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The Molecular Interfaces of Telomerase and Telomere Proteins in Yeast and Humans

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The Molecular Interfaces of Telomerase and Telomere Proteins in
Yeast and Humans

by

Andrew Benjamin Dalby

B.A., University of Colorado at Boulder, 2003

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
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This thesis entitled:
The Molecular Interfaces of Telomerase and Telomere Proteins in Yeast and Humans
written by Andrew Benjamin Dalby
has been approved for the
Department of Chemistry and Biochemistry

Thomas R. Cech

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The final copy of this thesis has been examined by the signatories, and we find that both the content and form meet the acceptable presentation standards of scholarly work in the above mentioned discipline.
Telomeres are the nucleoprotein endcaps of linear chromosomes. Telomeres shorten with each cell division, limiting the capacity of telomeres to protect chromosomal DNA. The enzyme telomerase counteracts telomere attrition by synthesizing new telomeric DNA. Telomerase is a ribonucleoprotein complex comprised of TElomerase Reverse Transcriptase (TERT) and Telomerase RNA (TR). To maintain a homeostatic telomere length, telomerase must assemble, traffic to the telomere, and interact with a host of protein cofactors. Since the discovery of telomerase, many key components of the telomerase holoenzyme and telomeric protein cap have been identified. This thesis presents detailed investigations of the molecular interactions of telomerase and associated proteins.

In *Saccharomyces cerevisiae* the Ku heterodimer binds directly to a hairpin of TR to promote the nuclear localization of telomerase. Footprinting experiments examined the Ku binding site and chemical probing investigated the hairpin secondary structure. Heterologous mixing experiments and RNA mutagenesis tested which stem-loop elements mediate Ku binding. These experiments suggest that Ku binds to the terminal loop and proximal stems of the hairpin, recognizing a critical bulge motif in a sequence-independent but structure-specific manner.

The TEL-patch consists of amino acids on the surface of the human telomere protein TPP1 that are necessary for telomerase recruitment and processivity stimulation, but the TEL-patch contributions are not fully understood. Single-turnover translocation, substrate-competition, and substrate-telomerase dissociation rate assays tested the impact of the TEL-patch on telomerase catalysis. The competition experiments constitute a step in developing an *in vitro* telomerase recruitment assay. Furthermore, the TEL-patch increases the apparent rate and efficiency of telomerase translocation,
slows the rate of substrate dissociation, and contributes to the preferential binding and extension of TPP1-bound substrates by telomerase.

The exact component of telomerase that interacts with the TEL-patch of TPP1 has been unknown. Direct telomerase extension assays identified human TERT separation-of-function alleles that disrupt the telomerase-TPP1 interaction. Perturbation of the interaction resulted in compromised telomere maintenance in cells. A deleterious mutation in the TEN-domain of hTERT was rescued by introducing a compensatory charge-swap mutation in the TEL-patch of TPP1 that restored telomerase stimulation in vitro, telomere maintenance in vivo, and suggests that these proteins interact directly.
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Chapter I: Background on Telomeres and Telomerase

Introduction

The proper transmission of genetic material is necessary for life. When a cell or organism divides, the cell’s DNA must be faithfully replicated and sound copies of the genome must be properly segregated between two daughter cells. Dysfunction in genetic transmission is linked to a wide range of human diseases, and cells have a number of mechanisms to ensure fidelity of the process and to protect the genetic material. Telomeres are nucleoprotein caps found at both ends of linear chromosomes in eukaryotic cells; these caps help to ensure the integrity of intervening chromosomal DNA (Blackburn and Gall 1978; Szostak and Blackburn 1982; Palm and de Lange 2008). Despite their protective role, telomeres are not static but dynamic in length, which necessitates telomere maintenance. In most eukaryotes, telomere length is maintained by the enzyme telomerase. Telomerase is comprised of a TElomerase Reverse Transcriptase (TERT) subunit that copies an internal template sequence within the Telomerase RNA (TR) subunit to lengthen the ends of telomeres (Greider and Blackburn 1987; Lingner et al. 1997b). This chapter will review the composition and function of telomeres and telomerase as well as the process of telomerase recruitment to telomeres in both humans and budding yeast. Comparisons to the process of telomere maintenance in fission yeast will be made when instructive.

The cellular importance of telomeres and telomerase

Telomeres, the end-replication problem, and senescence

One contributor to the dynamic nature of telomere length is a phenomenon known as the end-replication problem – a side effect of semi-conservative DNA replication (Figure 1.1) (Watson 1972; Olovnikov 1973; Levy et al. 1992). In short, the end-replication problem refers to the loss of telomeric
DNA from the 3’ termini of each newly synthesized DNA strand. During replication of the leading strand, DNA polymerase is able to completely replicate to the 5’ terminus of the template. However, on the lagging strand the RNA primer is removed after initiating DNA polymerase synthesis from the chromosome terminus. Removal of this primer results in a recessed 5’ end on the lagging strand of the chromosome, which cannot be filled by conventional DNA polymerases. Were DNA replication left to these polymerases alone, iterative cell divisions would lead to the progressive attrition of telomeres (Levy et al. 1992). In addition, 3’ overhangs are present on termini of telomeres synthesized by leading strand synthesis; the overhangs are generated by nucleolytic resection of the template strand (Palm and de Lange 2008). Thus an enzyme is necessary to combat the loss of telomeric DNA.

Figure 1.1. The DNA end-replication problem. Replication bubbles form at multiple sites of initiation of semi-conservative DNA replication; only one origin is shown in this simplified version. Leading and lagging strand DNA synthesis initiates from RNA primers (red lines) which are extended by DNA polymerase (arrows). After replication, the RNA primers are removed and missing sequence is replaced by fill in synthesis, except at the 5’ termini of the lagging strands. 3’ overhangs on the leading strand are generated by C-strand resection (orange dashes). The
simplified diagram highlights the loss of DNA associated with each division; however in reality DNA loss occurs in the context of 3’ overhangs are already present at telomeres.

Progressive telomere shortening serves as a molecular clock for dividing cells. In culture, normal human fibroblasts can undergo 50-70 doublings before entering senescence which prevents further proliferation (Hayflick and Moorhead 1961); this cellular ageing phenomenon is known as the Hayflick limit. Telomeres in human sperm cells are roughly 10-15 kb in length but shorter in somatic cells (8-10 kb), suggesting that telomere erosion accompanies development (de Lange et al. 1990; Palm and de Lange 2008). Telomeres in cultured fibroblasts progressively erode at a rate of 50-100 base pairs (bp) per division until they senesce, which coincides with an average telomere length of ~6 kb (Harley et al. 1990; Allsopp et al. 1992; Levy et al. 1992). In some cases, cells may bypass senescence. Escape from senescence is accompanied by resumed shortening of telomere length until the cells enter a state termed “crisis”, which coincides with an average telomere length of ~3 kb (Counter et al. 1994). Chromosome fusion- and-breakage cycles followed by apoptosis characterize the state of crisis for most cells (Stewart and Weinberg 2006). Thus, telomeres shorten as cells age, and when telomeres become critically short the cells die.

The synthesis of additional telomeric sequence by the enzyme telomerase counteracts telomere attrition. Telomerase is essential for both germ cells and stem cells to retain proliferative capacity (Batista and Artandi 2013; Sexton et al. 2014). Indeed, telomerase activity can be detected in both stem cells and progenitor cells, but this activity decreases as cells progress towards a differentiated state (Wright et al. 1996; Batista and Artandi 2013). In cells expressing telomerase, the number of active telomerase enzymes per cell roughly equals the number of telomere ends during S-phase (~240 in HEK 293T and HeLa cells) (Xi and Cech 2014). In humans, assembly of active telomerase complexes is limited...
by number of hTERT molecules which is 1.5 to 6 fold lower than the number of hTR molecules (Xi and Cech 2014). Telomerase is thought to extend each telomere by about 60 nucleotides (nt) per cycle (Zhao et al. 2011), mitigating the telomere shortening rate of 50-100 bp per cycle (Harley et al. 1990). Therefore, a precarious balance between telomere erosion and telomerase action exists, and small changes in telomerase activity or recruitment may have significant impacts on telomere length in cells (Nandakumar et al. 2012; Zaug et al. 2013; Sexton et al. 2014).

**Telomerase and cellular immortalization**

Occasionally, cells that have bypassed senescence can evade entering the state of crisis, usually by reactivating telomerase. Telomerase reactivation results in renewed telomere length maintenance and cellular immortalization and is a hallmark of 80-95% of cancers (Stewart and Weinberg 2006; Shay and Wright 2011). Escape from crisis is a rare event, and the odds of a cellular immortalization are thought to be approximately one in $3 \times 10^7$ (Wright and Shay 1992). Re-activation of telomerase is not the only the driving factor in cancer, and transformation requires other cellular events (e.g., the inactivation of tumor suppressor pathways and activation of auto-growth signaling) (Hahn et al. 1999a; Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). Ectopic expression of human TERT (hTERT) is sufficient to immortalize cells and maintain telomeres (Bodnar et al. 1998; Hahn et al. 1999b).

However, cells can also utilize an Alternate Lengthening of Telomeres (ALT) mechanism that relies on recombination and does not require telomerase to achieve immortalization (Bryan et al. 1997; Cesare and Reddel 2010). Cellular immortalization is required for cancers to progress to malignancy (Hanahan and Weinberg 2000). Although the exact mechanism by which telomerase is reactivated in cancers is unknown, recent work suggests one major mechanism: cancer-associated mutations in the promoter of hTERT may create additional E-Twenty-Six (ETS) transcription factor binding sites that result in increased hTERT expression (Horn et al. 2013; Huang et al. 2013). Evidence also suggests that reactivated
Telomerase utilizes canonical pathways, such as TPP1-dependent recruitment, to maintain telomeres (Nakashima et al. 2013).

