stock will be ready after 72h incubation at 27°C. P2 viral stock will be generated according to manufacturer's guidelines (Invitrogen). For large scale expression of the hTpp1-hPot1 fusion protein, 100 ml of Sf21 cells grown in suspension in Grace's Media (GibcoSFM) will be inoculated with virus at MOI = 10. After 48 hours further incubation at 27°C, cells will be collected by spinning at 2000 rpm for 10 min at 4°C, and protein expression will be tested by Western blotting with monoclonal mouse His-antibody (Qiagen). The cell pellet will be washed once with ice cold 1X PBS, frozen and kept at -80°C until further use.

**1.6.2.3. Recombinant hPot1 and hTpp1-hPot1 fusion protein purification.** Both N-terminal-[His]<sub>6</sub>-tagged hPot1 and N-terminal-[His]<sub>6</sub>-tagged hTpp1-hPot1 fusion protein will be purified using Talon<sup>TM</sup> metal affinity resin (Clontech, Palo Alto, CA) as previously

described (38). SDS-PAGE and Coomassie blue staining will be used to analyze the purity of the proteins.

**1.6.2.4. Recombinant hTrf2, hRap1, and hTin2 expression vectors.** N-terminal-[His]<sub>6</sub>-tagged insect expression vectors have already been constructed in our laboratory by cloning human Trf2, hRap1, and Tin2 encoding cDNAs into pFastBacHT (Invitrogen) after tagging them with six-histidine using PCR.

**1.6.2.5. Recombinant hTrf1, hTrf2, hRap1, and hTin2 purification.** N-terminal-[His]<sub>6</sub>-tagged hTrf1, hTrf2, hRap1, and hTin2 were already purified using Talon<sup>TM</sup> metal affinity resin (Clontech, Palo Alto, CA) as previously described (38). The purity of each protein was analyzed by SDS-PAGE and Coomassie blue staining.

**1.6.2.6. Generation of Telomeric DNA Templates.** Model telomere substrates will be generated as previously described (55). Briefly, pRST5 plasmid with ~3kb of non-telomeric DNA and a 500 bp region of telomeric repeat will be linearized using BbsI and BsmI restriction sites. This restriction digestion will locate 500 bp telomeric repeat tracts to one end of the linearized molecule. A 54-nt single stranded (TTAGGG)<sub>6</sub> overhang telomeric repeat tract will be generated by ligating a 58-nt oligonucleotide onto the 3' telomeric end (Figure 1.3). Different telomeric substrates will be generated using 3'overhang in different length, or sequence. Substrates with altered single-stranded double strand junction sequences will also be generated (55).

**1.6.2.7. Electron Microscopy.** Model telomere templates will be incubated with 100 ng of each protein for 30 min in EM buffer (20 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.5 mM dithiothreitol, 100 mM KCl) on ice. Protein-DNA complexes will be fixed with 0.6% glutaraldehyde and fractionated using a 2.5 ml BioGel A15M column. The complexes will be incubated with spermidine just before directly adsorbed to glow charged carbon foil grids, dehydrated by water and ethanol washes and rotary shadow cast with tungsten. Electron micrographs will be taken using an FEI Tecnai 12 Electron Microscope and Gatan Ultrascan US400SP digital camera with Gatan Digital Micrograph software (9, 58).

**1.6.2.8. Gel retardation Assays.** Proteins will be incubated with the aforementioned model substrates labeled with  $^{32}\text{P}$  in EM buffer for 20 min at room temperature. Then, the mixtures will be directly loaded onto a 3.5% (w/v) nondenaturing polyacrylamide gel in 45mM Tris borate, 1mM EDTA. The gels will be run at 140 V for 1 h at 4 °C, dried, analyzed by autoradiography, and quantified using a Storm 840 PhosphorImager (GE Healthcare) (38).