**Telomerase: a reverse transcriptase with an internal RNA template**

Telomerase is a ribonucleoprotein complex that is minimally comprised of a templating TR and the enzymatically active TERT (Greider and Blackburn 1989; Lingner et al. 1997b). Telomerase sequentially adds nucleotides to the 3’ end of a DNA substrate (primer) that is base-paired with the TR template (Greider and Blackburn 1987); the addition of multiple nucleotides after a single primer-binding event is known as Nucleotide Addition Processivity (NAP). Telomerase is also capable of adding multiple telomeric repeats prior to dissociating from its substrate, known as Repeat Addition Processivity (RAP). TERT and TR are sufficient to reconstitute telomerase enzymatic activity in vitro (Weinrich et al. 1997; Beattie et al. 1998; Zappulla et al. 2005). Although telomerase has been proposed to dimerize in budding yeast, ciliates, and humans (Prescott and Blackburn 1997; Fouche et al. 2006; Sauerwald et al. 2013), its activity and processivity are not dependent upon dimerization (Bryan et al. 2003). This section will give a general introduction to TERT, TR, and the telomerase catalytic cycle common to most eukaryotic organisms.

**The structure and function of telomerase reverse transcriptase**

TERT is organized into three distinct domains: the N-TERminal TEN-domain, a TR-Binding Domain (TRBD), and a conserved Reverse Transcriptase domain (RT) which has a C-Terminal Extension (CTE) – the polymerase thumb (Figure 1.2) (Nakamura et al. 1997; Nandakumar and Cech 2013). The initial identification of the TERT genes revealed that TERT contained conserved reverse transcriptase motifs (Lingner et al. 1997b; Nakamura et al. 1997). Mutation of select conserved aspartic acid residues in the RT domain resulted in loss of telomerase activity as well as a failure to maintain telomeres in vivo
(Lingner et al. 1997b). Crystal structures of the RT domain of a putative TERT, which lacks a TEN-domain, from Tribolium castaneum reveal that the conserved RT motifs dictate the common polymerase hand structure, which interacts with the TRBD to form a closed ring structure (Gillis et al. 2008). The palm, fingers, thumb, and TRBD of TERT form a closed ring structure thought to encircle TR and hybridized DNA substrate (Gillis et al. 2008; Mitchell et al. 2010). The RT domain of TERT binds to the template/pseudoknot domain, and the TRBD is thought to associate with other RNA elements, some of which may be specific to various eukaryotic lineages (O’Connor et al. 2005; Robart and Collins 2011; Huang et al. 2014).

Figure 1.2. The domain structure of hTERT. hTERT and other eukaryotic TERTs are organized into three main domains: the TEN-domain (red), TRBD (blue), and RT domain (yellow), which includes the CTE. Note separate brackets delineate each domain, while a connected bracket emphasizes the CTE portion of the RT domain. TRBD motifs shown. Conserved reverse transcriptase motifs are denoted in the RT domain. The N-DAT and C-DAT (Disassociates Activities of Telomerase) are regions within the TEN domain and CTE of the RT domain of hTERT, indicated by lines. Mutations in DAT regions were reported compromise the in vivo function of telomerase. Domain structure to scale, bar represents 100 amino acids. Figure modified from (Zaug et al. 2013).

The three domains of TERT stabilize DNA substrates and act as platform for association with other proteins. The TEN-domain contains the so called “anchor-site”. This site contains conserved surface residues that can be cross-linked to the DNA primer and are thought to bind to the 5´ end of the
primer to add stability during synthesis and translocation (Morin 1991; Collins and Greider 1993; Hammond et al. 1997; Jacobs et al. 2006). A recent report suggests that the TEN-domain may instead function to increase occupancy and extension of the primer/template hybrid within the active site rather than acting as an anchor for single-stranded DNA (Wu and Collins 2014). Surfaces distinct from the anchor site on the TEN-domain have been proposed to interact with the primer to facilitate NAP (Jurczyluk et al. 2011), to provide stability through protein-protein interactions during RAP (Zaug et al. 2008), and to interact with the TRBD to better stabilize substrates (Finger and Bryan 2008). Specific motifs within the RT domain interact with the primer to provide stability during RAP (Bryan et al. 2000; Xie et al. 2010). The RT domain, which includes the CTE, has been proposed to interact with the telomeric DNA substrates to provide stability and/or in some cases promote RAP (Huard et al. 2003; Finger and Bryan 2008; Xie et al. 2010; Jurczyluk et al. 2011; Robart and Collins 2011; Wu and Collins 2014). A portion of the human CTE was proposed to Dissociate Activities of Telomerase (C-DAT) not essential for enzymatic activity (Figure 1.2) (Banik et al. 2002). However, the CTE likely comprises the thumb of polymerase (Hossain et al. 2002; Gillis et al. 2008). Mutation of the CTE can disrupt both processivity and activity, suggesting that the CTE is indeed essential to catalysis (Hossain et al. 2002; Huard et al. 2003; Jurczyluk et al. 2011).

The TEN-domain of TERT was also shown to be important for the interaction of telomerase with various co-factors that stimulate activity, processivity, recruitment, or activation. The TEN-domain of TERT in budding yeast interacts with Ever Shortening Telomeres-3 (Est3), a telomerase holoenzyme component (Friedman et al. 2003; Talley et al. 2011; Yen et al. 2011). The DAT region of the human TEN-domain (N-DAT) separates telomerase enzymatic activity from in vivo function (Figure 1.2) (Armbruster et al. 2001) by interacting with TPP1, another telomerase co-factor (Zaug et al. 2010; Zhong et al. 2012; Schmidt et al. 2014). The C-DAT region of the human RT domain is also proposed to interact
with TPP1 (Zhong et al. 2012); however, there is little direct evidence of this. Thus multiple domains in TERT contribute to association with TR, to enzymatic activity, and to binding of various co-factors.

The structure and function of telomerase RNA

TRs contain an internal template and various domains that interact with TERT and other holoenzyme components to lengthen telomeres. TRs of various eukaryotes can vary by up to a thousand nt in length. Ciliate TRs are the smallest at ~150 nt, vertebrate RNAs are intermediate in size, and fungal TRs are often 1,000 nt or larger (Figures 1.3,1.4) (Greider and Blackburn 1989; Singer and Gottschling 1994; Chen et al. 2000; Qi et al. 2012a). Despite the fact that the sequences and secondary structures of TRs are highly divergent amongst eukaryotes, a number of conserved RNA features emerge from phylogenetic comparative analysis and biochemical studies. The template/pseudoknot domain is highly conserved in all eukaryotic TRs (Lingner et al. 1994; Chen and Greider 2004; Lin et al. 2004). This domain contains a triple-helix that is important for activity (Theimer et al. 2005; Qiao and Cech 2008). A second highly conserved structural element is the Template Boundary Element (TBE), which is typically a paired stem adjacent to the template (Lai et al. 2002; Chen and Greider 2003; Seto et al. 2003; Zappulla and Cech 2004). Finally yeast and vertebrate TRs contain a conserved Three-Way Junction (TWJ) that is critical for enzymatic activity thought to mediate the interaction with the TRBD of TERT (Brown et al. 2007; Huang et al. 2014).
Figure 1.3. The human telomerase holoenzyme. Predicted hTR secondary structure. Secondary structure supported by strong co-variation and experimental data. Important domains labeled in red including Template Boundary Element (TBE) and three-way junction (TWJ). Box H motif (green), CAB Box (purple), and ACA motif (blue) denoted within the ScaRNA domain of hTR. Protein subunits hTERT, dyskerin complex, and TCAB1 shown near associated RNA domains. RNA secondary structure from (Podlevsky et al. 2008).
Figure 1.4. *The S. cerevisiae telomerase holoenzyme*. Predicted TLC1 secondary structure. Secondary structure based on limited co-variation and experimental data. Important domains labeled in red including Template Boundary Element (TBE) and three-way junction (TWJ). Protein subunits Est2, Est1, Sm7 complex, and Ku heterodimer shown near associated RNA domains. Est3 associates with the holoenzyme through interactions with Est1 and Est2. RNA secondary structure from (Podlevsky et al. 2008).

Outside of the template/pseudoknot domain and template boundary element, eukaryotic TRs contain additional domains that are partially conserved within individual eukaryotic lineages, but not
between lineages (Chen et al. 2000; Zappulla and Cech 2004; Qi et al. 2012a). Although the sequences and secondary structures of additional TR modules vary drastically, one unifying feature is that they enable association with protein co-factors. Protein partners that associate with TR facilitate maturation of the RiboNucleoProtein particle (RNP), cellular trafficking, intracellular localization, and modulation of enzymatic activity. For example, vertebrate RNAs contain a conserved H/ACA box motif for association with dyskerin, which is important for TR biogenesis (Figure 1.3) (Mitchell et al. 1999a; Chen et al. 2000; Egan and Collins 2012a). In contrast, TRs mature differently in yeast (discussed below).

**The telomerase catalytic cycle**

Telomerase is not a particularly robust enzyme; it possesses a specific activity approximately one to three orders of magnitude lower than that of other common polymerases (Xi and Cech 2014). As mentioned above, the telomerase catalytic cycle initiates when a single-stranded DNA anneals to the RNA template and the RT domain of TERT catalyzes the sequential addition of nucleotides to the 3’ end of the substrate (Figure 1.5) (Greider and Blackburn 1987). During nucleotide incorporation, the number of base-pairs between the template-substrate duplex is thought to remain constant (Forstemann and Lingner 2005). When TERT reaches the end of the RNA template, the template boundary helix halts TERT from incorporating additional nucleotides (Lai et al. 2002; Seto et al. 2003). The telomeric DNA then repositions on the template, enabling another repeat to be added; this is referred to as translocation (Figure 1.5A).
Figure 1.5. The catalytic cycle of human and *S. cerevisiae* telomerases. A. i) TERT utilizes the internal template of hTR to synthesize telomeric repeats ii) telomeric DNA associates with the template iii) TERT catalyzes the addition of nucleotides to the 3′ end of the DNA until the TBE is reached. iv) translocation of the telomeric substrate enables another repeat to be added. Substrate dissociation can occur any stage of the catalytic cycle, but usually coincides with translocation. (Right) Example of human telomerase direct extension assay showing repeat addition processivity. B. i) Est utilizes the internal template of TLC1 to synthesize a single telomeric repeat *in vitro* ii) telomeric DNA anneals to the TLC1 template iii) Est2 catalyzes the addition of nucleotides to the 3′ end of the DNA until the TBE is reached. iv) Substrate dissociation may occur any stage of the catalytic cycle, but product dissociation was proposed to be rate-limiting for *S. cerevisiae* telomerase *in vitro* (Prescott and Blackburn 1997). (Right) Example minimal *S. cerevisiae* telomerase (Mini T) direct extension assay showing the addition of a single telomeric repeat.
Translocation requires that the single-stranded RNA on either side of the TR template is intact or connected (Miller and Collins 2002; Mason et al. 2003) Single molecule experiments suggest that the single-stranded RNA surrounding the template appears to compress and extend during nucleotide addition to make translocation more energetically favorable (Berman et al. 2011). Alternatively, translocation may involve template realignment outside of the active site (Qi et al. 2012b). Translocation is an inefficient process and the step at which most telomeric DNA substrates dissociate from the enzyme, leading to the characteristic multiple nucleotide banding pattern seen for processive telomerases (Greider 1991). Higher translocation efficiency (i.e. the fraction of total substrates that translocate) correlates with increased RAP (Qi et al. 2012b). Most eukaryotic telomerases are capable of RAP (Figure 1.5A) (Greider 1991; Autexier et al. 1996; Lingner and Cech 1996). One exception is telomerase from S. cerevisiae which has little or no capability for multiple repeat addition in vitro, possibly due to extremely slow product dissociation step after the synthesis of a full repeat (Figure 1.5B) (Cohn and Blackburn 1995; Lingner et al. 1997b; Prescott and Blackburn 1997; Bosoy and Lue 2004). Interestingly, this does not appear to be a general feature of all fungal or even budding yeast telomerases (Cohn and Blackburn 1995; Qi et al. 2012a)

**Telomere maintenance in budding yeast**

**The telomerase holoenzyme of *Saccharomyces cerevisiae***

This section begins with an overview of the budding yeast telomerase holoenzyme and telomere-end protection followed by a discussion of the interaction between telomerase and the telomere to mediate recruitment, cellular trafficking, and biogenesis of telomerase in budding yeast. The telomerase core in the budding yeast *Saccharomyces cerevisiae* consists of a large 1.1 kb TLC1 RNA
and yeast TERT (Singer and Gottschling 1994; Lingner et al. 1997b). TLC1 is present at approximately 30 copies per haploid cell which is less than the number of telomeric ends (Mozdy and Cech 2006). Thus, only a subset of telomeres is extended by a few repeats each cell cycle, with preferential extension at telomeres with shorter tracts (Teixeira et al. 2004). Structurally, TLC1 contains three long helical arms that emanate from a conserved pseudoknot (Figure 1.4) (Dandjinou et al. 2004; Lin et al. 2004; Zappulla and Cech 2004). Yeast TERT binds to the central template/pseudoknot region and each of the arms serves as a scaffold for protein co-factors that comprise the holoenzyme (Zappulla and Cech 2004). In contrast to human telomerase, the template/pseudoknot domain and yeast TERT are sufficient to reconstitute enzymatic activity in vitro (Qiao and Cech 2008; Mefford et al. 2013).

Three of the yeast telomerase holoenzyme components were identified through genetic screens. The observation that the deletion of specific genes resulted in progressive telomere shortening, eventually leading to cell death, led to the articulation of the so called “Ever Shorter Telomeres” phenotype (Est) and the discovery of the Est1, Est2, and Est3 genes (Lundblad and Szostak 1989; Lendvay et al. 1996). Subsequently, Est2 was identified as the gene that encodes yeast TERT (Lingner et al. 1997b). In addition to Est1 and Est3, the Sm7 complex and Ku heterodimer were later identified as subunits of the yeast telomerase holoenzyme; although all of these components are essential for telomerase function in vivo, they are dispensable for catalysis in vitro (Lingner et al. 1997a; Seto et al. 1999; Peterson et al. 2001; Zappulla et al. 2005; Qiao and Cech 2008). The function of each holoenzyme component and its association with telomerase is discussed in more detail below.

The Est1 protein associates with telomerase through an interaction with TR (Figure 1.4). Est1 appears to be specific to yeast holoenzymes and essential for telomerase recruitment to telomeres. Est1 binds directly to an internal loop and proximal helices on one arm of TLC1, distinct from the pseudoknot (Livengood et al. 2002; Seto et al. 2002). Perturbation of the bulged stem structure in this
arm results in telomere shortening and loss of Est1 binding (Seto et al. 2002). Additional portions of the TLC1 Est1-binding arm also participate in the association of Est1. Although Est1 over-expression does not suppress deletion of RNA structural elements adjacent to the bulged stem (Lubin et al. 2012; Laterreur et al. 2013), interestingly, deletion of the bulge in the originally described Est1 binding site is compensated for upon Est1 overexpression (Seto et al. 2002). Collectively, these data indicate that Est1 association requires additional RNA elements and that loss of the bulge stem can be compensated by overexpression due to the additional Est1 TLC-1 interactions. Although the local RNA structure of the Est1 binding site is critical for telomerase function, the Est1 arm of TLC can be relocated in TLC1 without affecting telomere maintenance, suggesting that TLC1 serves as a flexible scaffold for protein co-factors (Zappulla and Cech 2004). The physical association of TLC1 and Est1 is essential for the localization of TLC1 to telomeres in the late S/G2 phase and for nuclear retention of TLC1 (Chan et al. 2008; Laterreur et al. 2013). Est1 may also play a role in telomerase activation (Evans and Lundblad 1999), and it is necessary for the recruitment of Est3 to the telomerase holoenzyme (Osterhage et al. 2006; Tuzon et al. 2011; Tucey and Lundblad 2014). Est1 is also proposed to localize telomerase to the nuclear periphery during S-phase (Schober et al. 2009).

In contrast to Est1, Est3 does not bind to TLC1 but makes protein-protein contacts with the telomerase holoenzyme. Est3 does not appear to be necessary for telomerase recruitment, but is thought to stimulate telomerase activity (Talley et al. 2011). Est3 is thought to directly interact with the TEN-domain of Est2 (Hughes et al. 2000; Friedman et al. 2003; Talley et al. 2011; Yen et al. 2011), as well as with Est1 (Tuzon et al. 2011). The recent structure determination of Est3 demonstrated that it is a structural homolog of the N-terminal oligonucleotide/Oligosaccharide-Binding fold (OB-domain) of TPP1 and showed that Est3 also contains an acidic loop corresponding to the TPP1 glutamate (E) and leucine (L)-rich patch (TEL-patch), a group of conserved surface amino acids on the of TPP1 (Rao et al. 2014).
Mutation of the Est3 loop that corresponds to the TEL-patch of TPP1 or mutations of the TEN-domain of Est2 each disrupt telomere maintenance (Friedman et al. 2003; Rao et al. 2014). Est3 appears to have both positive and negative regulatory functions in telomere maintenance. Mutation of basic amino acids, outside of the TEL-patch, resulted in significant telomere shortening (Rao et al. 2014). Conversely, mutation of a serine adjacent to the TEL-patch resulted in a telomere lengthening phenotype (Tucey and Lundblad 2014). Finally, Est3 is also required for the nuclear retention of TLC1, although this requirement can be bypassed by overexpression of Est2 (Gallardo et al. 2008).

The observation that TLC1 has a 5´ trimethylguanosine cap led to discovery that the telomerase holoenzyme associates with the ring-like hetero-heptameric Sm (Sm7) complex (Figure 1.4) (Seto et al. 1999). TLC1 contains a single-stranded RNA tail near its 3´ end that contains a canonical AU-rich Sm consensus binding sequence (Seto et al. 1999). Sm proteins are widely known as regulators of RNA metabolism. The association of Sm7 with TLC1 is thought to act as a protective barrier during exosome-mediated removal of the poly-A tail, to form the mature TLC1 RNA (Seto et al. 1999; Coy et al. 2013). This model is consistent with the observation that the deletion of Sm proteins results in accumulation of the poly A+ form of TLC1, loss of mature TLC1 RNA, and telomere shortening (Seto et al. 1999). The Sm protection mechanism is conserved in fission yeast. However, maturation of the S. pombe TR depends on a Sm-dependent splicing step, after which the Sm cap is replaced by a closely related Lsm cap (Tang et al. 2012). Thus, Sm7 is important for maintaining steady state-levels of various yeast telomerase RNAs, but does not directly impact telomerase action.