### **1.6.3. Expected Results**

#### **1.6.3.1. Expected Results for Specific Aim 1**

Pot1 is known to recognize and bind specifically to single-stranded 3'-end and internal 5'-(T)TAGGGTTAG-3' sequences via its DNA binding domain (34, 35). Tpp1 does not directly bind to telomeric DNA; it associates with Pot1. Therefore, hPot1 and hTpp1-hPot1 fusion protein will associate with single-stranded 3'overhang, whereas hTpp1 alone will not be able to bind to model telomeres. I expect to see diminished interaction of hPot1, and hTpp1-hPot1

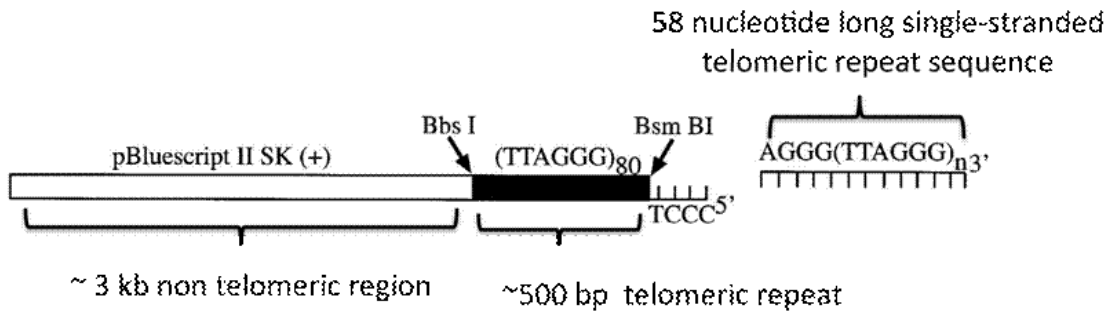


Figure 1.3: Schematic of model telomere substrates (adapted from Stansel, RM, *et.al.*, 2001.) pRST5 plasmid with 3kb of non- telomeric DNA and a 500 bp region of telomeric repeat will be linearized using denoted restriction sites. 500 bp telomeric repeat tracts will be positioned at one end of the linearized molecule. A 54-nt single stranded (TTAGGG)<sub>6</sub> overhang telomeric repeat tract of the same length will be generated by ligation of a 58-nt oligonucleotide onto the 3' telomeric end. Different telomeric substrates will be generated using 3'overhang in different length and sequence. Substrates with altered single-stranded double stranded junction sequences will also be generated (55).

with altered 3'-TTAGGG repeat sequence containing templates. I will also be able to score these proteins according to their localization on model substrates. A protein can interact with non-telomeric DNA, telomeric double-stranded (ds) DNA, telomeric single-stranded (ss) DNA, or to ss-ds DNA junction of the telomeric substrates in this experimental system. pRST5 plasmid without any TTAGGG repeat sequences will be used as a negative control. hTpp1 protein alone will be another negative control for these experiments due to its inability to associate with telomeric TTAGGG sequences on its own. Telomeric DNA substrates without proteins, proteins without telomeric DNA substrates will also be used as the negative controls for this study. BSA, which cannot bind to DNA, will also be a negative control. hTrf2 can be used as a positive control for DNA remodeling under the same experimental conditions, since it has already been shown to facilitate t-loop formation on its own *in vitro*. Similarly, p53 can also be a positive control, since it is known to bind to 3'overhangs and ss-ds junctions without any sequence preference (57). Any requirement for ATP or reaction buffer of different composition (ion, salt, etc.) will also be tested.

### **1.6.3.2. Expected Results for Specific Aim 2**

For this aim, I will test the binding and DNA remodeling activities of (1) Trf2, hRap1, and (2) Trf2, hRap1, Tin2 subcomplexes on model telomere substrates. I expect to see t-loop formations upon the interaction of Trf2 with model telomeres (55), if the purified Trf2 protein is active. Since hRap1, and Tin2 cannot directly interact with TTAGGG sequences, the effect of these proteins on telomeric DNA remodeling can be tested in two ways. Firstly, either hRap1, or Tin2 can be incubated simultaneously with Trf2, and then with model DNA. Alternatively, Trf2-hRap1, Trf2-Tin2, hRap1-Tin2 or Trf2-hRap1-Tin2