The Ku heterodimer (see below) also incorporates into the telomerase holoenzyme through association with TLC1 (Figure 1.4). Ku binds to the template boundary arm, which is distinct from the Est1-binding arm. The association of Ku with TLC1 was originally identified in an overexpression screen for disruption of telomeric silencing, and deletion analysis localized the Ku Binding Site (KBS) to a 48 nt

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stem-loop at the distal end of the template boundary arm (Peterson et al. 2001). The terminal KBS (288nt-312nt) is predicted to form a hairpin with a two-base bulge at the center. The fold of the terminal KBS is retained in subsequent structural modeling of TLC1, but the conformation of the helix adjacent to the KBS varies among the proposed structures (Dandjinou et al. 2004; Zappulla and Cech 2004). Disruption of the KBS structure perturbs Ku binding both in vivo and in vitro, resulting in a telomere shortening phenotype (Peterson et al. 2001; Stellwagen et al. 2003). In addition, the Ku heterodimer binds DNA and RNA in a competitive manner, suggesting that they may bind at the same site on Ku (Pfingsten et al. 2012). DNA binds to an electronegative pre-formed ring at the interface of the Ku subunits (Figure 1.6A) (Walker et al. 2001), leading to the proposal that the RNA may mimic B-form DNA in order to bind the channel (Pfingsten et al. 2012). In addition, Ku and the KBS are both necessary for the nuclear retention of TLC1 (Gallardo et al. 2008; Pfingsten et al. 2012).
**Figure 1.6. Duplex DNA bound to the Ku heterodimer.**  
A. Ku70 (blue) and Ku80 (yellow) associate to create a pre-formed ring which binds duplex DNA (pink) in a sequence independent manner.  
B. Cross-section and 90° rotation from A. Side-chains protrude from the base of the ring to contact phosphate backbone and deoxyribose moieties. Coloring as in A.  
C. Bird’s eye view of the preformed ring encircling the DNA duplex with a three-way junction. Junction constrains DNA binding to a single mode. Coloring as in A. PDB: 1JEY (Walker et al. 2001).
**Telomere structure and function in budding yeasts**

Telomere structure and end-binding proteins in budding yeast differ significantly from those in other eukaryotic organisms. In contrast to other telomeric DNAs, *S. cerevisiae* telomeres are comprised of irregular TG$_{1-3}$ repeats (Shampay et al. 1984), although the regularity and sequence of the repeats varies significantly even between budding yeasts (Podlevsky et al. 2008). Telomeres in *S. cerevisiae* are much shorter than human telomeres and the average telomeric duplex tract is ~300 to 400 base-pairs (Figure 1.7A) (Palm and de Lange 2008; Wellinger and Zakian 2012). The single-stranded overhang is 12-15 nt during G1 and lengthens as a result of C-strand resection to 30-100 nt during late S/G2, presumably to facilitate increased telomerase accessibility (Wellinger and Zakian 2012). Both double- and single-stranded DNA regions are bound by protective capping proteins (Figure 1.7B). Groups of degenerate repeat sequences are found adjacent to the telomere (subtelomeric region). Subtelomeric sequence elements, X and Y' in budding yeast, can be many kb in length and vary in number and presence from chromosome to chromosome (Wellinger and Zakian 2012). Genes adjacent to telomeres are transcriptionally silenced in yeast, a phenomenon known as Telomere Position Effect (TPE) (Gottschling et al. 1990). Yeast telomeres are not believed to form t-loops like human telomeres (discussed below); however, one model which has not been rigorously validated, suggests that telomeres fold back upon themselves to form a hairpin structure mediated by protein-protein contacts (Figure 1.7C) (Strahl-Bolsinger et al. 1997).
Figure 1.7. The structure of telomeres in *S. cerevisiae*. A. Linear organization of yeast telomeric DNA. Subtelomeric repeats (yellow) are followed by duplex telomeric tract and 3' single-stranded G-strand overhang (black), C-strand shown (grey). B. Telomeric capping proteins in *S. cerevisiae*. RAP1 coats the double-stranded telomeric DNA. The Sir complex mediates TPE, complex comprised of Sir3 (S3), Sir4 (S4), and Sir 2 (cyan). Rif1/2 (R1 and R2 respectively) also associate with RAP1 and are negative regulators of telomere length. The Ku heterodimer (Ku) binds at the transition from duplex to single-stranded telomeric DNA. Cdc13 is the avid single-stranded DNA binding protein and Stn1 and Ten1 form a complex with Cdc13 (CST complex). C. Budding yeast telomeres are proposed to form a fold-back hairpin structure. Protein identities the same as shown in B.
The telomere binding proteins of *S. cerevisiae*

In contrast to both fission yeast and human telomeres, budding yeast telomeric DNA is not bound by a shelterin complex, but instead relies on a different set of capping proteins (Figure 1.7B) (Nandakumar and Cech 2013). Rap1 is the predominant double-stranded DNA binding protein found at telomeres in *S. cerevisiae*. Rap1 contains MYleomBlastosis (myb) transcription factor-like domains that recognize telomeric DNA in a sequence specific manner (Konig et al. 1996). TRF1 and TRF2, the human telomeric DNA duplex binding proteins, each have a single myb domain (discussed below). Roughly 20 Rap1 molecules are thought to coat the average double-stranded telomere in budding yeast (Gilson et al. 1993). Additional domains on Rap1 enable interactions with other telomeric proteins (Figure 1.7B). Rif1 and Rif2 associate with Rap1 to contribute to telomere length regulation through a protein counting mechanism where telomere length is inversely proportional to the number of bound Rif proteins (Marcand et al. 1997; Levy and Blackburn 2004). The Sir2-Sir3-Sir4 complex also associates with Rap1 to maintain transcriptional silencing at telomeres (Aparicio et al. 1991; Hardy et al. 1992). Loss of Rap1 results in telomere shortening as well as end-to-end chromosome fusion by NonHomologous End-Joining (NHEJ) (Lustig et al. 1990; Marcand et al. 2008). Rap1 binding partners Rif2 and Sir4 also contribute to the prevention of telomere end-fusion (Marcand et al. 2008). In summary, yeast telomeres are coated by Rap1 which contains binding sites for multiple proteins which help to regulate telomere homeostasis.

A second double-stranded DNA end-binding protein, the Ku heterodimer, also associates with telomeric chromatin in budding yeast (Figure 1.7B), although it is best known for its role in NHEJ. The Ku heterodimer is comprised of two subunits that share the same overall topology, namely an N-terminal \(\alpha/\beta\) domain followed by a \(\beta\)-barrel domain and a variable C-terminal arm (Walker et al. 2001). The structural basis for the recognition of duplex DNA by Ku is illustrated in the co-crystal structure of
human Ku bound to DNA (Figure 1.6A) (Walker et al. 2001). The two β-barrel domains of each subunit associate at a large dimerization interface suggesting why the Ku monomers are individually unstable (Figure 1.6A,B) (Walker et al. 2001). A preformed ring structure is formed by two loops protruding from each subunit to bridge the interface. DNA binds in the central cavity of the ring and makes sequence-independent electrostatic contacts with the surfaces of both subunits adjacent to the dimerization interface (Figure 1.6 B,C).

In S. cerevisiae, both Ku subunits are approximately 70 kDa and show sequence similarity with the subunits of the human Ku heterodimer (Feldmann et al. 1996). The observation that disruption of the Ku heterodimer leads to telomere shortening and lengthened 3’ telomeric overhangs in budding yeast provided the first evidence that Ku is involved in telomeric capping (Boulton and Jackson 1996; Gravel et al. 1998; Polotnianka et al. 1998). Chromatin immunoprecipitation (ChIP) experiments led to the proposal that Ku directly binds to telomeric and subtelomeric DNA throughout the cell cycle (Gravel et al. 1998; Fisher et al. 2004). However, telomeric association through protein-protein interactions and direct binding of telomeric DNA by Ku cannot be distinguished by ChIP due to a non-specific crosslinking step.

Despite shortcomings associated with Ku ChIP experiments, the phenotypes observed upon Ku knockout are consistent and most easily explained by Ku directly binding to telomeric DNA; multiple lines of evidence support this conclusion. The Ku heterodimer binds DNA in a directional manner (Walker et al. 2001). Mutational analysis indicates that the Ku70 subunit, which faces towards the DNA terminus, is crucial for NHEJ function, and the Ku80 subunit, which faces away from the terminus, maintains telomeric silencing through interaction with Sir4 (Bertuch and Lundblad 2003; Roy et al. 2004; Ribes-Zamora et al. 2007). Thus, functions mediated by each Ku subunit in vivo are consistent with the polarity of Ku-DNA binding. In addition, it is well established that Ku directly binds to DNA ends to
mediate the repair of double-stranded DNA breaks during NHEJ, reviewed (Dynan and Yoo 1998). In budding yeast, end-binding by Ku at double-stranded breaks serves to limit Exo1 dependent single-stranded nucleolytic resection, preventing the formation of a single-stranded DNA tail that is necessary for the initiation of homologous recombination (Mimitou and Symington 2010; Foster et al. 2011). Likewise, Ku is required to prevent Exo1 dependent C-strand resection at telomeres (Maringele and Lydall 2002; Bertuch and Lundblad 2004; Vodenicharov et al. 2010). Thus, the simplest explanation is that Ku employs direct DNA binding to mediate analogous functions at both double-stranded breaks and telomeres (Dewar and Lydall 2012; Balestrini et al. 2013).

Perhaps the strongest evidence that Ku directly binds DNA at telomeres is the similarity in phenotype of mutations that impact the ability of Ku to bind DNA. The deletion of amino acids within the DNA binding loop of Ku leads to decreased telomeric silencing, shorter telomere lengths, decreased NHEJ, and reduced affinity for duplex DNA (Pfingsten et al. 2012). Point mutations on the surface of the DNA binding interface also result in reduced affinity for DNA and compromise telomere homeostasis (Lopez et al. 2011). In some cases, mutant Ku heterodimers, which have reduced affinity for DNA, slide inward on DNA and compromise the ability of Ku to antagonize Exo1 resection in vitro, and the mutants are defective for telomere end-protection in vivo (Balestrini et al. 2013). Thus, multiple independently derived mutations in Ku reduce affinity for DNA and compromise telomeric capping in vivo, suggesting that Ku does bind telomeric DNA directly. A direct DNA binding model does not preclude the possibility that Ku also associates with telomeres by multiple mechanisms, for instance via protein-protein interactions (Roy et al. 2004).

In budding yeast, single-stranded telomeric DNA is bound by the Stn1-Ten1-Cdc13 (CST) complex (Figure 1.7 B). CST is proposed to be a telomeric RPA complex (i.e. single-stranded DNA binding complex that facilitates DNA replication) (Gao et al. 2007). Indeed, both Stn1 and Ten1 share structural
homology with RPA complex proteins (Gelinas et al. 2009; Sun et al. 2009); although, an additional winged helix-turn-helix motif on Stn1 contributes to its telomeric function (Gelinas et al. 2009) Cdc13 mediates high affinity DNA-binding for the CST complex, as discussed below. Loss of either Stn1 or Ten1 results in compromised telomeric capping and resection of the C-strand (Grandin et al. 1997; Grandin et al. 2001). The CST complex was originally thought to functionally replace POT1 (discussed below) in budding yeast. However, the discovery of CST homologs in human and fission yeast, which both possess POT1, suggests otherwise (Martin et al. 2007; Miyake et al. 2009). In addition to end-protection, the CST complex functions to prevent telomere over-elongation and to coordinate telomere fill-in synthesis (Qi and Zakian 2000; Chen and Lingner 2013).

Cdc13 is the avid single-stranded DNA-binding protein within CST. Cdc13 contains multiple OB-domains and a telomerase recruitment domain. The CDC13 DNA-binding-domain has picomolar affinity for telomeric DNA (Nugent et al. 1996; Anderson et al. 2002). Aromatic amino acids on the surface of the DNA-binding-domain make specific contacts with the bases of the single-stranded DNA (Mitton-Fry et al. 2002). The remaining OB-domains and are thought to mediate Cdc13 dimerization as well as the interactions with Stn1, Ten1, and DNA polymerase α (Qi and Zakian 2000; Sun et al. 2011). Cdc13 was previously thought to be vital telomere capping in budding yeast. Perturbation of Cdc13 led to extensive lengthening of the single-stranded overhang, telomere shortening, and eventually senescence (Garvik et al. 1995; Lendvay et al. 1996). However, the recent identification of novel Cdc13 temperature sensitive alleles that do not have additional temperature-dependent defects suggests that the current model for Cdc13 in capping merits further investigation (Paschini et al. 2012). In addition, the telomeric capping role for CST appears to be dispensable during G1, but necessary during S-phase, further supporting its role in the coordination of telomerase action with semi-conservative DNA replication (Vodenicharov et
Finally, Cdc13 plays a foundational role in telomerase recruitment in budding yeast, as discussed below.

**Two pathways for telomerase recruitment in budding yeasts**

Prior to action at the telomere, telomerase must be properly assembled. In order for telomerase to extend telomeres, the holoenzyme must traffic to and physically interact with capping proteins at the telomere – the recruitment process (Figure 1.8). This section will give an overview of recruitment interactions, telomerase biogenesis, and cellular localization of telomerase in budding yeasts. Two sets of interactions between telomerase and the telomere have been proposed to mediate recruitment in *S. cerevisiae*: i) the interaction between the telomere binding protein Cdc13 and the telomerase subunit Est1 and ii) an interaction between TLC1 RNA and the Ku heterodimer, which also binds to telomeric DNA. Although there is evidence to support each of these models, studies which track TLC1 localization in various genetic backgrounds suggest that nuclear retention may be epistatic to recruitment interactions between telomerase and the telomere (Gallardo et al. 2008; Pfingsten et al. 2012). The two models for recruitment, cell-cycle regulation, and telomerase maturation and localization are discussed below.
Figure 1.8. Telomerase biogenesis, cellular localization, and recruitment to the telomere in *S. cerevisiae*. Schematic to emphasize select steps of telomerase processing, assembly, and nuclear shuttling. Holoenzyme stoichiometry and additional co-factors which associate with telomerase at various points are not indicated. Processing is in part regulated in part by the Sm7 complex (red ring structure). Cytoplasmic shuttling results in assembly of TLC1 with Est2, Est1 (E1), and Est3 (E3), and Ku which are necessary for nuclear retention. Yeast telomerase is thought to freely diffuse through the nucleus until tethering alongside of telomeres to the nuclear envelope.
during S phase. Tethering of telomerase or telomeres to the nuclear envelope is denoted by jagged black lines. Although not shown in the figure, release of telomerase and short telomeres from the envelope may function in the regulation of extendable (cytoplasmic) and less-extendable (envelope bound denoted) telomeres. Est1-Cdc13 recruitment interaction shown. Telomeric proteins same as figure 1.7.

**Est1-Cdc13 interactions recruit telomerase**

The demonstration that two *Cdc13* alleles each had distinct telomeric phenotypes, one which hampered telomerase action and another which resulted in the loss of telomeric capping, led to the hypothesis that Cdc13 plays a role in both processes (Nugent et al. 1996; Qi and Zakian 2000). Two main lines of evidence support the Cdc13–Est1 recruitment pathway. First, fusion of Cdc13 and Est1 results in telomere elongation in cells, complementing both *Cdc13* and *Est1* knockout strains (Evans and Lundblad 1999). Second, charge-swap experiments strongly suggest direct protein-protein interactions between Est1 and Cdc13 (Pennock et al. 2001). A mutation of glutamate to lysine at position 252 of Cdc13 (*Cdc13*-2) causes telomere shortening and eventual senescence (Nugent et al. 1996). Likewise, a mutation of lysine to glutamate at position 444 of Est1 results in the same phenotype. However, when these two alleles are combined in a Cdc13-Est1 double mutant strain, they complement one another and telomere length is rescued (Pennock et al. 2001). The compensatory nature of the charge-swap rescue provides strong genetic evidence that these two proteins directly interact to physically link telomerase and the telomere.

The direct Est1-Cdc13 interaction is further supported by a number of other studies. Est1 and Cdc13 physically associate *in vitro*, and a Cdc13 domain known as the recruitment domain is sufficient for the interaction with Est1 (Wu and Zakian 2011). A significant increase in Est1-dependent *de novo* telomere addition is observed when Cdc13 is tethered near an artificially induced double-stranded break (Bianchi et al. 2004). Telomeres shorten in strains bearing the *Cdc13*-2 allele, in which telomeric capping
is not compromised but telomeres shorten due to a telomerase recruitment defect (Nugent et al. 1996). Consistent with the telomere shortening phenotype observed in Cdc13-2 strains, telomerase recruitment to telomeres during S-phase is compromised in these strains as determined by live-cell imaging (Gallardo et al. 2011). Based on the above data, the Cdc13-Est1 telomerase recruitment pathway is well established (Figure 1.8).

The role of the Ku heterodimer in telomerase recruitment

A Ku-dependent recruitment process is thought to act in concert with the established Est1-Cdc13 mechanism (Fisher et al. 2004). Because Ku binds to both telomeres and to TLC1 RNA, it has been proposed to serve as a bridging factor to recruit telomerase to the telomere during G1 of the cell cycle and thereby help to facilitate telomerase action during late S-phase (Peterson et al. 2001; Stellwagen et al. 2003; Fisher et al. 2004). Loss of Ku or TLC1 results in a substantial decrease in de novo telomere addition upon DNA damage, adding support to the Ku-dependent telomerase recruitment hypothesis (Evans and Lundblad 1999; Myung et al. 2001; Stellwagen et al. 2003). Tethering of Ku adjacent to an artificially induced double-stranded break results in increased Est2 association (Bianchi et al. 2004). Notably, deletion of the KBS within TLC1 does not affect telomere capping or telomeric silencing suggesting that Ku association with the telomere is not dependent upon its interaction with TLC1 (Peterson et al. 2001; Vodenicharov et al. 2010).

More recent experiments indicate that the Ku-TLC1 interaction may play a more complex role in telomerase recruitment than originally thought. Fluorescent in situ hybridization experiments demonstrate that the deletion of the KBS or either of the Ku subunits results in the loss of TLC1 nuclear localization (Figure 1.8), explaining in part the telomere shortening phenotype (Gallardo et al. 2008; Pfingsten et al. 2012). In contrast to the stable tethering of telomerase to telomeres in G1, suggested by
ChIP experiments (Fisher et al. 2004), live cell imaging experiments indicate that telomerase only transiently associates with telomeres during G1 (Gallardo et al. 2011). Additionally, Ku binds to KBS RNA and duplex DNA in a mutually exclusive and competitive manner in vitro (Pfingsten et al. 2012). These experiments call into question the hypothesis that Ku serves as a simple bridging factor to recruit telomerase.

Nonetheless, the insertion of an additional KBS into the TLC1 RNA results in a telomere hyper-elongation phenotype, indicating that the Ku-TLC1 interaction may contribute to telomere homeostasis in capacities beyond nuclear retention (Zappulla et al. 2011). For example, the Ku contributes to anchoring of telomeres and possibly telomerase to the nuclear envelope during S-phase, and critically short telomeres are proposed to be released from the envelope for extension while long telomeres remain tethered (Figure 1.8) (Schober et al. 2009; Ferreira et al. 2011). Furthermore, a recent report suggests that Ku functions to promote the association of Est1 with the telomere, and that Ku mediates telomere lengthening mainly through the interaction with Est1 (Williams et al. 2014). The Ku-Est1 genetic interaction is independent of nuclear retention (Williams et al. 2014). An interaction between Ku and Est1 raises an intriguing possibility for how the well-established Est1-Cdc13 and Ku recruitment pathways might function synergistically, perhaps in the capacity of nuclear anchoring during S-phase (Fig 1.8).

**Cell-cycle regulation of telomerase recruitment in S. cerevisiae**

Telomerase action is tightly coupled to cell cycle progression, and the mechanisms of coupling likely involve multiple layers of regulation that are not fully understood. Telomeres are replicated during late S-phase in budding yeast. However, cell cycle progression is not dependent upon telomerase as cells continue to divide in ΔTLC1 strains (Marcand et al. 2000). Telomeric synthesis is coordinated with
semi-conservative DNA replication and is dependent upon interaction with DNA polymerases α and δ (Diede and Gottschling 1999). One important physical link appears to be the CST complex, in which Cdc13 interacts with both DNA polymerase-α and telomerase through Est1 (Qi and Zakian 2000; Sun et al. 2011). Cell cycle-dependent regulation of Est1 expression may in turn contribute to both Est3 and telomerase recruitment, as high Est1 expression levels are high during S-phase and low during other phases of the cell cycle (Osterhage et al. 2006; Tuzon et al. 2011). Although more recent co-immunoprecipitation experiments suggest that a TLC1-Est1-Est2 subcomplex forms early in the cell cycle and that the association of Est3 regulates the formation of the active telomerase which coincides with S-phase (Tucey and Lundblad 2014). TLC1 transcription is also proposed to be regulated in a cell cycle-dependent manner (Dionne et al. 2013); although, steady-state levels of TLC1 remain constant (Mozdy and Cech 2006). In addition, tightly regulated telomerase pre-assembly and dis-assembly processes coincide with the timing of replication and are thought to prevent telomerase activity outside of late S-phase (Tucey and Lundblad 2014).