can be pre-incubated before their interaction with DNA substrates. This will reveal whether hRap1, Tin2, or hRap1 with Tin2 has an effect on binding and DNA remodeling activities of Trf2. Secondly, order of addition experiments can be carried out in order to test whether there is a preferred sequence of protein-protein interactions for efficient telomeric DNA remodeling. In addition, localization of these shelterin subunits and subcomplexes on model telomeres will also be scored. Similar to Specific Aim 1, pRST5 plasmid without any TTAGGG repeat sequence, hRap1, and Tin2 proteins alone, telomeric DNA substrates without proteins, proteins without telomeric DNA substrates, and BSA will be the negative controls. p53 will be used as positive control for binding to ss-ds junctions (57). For testing the possible role of hRap1, or Tin2 in facilitation/inhibition of t-loop formation, hTrf2 will be the positive control. Any requirement for ATP or reaction buffer of different composition (ion, salt, etc.) will also be tested.

#### **1.6.4. Potential Pitfalls**

##### **1.6.4.1. Potential Pitfalls for Specific Aim 1**

It may not be possible to express N-terminal-[His]<sub>6</sub>-tagged hTpp1-hPot1 fusion protein from insect cells. Even if expressed, the fusion protein may be insoluble and cannot be purified. In this case, position of the His-tag and the relative position of the cDNA sequences encoding these two proteins will be switched in the insect. Alternatively, a mammalian expression system for hTpp1-hPot1 can be generated. Even if purified successfully, the fusion protein may not be active due to the restricted three-dimensional diffusion of the protein. Another trial of cloning, expression, and purification can be employed by the



addition of a linker region in between these two proteins during cloning step. In any case, we have already purified Tf1-Pot1 fusion (58), chicken Pot1 (cPot1), and hTpp1 proteins in our laboratory. cPot1 has 61% sequence identity to hPot1 and DNA binding domains of cPot1, and hPot1 are 75% identical (59). Alternatively, individually purified hTpp1 and hPot1, or hTpp1 and chicken Pot1 proteins can be tested for their binding and DNA remodeling activities *in trans*, instead of *in cis*.

#### **1.6.4.2. Potential Pitfalls for Specific Aim 2**

hTrf2, hRap1, and hTin2 are readily available in our laboratory. EM analysis and gel retardation assays will be carried to make sure that these proteins are biologically active. For instance, Trf2 should be interacting with double-stranded TTAGGG repeats, if it is active. If the interactions of these proteins are cell-cycle regulated, and requires additional proteins, it is possible to co-express them *in vivo* and purify these subcomplexes from insect cells. On the other hand, protein expression in insect cells may be a problem, if these proteins require posttranslational modification(s), which occurs only in mammalian but not insect cells, for interacting with each other and, interacting with DNA, or for their biological function. This may also be a potential problem for Specific Aim 1. Under such circumstances, it is possible to construct mammalian expression vectors for these proteins, and test their expression in mammalian cells.

### **1.6.5. Summary and Future Directions**

Telomeres are extremely important due to the role they play in both cancer and aging. Their proper maintenance and regulation are central to the protection of genomic stability and viability of the cell. The recent discovery of t-loops by use of electron microscopy has provided very significant information on the structural folding of the DNA component of telomeres. However, the exact structure of telomeres is still unknown. Therefore, understanding telomere structure will be of great value for the discovery of the mechanisms responsible for telomere function regulation. In this process, the structure of telomere-specific factors and structures generated upon their binding to telomeres are equally of great value.

Therefore, I aim to test the DNA binding and remodeling activities of the shelterin complex subunits and subcomplexes in this study. I will focus on the activities of single-stranded and double-stranded TTAGGG repeat binding proteins, separately. In the continuation of this study, the next will be the examination of binding and DNA remodeling activities of Trf1 containing subcomplexes, and the whole shelterin complex. In the long term, the structural information provided by the characterization of the whole shelterin complex in terms of exact localization and copy number of subunits bound to telomeres, will be a milestone in telomere biology.

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