Post-translational modifications also play a role in the timing and location of telomerase recruitment. Cdk1 phosphorylates Cdc13, and phosphorylation near the recruitment domain in late S-phase is thought to regulate the association of telomerase through Est1 binding (Li et al. 2009). In budding yeast, deletion of the Tel1 kinase (the ATM homolog, discussed below) results in short telomeres, although cells do not senesce (Lustig and Petes 1986). Tel1 was also proposed to phosphorylate Cdc13 and thereby enhance Est1 association. However, this does not appear to be the case as telomere maintenance is not compromised by simultaneous mutation of all Tel1 consensus sites within Cdc13 (Gao et al. 2010). Instead, Tel1 may instead regulate C-strand resection or other telomeric proteins which have a less direct effect on recruitment (Gao et al. 2010). In addition to phosphorylation, both Cdc13 and Ku 80 are sumoylated and this modification appears to impact telomerase action and
tethering of telomeres to the nuclear periphery (Ferreira et al. 2011; Hang et al. 2011). Thus, cell cycle-dependent regulation of telomerase action is only partially understood in budding yeast.

**Budding yeast telomerase biogenesis, localization, and recruitment**

Budding yeast telomerase undergoes a maturation and localization process that is distinct from humans. Although the processes are seemingly distinct in the two organisms some similarities and differences are apparent. Maturation of human telomerase, for example, includes association with Cajal bodies. Yeast on the other hand do not have Cajal bodies, and mounting evidence suggests that budding yeast telomerase undergoes a maturation process in which cytoplasmic shuttling is a vital step (Figure 1.8). This section overviews the maturation and localization process in budding yeast and will be compared to human telomerase maturation and cellular trafficking in a later section.

TLC1 is transcribed in the nucleus by RNA polymerase II and then polyadenylated (Chapon et al. 1997). The poly-A tail of the nuclear precursor TLC1 is then resected by the exosome (Coy et al. 2013). The degree of resection is controlled by the Sm7 complex which halts resection at the 3’ end of the mature TLC1 (Seto et al. 1999; Coy et al. 2013). A TMG cap is added to the 5’ end of TLC1 by the TriMethylguanosine Synthetase-1 (Tgs1), prior to export from the nucleus (Seto et al. 1999; Gallardo et al. 2008). TLC1 is then exported from the nucleus by both the non-coding RNA Xpo1-Crm1 export machinery as well as the Mex67/Mtr2 mRNA export machinery (Gallardo et al. 2008; Wu et al. 2014). TLC1 is thought to assemble with the Est1, Est2, Est3 and Ku heterodimer subunits in the cytoplasm (Figure 1.8) (Gallardo et al. 2008). TLC1 is re-imported into the nucleus by the importins Kap122 and Mtr10, where nuclear retention is dependent upon association with Est1, Est2, Est3, and the Ku heterodimer (Ferrezuelo et al. 2002; Gallardo et al. 2008). Upon maturation, telomerase diffuses through the nucleus, associating only transiently with telomeres during G1. During S-phase, telomerase
aggregates at stationary telomere ends (Gallardo et al. 2011). Ku, Est1, Mps3, and Sir4 are proposed to regulate anchoring and release of telomerase from the nuclear envelope to facilitate action at short telomeres during S-phase (Schober et al. 2009; Ferreira et al. 2011). Thus, a number of maturation and localization parameters requiring nuclear/cytoplasmic shuttling must be met for telomerase to act on telomeres (Ferrezuelo et al. 2002; Teixeira et al. 2002; Gallardo et al. 2008). For instance, preventing nuclear export/re-import of TLC1 results in decreased telomere length (Ferrezuelo et al. 2002; Wu et al. 2014).

As mentioned in the beginning of the recruitment section, the cellular localization of telomerase has an epistatic effect on telomerase recruitment. For the purposes of this discussion, recruitment includes the molecular interactions that link telomerase to the telomere, and the trafficking of telomerase from a defined cellular compartment to the telomere. Given the maturation process described above, it is clear that perturbation of Est1 or the Ku heterodimer may not only disrupt telomerase recruitment but also holoenzyme assembly, nuclear shuttling, and nuclear anchoring. Given the difficulty of in vitro expression and reconstitution of the budding yeast telomerase holoenzyme, the genetic identification of separation-of-function alleles will be invaluable in distinguishing between recruitment and other roles in biogenesis and/or localization that these proteins play.

**Telomere maintenance in humans**

**The human telomerase holoenzyme**

This section introduces the telomerase holoenzyme, telomerase biogenesis, telomere-end protection, and telomerase recruitment from the Cajal body to the telomere in humans. Telomere maintenance in humans and fission yeast will be compared and contrasted in a number of instances. Finally, this chapter will end with a discussion of diseases associated with telomere dysfunction in
humans. In particular, this section of the introduction provides background for the latter chapters of the thesis which focus on understanding the nature and impact of the telomerase interaction with the TPP1 TEL-patch that mediate telomerase recruitment and processivity.

The 450 nt human TR (hTR) is smaller than its yeast counterparts and contains three main domains, the aforementioned canonical pseudoknot/template and template boundary elements, a partially conserved Box H/ACA motif, and a three-way junction (CR4/5) (Chen et al. 2000). The pseudo/knot template domain and CR4/5 both contact hTERT and are necessary for enzymatic activity (Tesmer et al. 1999). The box H/ACA is recognized by dyskerin (described below) and other Small CAjal body-specific RNA (scaRNA) associated proteins. One of the hairpins in the H/ACA also contains a CAB box motif that is recognized by TCAB1 to promote Cajal body localization (Figure 1.3) (Mitchell et al. 1999a; Mitchell et al. 1999b; Jady et al. 2004; Venteicher et al. 2009).

The dyskerin complex associates with human telomerase through RNA-protein interactions with hTR. Dyskerin is the catalytic subunit of a pseudouridylase complex that also includes GAR1, NOP10, and NHP2 (Mitchell et al. 1999a; Cohen et al. 2007). The multi-protein complex recognizes hTR secondary structure elements consisting of a hairpin followed by a single-stranded hinge, and a second hairpin followed by a single-stranded ACA motif (Figure 1.3). In the case of snoRNPs, dyskerin utilizes loops in the box H/ACA to site-specifically pseudouridylate other RNAs (Kiss et al. 2010). RNA targets of the hTR dyskerin complex have not been identified, suggesting that dyskerin may not function as a pseudouridylase when bound to hTR (Egan and Collins 2012a). Instead, dyskerin contributes to hTR stability, 3’ end-processing, cellular localization, and biogenesis (Mitchell et al. 1999a; Lukowiak et al. 2001). Perturbation of the telomerase-dyskerin interaction has been associated with loss of hTR accumulation in vivo and with telomere shortening (Mitchell et al. 1999b). Mutations in the H/ACA box
motif, Dyskerin, NHP2, and NOP10 are all associated with telomere-shortening diseases in humans (Podlevsky et al. 2008; Holohan et al. 2014).

TCAB1 is another RNA-binding component of the human telomerase holoenzyme. TCAB1 binds to the CAB box motif in hTR to promote localization to Cajal bodies (Jady et al. 2004; Zhu et al. 2004; Tycowski et al. 2009; Venteicher et al. 2009). The CAB box in hTR has a consensus sequence located in the terminal loop of the helix located between the single-stranded hinge and ACA regions (Figure 1.3) (Jady et al. 2004). TCAB1, also known as WDR79 and Wrap53, is a member of the WD40 repeat-containing family of proteins which adopt β-propeller folds. This motif is known to mediate a wide range of protein-protein interactions and is thought to play a role in intracellular scaffolding (Stirnimann et al. 2010). TCAB1 is proposed to function by concentrating low abundance telomerase into Cajal bodies, and it likely makes additional contributions to telomerase recruitment (Stern et al. 2012). Disruption of the TCAB1-hTR interaction results in mislocalization of telomerase to the nucleolus and telomere shortening (Tycowski et al. 2009; Venteicher et al. 2009; Zhong et al. 2011). Mutations in TCAB1 are implicated in telomere shortening diseases (Zhong et al. 2011). Collectively these data suggest that telomerase must localize to the Cajal body in order to be competent for subsequent recruitment to telomeres.

**TPP1 is a telomere-bound processivity factor for telomerase**

Although both TPP1 and POT1 are telomere-associated proteins and not telomerase holoenzyme components (Figures 1.9, 1.10), the TPP1-POT1 complex is a processivity factor for telomerase. The addition of TPP1-POT1 to primers in direct telomerase extension assays stimulates RAP (Wang et al. 2007; Zaug et al. 2010). TPP1-POT1 interacts with telomerase to stimulate processivity through at least two mechanisms: i) decreasing the rate of primer dissociation from the enzyme, and ii)
increasing the apparent translocation rate and efficiency (Latrick and Cech 2010; Hwang et al. 2014).

Mutations to the TPP1 glutamate (E) and leucine (L)-rich patch (TEL-patch), a group of conserved surface amino acids on the N-terminal oligonucleotide/oligosaccharide-binding fold (OB-domain) of TPP1, also decrease TPP1-POT1-dependent RAP stimulation of telomerase (Figure 1.9) (Nandakumar et al. 2012). Moreover, RAP stimulation defects of TPP1 TEL-patch mutants can be rescued by a compensatory charge-swap mutation in the TEN-domain of hTERT, suggesting a direct interaction (see chapter 3) (Schmidt et al. 2014). It is not yet clear, however, whether POT1-TPP1 remains at a fixed position on the primer to stimulate telomerase (Latrick and Cech 2010), or slides behind telomerase along the single-stranded DNA in a sequence-independent manner (Hwang et al. 2012; Hwang et al. 2014).
Figure 1.9. The domain structure of human telomere proteins TPP1 and POT1. Top, crystal structure of the human TPP1 N-terminal OB domain (PDB: 2I46). TEL-patch residues, which are conserved in mammals and shown to affect telomerase function in vitro (Nandakumar et al. 2012), are shown. TEL-patch residues colored according to identity, acidic residues (red), basic residues (blue), leucine (cyan). Middle, linear domain organization for TPP1 including N-terminal
OB (OB), POT1 binding domain (PBD), and TIN2 binding domain (TIN2 BD). TEL-patch indicated with a line. POT1 associates with TPP1 through a C-terminal TPP1 binding domain (TPP1 BD) and binds to single-stranded telomeric DNA with two N-terminal OB domains. Below, crystal structure of the two hPOT1 OB-domains bound to a single-stranded telomeric repeat (orange), POT1 residues involved in stacking interactions with the DNA nucleobases shown (olive), (PDB: 1XJV).
Figure 1.10. The architecture of human telomeres. A. Linear organization of human telomeric DNA. Subtelomeric repeats (yellow) are followed by duplex telomeric tract and 3’ single-stranded G-strand overhang (black), C-strand shown (grey). B. Cartoon of telomeres coated by shelterin. TRF1/2 bind the double-stranded telomeric DNA, TRF2 associates with Rap1 (R1). TIN2 bridges TRF1 and TRF2, and also serves as a bridge duplex telomeric tract and the single-stranded overhang by also associating with the TPP1-POT1 complex which protects the 3’ telomeric overhang. Shelterin components are non-stoichiometric (Takai et al. 2010). C. Mammalian telomeres are proposed to form T-loops in which the single-stranded G-rich overhang invades the duplex telomeric tract and base-pairs to the C-strand, in turn displacing the duplex G-strand to form a D-loop. Protein identities the same as shown in B. Artistic renderings modified from (Schmidt et al. 2014).

The biogenesis of human telomerase

hTR is transcribed by RNA Polymerase II and undergoes an RNA processing event to generate a uniform 3’ end (Zaug et al. 1996; Fu and Collins 2003). Sequences in the H/ACA domain of hTR (Bio box
motif and ACA) are necessary for assembly of hTR with dyskerin and other H/ACA protein cofactors; these proteins in turn promote hTR stability and determine the position of the mature 3´ end, which is necessary for localization to the Cajal body (Figure 1.11) (Mitchell and Collins 2000; Fu and Collins 2003; Jady et al. 2004; Theimer et al. 2007; Egan and Collins 2012b). hTR H/ACA RNP assembly and subsequent hTR accumulation are dependent upon NUFIP, RUVBL1, and RUVBL2, which are RNP chaperones (Boulon et al. 2008). Like budding yeast TR, hTR has a 5´ TMG cap, which is added by TGS1 (Jady et al. 2004; Girard et al. 2008). Mature hTR is actively transported to Cajal bodies (Figure 1.11) (Boulon et al. 2004), where assembly of the dyskerin complex is completed (Darzacq et al. 2006). TCAB1 tethers hTR within the Cajal body (Tycowski et al. 2009; Venteicher et al. 2009) and may play additional roles in the maturation of hTR (Cristofari et al. 2007).
Figure 1.11 Telomerase biogenesis, cellular localization, and recruitment to the telomere in humans. Cartoon of select steps of telomerase biogenesis and assembly. Dyskerin (Dys) is necessary for hTR maturation and accumulation. TCAB1 (T1) promotes localization to the Cajal body, which is important for recruitment of telomerase to the telomere in S-phase. Telomerase is recruited to the telomere by a direct interaction between the TEN domain of hTERT and the TEL-patch of TPP1. Telomeric proteins same as figure 1.10. Cartoon modified from (Schmidt et al. 2014).

Although hTERT mRNA is virtually undetectable in senescent cells, promoter mutations appear to increase hTERT mRNA expression in a variety of cancers (Horn et al. 2013; Huang et al. 2013), suggesting that hTERT transcription is a tightly regulated process. Twenty-two alternatively spliced hTERT mRNA isoforms have been identified to date. Although the roles for all isoforms are not known, the β-deletion isoform has also been proposed to inhibit telomerase activity (Saebø-Larssen et al. 2006; Listerman et al. 2013; Wong et al. 2014). In addition to regulation at the transcriptional and posttranscriptional levels, hTERT must properly assemble into the telomerase RNP. Nuclear-localization signals encoded within hTERT facilitate nuclear import by importin 7 and nucleoporin Nup385 (Frohnert et al. 2014). Although the exact site and timing of hTERT-hTR assembly are not well understood, production of active telomerase also requires the presence of chaperones (Holt et al. 1999; Forsythe et
al. 2001). Cell cycle-dependent assembly and disassembly of the telomerase holoenzyme is proposed to occur in *S. cerevisiae* (Tucey and Lundblad 2014). Similarly, hTERT mRNA levels increase in S-phase while excess hTR levels remain relatively constant, suggesting that cell cycle-dependent assembly of human telomerase is also possible (Xi and Cech 2014), although the exact mechanism varies from *S. cerevisiae*.

**The architecture of human telomeres**

In contrast to the irregular repeats of yeast telomeres, human telomeres are comprised of the regularly repeating hexanucleotide sequence TTAGGG (G-strand) (Moyzis et al. 1988). The average human telomere starts at ~8-15 kb in length but undergoes shortening throughout the duration of an individual’s lifetime (Figure 1.10A) (de Lange et al. 1990; Palm and de Lange 2008). The 3’ overhang length in humans ranges from ~40 to 130 nt on average and is longer than the dynamic 15-100 nt overhang in *S. cerevisiae* (Chai et al. 2006; Wellinger and Zakian 2012). The double- and single-stranded regions of telomeric DNA are bound by the protective protein complex shelterin (Figure 1.10B).

Approximately one-quarter of human chromosomes contain subtelomeric repeats (degenerate repeat sequences that are distinct from telomeric repeats), which range in size from 4-200 kb (Figure 1.10A) (de Lange et al. 1990). In many eukaryotic cells, including humans, the termini of telomeres are thought to loop back to form lariat structures of variable length known as T-loops (Figure 1.10C) (Griffith et al. 1999; Murti and Prescott 1999; Palm and de Lange 2008). T-loops were first observed in electron micrographs of isolated telomeres and their existence has been supported by psoralen cross-linking data (Griffith et al. 1999). T-loop architectures were also recently observed in intact cells using super-resolution microscopy (Doksani et al. 2013). T-loop formation is thought to depend on the invasion of the 3’ single-stranded G-rich overhang into the adjacent duplex telomeric DNA. Upon invasion, the G-rich strand in the telomeric duplex is displaced by the 3’ overhang, forming a smaller D-loop (Figure 1.10C). The T-loops protect telomeric-ends from activating DNA damage response pathways and inhibiting
telomerase by sequestering the 3´ end (Griffith et al. 1999; Doksani et al. 2013). The formation of T-loops is dependent upon the telomeric protein TRF2, but not TRF1 or RAP1 (Griffith et al. 1999; Doksani et al. 2013). How T-loops are formed and disassembled in a cell-cycle dependent manner to protect the telomere as well as allow telomerase access during semi-conservative DNA replication remains unknown.

The shelterin complex: the telomere-binding proteins of humans and fission yeast

Human telomeric DNA is coated by shelterin which is comprised of six proteins and suppresses telomeric activation of DNA damage response, inhibits inappropriate fusion and recombination, and recruits telomerase (de Lange 2005; Nandakumar and Cech 2013). TRF1/TRF2 are the double-stranded DNA-binding proteins, RAP1 is a TRF1/2 accessory factor, POT1 binds the single-stranded telomeric 3´ overhang, and TIN2 and TPP1 serve as bridging factors between TRF1/2 and POT1 (Figure 1.10B). Depletion of the entire shelterin complex in mice leads to the activation of multiple DNA damage response pathways as well as significant telomere dysfunction (Sfeir and de Lange 2012). Fission yeast utilizes a related five membered shelterin complex to cap its telomeres (Nandakumar and Cech 2013).

TRF1/2 are the telomere double-stranded DNA-binding proteins in mammals. TRF1 and TRF2 share a high degree of structural similarity to one another and utilize myb transcription factor-like domains to recognize telomeric repeats in double-stranded DNA in a sequence-specific manner (Nishikawa et al. 2001; Hanaoka et al. 2005). Both TRF1 and TRF2 homodimerize via homology domains, but despite structural similarity between the domains of these two proteins, TRF1 and TRF2 do not heterodimerize (Fairall et al. 2001). TIN2 associates with both TRF1 and TRF2 (Kim et al. 2004; Ye et al. 2004a; Chen et al. 2008), while RAP1 associates only with TRF2 (Li et al. 2000). TRF1 and TRF2 each interact specifically with accessory proteins through F/YxLxP motifs in their homology domains (e.g.,
TRF2 interacts with the nuclease Apollo) (Chen et al. 2008; Palm and de Lange 2008). TRF1 is proposed to regulate telomere length through a counting mechanism similar to that proposed for budding yeast RAP1 (van Steensel and de Lange 1997). Removal of TRF1 results in decreased telomeric levels of TRF2 and Rap1 (Ye et al. 2004a). In mice, TRF2 is necessary to suppress the ATM kinase mediated DNA-damage response (Denchi and de Lange 2007). The ATM and ATR kinases coordinate the DNA damage response in eukaryotes: ATM is predominantly activated by double-strand breaks and ATR is activated by unprotected single-stranded DNA (Palm and de Lange 2008). Removal of TRF2 from telomeres results in substantially increased telomere end fusion and ATM-mediated cell cycle arrest (Denchi and de Lange 2007; Konishi and de Lange 2008), which is likely caused by a failure to form T-loops (Doksani et al. 2013). The fission yeast homolog for both TRF1 and TRF2 is a single protein – Taz1, which also dimerizes to bind double-stranded DNA (Cooper et al. 1997; Spink et al. 2000).

The role of human Rap1 within the shelterin complex remains somewhat enigmatic. Unlike its budding yeast counterpart, human RAP1 does not bind with high affinity to DNA (Li et al. 2000; Arat and Griffith 2012), but rather appears to increase the affinity of TRF2 for DNA (Arat and Griffith 2012). Human RAP1 binds to TRF2 through its C-terminal domain (Chen et al. 2011). RAP1 association with TRF2 appears to be necessary for suppression of homology-directed recombination at telomeres in mammalian cells (Sfeir et al. 2010; Chen et al. 2011). The RAP1 homolog in S. pombe is important for bouquet formation (clustering of telomeres at the nuclear envelope) during meiosis (Chikashige and Hiraoka 2001); however, this role in nuclear envelope attachment does not appear to be conserved in higher eukaryotes (Scherthan et al. 2011). Further elucidation of human RAP1 binding partners will likely be helpful in discerning additional RAP1 functions, such as regulating gene expression (Ye et al. 2014).
TIN2 is the bridging factor that connects single-stranded and double-stranded DNA-binding proteins at human telomeres. TIN2 plays a significant role in connecting, stabilizing, and recruiting various shelterin components. TRF1 binding is mediated through the N-terminal domain of TIN2 and the C-terminal domain of TRF2 (Chen et al. 2008); this connection is important for the stability of the TRF1/2 interaction (Kim et al. 2004; Ye et al. 2004a). TIN2 also connects the TRF1/2 bound duplex telomere with the single-stranded 3´ overhang through an association with the TPP1-POT1 complex (Houghtaling et al. 2004; Liu et al. 2004; Ye et al. 2004b). TIN2 is required for the recruitment or stabilization of POT1-TPP1 at telomeres, and depletion of TIN2 leads to loss of both POT1 and TPP1 at telomeres (Abreu et al. 2010; Takai et al. 2011). Deletion of TIN2 leads to activation of both the ATR- and ATM-mediated DNA damage responses and telomeric fusion, likely as a result of compromised TRF2-dependent ATM suppression and reduced POT1-TPP1 association with the telomeric overhang (Takai et al. 2011).

Additionally, the interactions between TIN2 and chromatin-associated proteins SA1 and HP1 promote cohesion of sister chromatids during mitosis, which impacts telomere length in a telomerase-dependent manner (Canudas et al. 2007; Canudas et al. 2011). The mechanistic details of how chromatid cohesion influences telomere length are not well understood. Poz1 is the TIN2 homolog in fission yeast and also acts as a bridging factor for single-stranded and double-stranded DNA-binding proteins (Nandakumar and Cech 2013).

POT1 binds the single-stranded 3´ overhangs of telomeres in humans and fission yeast. POT1 was first identified both in fission yeast and humans through sequence similarity to TEBPα (Baumann and Cech 2001), one of two single-stranded telomeric DNA-binding binding proteins in ciliates (Hicke et al. 1990). Crystal structures of the POT1 DNA-binding domains revealed that it utilizes two separate OB-folds to recognize telomeric DNA with moderately high affinity, predominantly by making direct contacts with nucleotides (Lei et al. 2003; Lei et al. 2004; Altschuler et al. 2011; Dickey et al. 2013). Human POT1
recognizes the sequence (TTAGGGTTAG) in a contiguous manner through stacking of aromatic amino acids and nucleobases (Lei et al. 2004). OB1 binds the first five nucleotides and OB2 binds nucleotides 7-10, inducing a sharp kink in the DNA at nucleotide six (Figure 1.9) (Lei et al. 2004). *S. pombe* Pot1, on the other hand, recognizes 12 nucleotides as a dimer (Nandakumar and Cech 2012), or 15 nucleotides as a monomer (Altschuler et al. 2011). The N-terminal OB-domain confers sequence specificity while the C-terminal OB-domain is more tolerant of sequence variation (Altschuler et al. 2011). This has led to the hypothesis that Pot1 in *S. pombe* has multiple binding modes, in which the 3′ end is either sequestered in the C-terminal OB-domain or accessible to telomerase for extension (Dickey and Wuttke 2014).

In humans, POT1 appears to be a negative regulator of telomere length as deletion of POT1 leads to telomere lengthening (Ye et al. 2004b). POT1 is sufficient to inhibit telomerase activity *in vitro* unless a sufficiently long 3′ single-stranded overhang is accessible (Lei et al. 2005; Wang et al. 2007). In mammals, POT1 is necessary to suppress the ATR-dependent DNA-damage response pathway, which is activated in response to single-stranded telomeric DNA (Hockemeyer et al. 2005; Denchi and de Lange 2007). Depletion of POT1 leads to the appearance of DNA damage foci at the telomere but does not result in substantial telomere end fusion (Hockemeyer et al. 2005; Denchi and de Lange 2007). Furthermore, removal of POT1 leads to loss of telomeric 3′ end uniformity, and telomeres terminate randomly (Hockemeyer et al. 2005). The phenotypes of POT1 loss in mammals contrast with those observed in fission yeast. Deletion of Pot1 in *S. pombe* results in rapid loss of telomeric DNA, death of most cells and the emergence of survivor cells that circularize their chromosomes to circumvent senescence (Baumann and Cech 2001). POT1 utilizes its C-terminal domain to recognize its binding partners TPP1 or TPZ1 in humans and fission yeast, respectively (Nandakumar and Cech 2013).

TPP1 was first identified through its interaction with POT1 (Houghtaling et al. 2004; Liu et al. 2004; Ye et al. 2004b). As POT1 is the structural homolog of TEBPα, TPP1 is the structural homolog of
TEBPβ, indicating that telomere-capping complexes are more conserved than previously thought (Liu et al. 2004; Wang et al. 2007). The TEBPαβ complex is responsible for single-stranded telomere end-protection in ciliates (Hicke et al. 1990; Gray et al. 1991). DNA contacts both subunits of the TEBP heterodimer, binding in the cleft between the two subunits (Horvath et al. 1998). Like TEBPβ, TPP1 alone does not have appreciable affinity for DNA; however, TPP1 increases the affinity of POT1 for DNA by roughly 10-fold in vitro (Lei et al. 2004; Wang et al. 2007). Based upon its homology to TEBPβ, TPP1 may also make transient contacts with single-stranded DNA (Hwang et al. 2012; Rajavel et al. 2014). In addition to the centrally located POT1 binding domain, TPP1 also contains a C-terminal TIN2 binding domain and an N-terminal OB-domain that contribute to telomeric function (Figure 1.9) (Wang et al. 2007).

In contrast to POT1, TPP1 appears to be a positive regulator of telomere length; however, this is somewhat complicated by multiple functions of POT1-TPP1, which include telomere end-protection, stimulation of telomerase processivity, and telomerase recruitment (telomerase recruitment discussed below) (Nandakumar et al. 2012). TIN2 is required for the recruitment of TPP1 to the telomere (Abreu et al. 2010; Takai et al. 2011), and despite the ability of POT1 to bind single-stranded DNA, TPP1 is necessary for the recruitment or stabilization of POT1 at the telomere. Depletion of TPP1 leads to telomere lengthening and activation of the ATR-dependent DNA-damage response in identical manner to POT1 depletion (Liu et al. 2004; Ye et al. 2004b; Denchi and de Lange 2007). Tpz1, the fission yeast homolog of TPP1, interacts in an analogous manner with S. pombe Pot1 to prevent telomere end-deprotection, but Ccq1, an additional shelterin co-factor, mediates telomerase recruitment. (Miyoshi et al. 2008; Moser et al. 2011)

Although telomeric roles for the Ku heterodimer have not been extensively studied in humans, evidence suggests that it makes vital contributions to telomere homeostasis. Human Ku is a
heterodimer consisting of Ku70 and Ku86 subunits, which together play an integral role in the NHEJ pathway for repairing double-stranded DNA breaks (Dynan and Yoo 1998). In humans, Ku is the DNA end-binding protein for DNA-dependent Protein Kinase C (DNA-PKc), which initiates a signaling cascade in response to DNA damage. Budding yeast, on the other hand, lack DNA-PKc (Dynan and Yoo 1998). At telomeres, Ku plays a role in preventing telomere dysfunction by preventing sister chromatid exchange and alternative NHEJ pathways in mice (Celli et al. 2006; Sfeir and de Lange 2012). Deletion of Ku86 results in massive telomere deletion and loss of viability (Wang et al. 2009). Whether the Ku heterodimer directly binds to human telomeric DNA is unclear. T-loop formation has been implicated in restricting Ku access to DNA and, consistent with this, Ku was recently shown to associate with TRF1/TRF2 in vivo (Celli et al. 2006; Ribes-Zamora et al. 2013). Ku also plays a role in telomere end protection in S. pombe and prevents nucleolytic resection as well as telomeric recombination (Baumann and Cech 2000). Future work should shed light on the role of Ku at telomeres in human cells.

**TPP1-dependent recruitment of human telomerase to telomeres**

In contrast to budding yeast, a single telomerase recruitment pathway appears to dominate in humans. As noted earlier, telomerase is sequestered in Cajal bodies for most of the cell cycle through an interaction with TCAB1 (Jady et al. 2006; Tomlinson et al. 2006). During S-phase, telomerase is recruited to telomeres through an interaction with TPP1 (Figure 1.11) (Xin et al. 2007; Abreu et al. 2010). TIN2 is necessary for the recruitment of TPP1 to shelterin, and consequently telomerase recruitment is compromised upon depletion of TIN2 (Abreu et al. 2010). Although POT1 also associates with TPP1, POT1 is not required for telomerase recruitment in vivo (Xin et al. 2007; Abreu et al. 2010). Thus, the link between TIN2 and TPP1 is the critical factor for telomerase recruitment in vivo. In contrast, POT1 is necessary for RAP stimulation of telomerase in vitro (as discussed above) (Wang et al. 2007); this discrepancy is likely due to an inability to fully reconstitute telomeres in vitro.
In addition to the TIN2-binding domain of TPP1, its N-terminal OB-domain is necessary for telomerase recruitment (Figure 1.9, 1.11). Deletion of the TPP1 OB-domain results in loss of telomerase recruitment (Xin et al. 2007; Abreu et al. 2010), and the TPP1 OB-domain is sufficient to recruit telomerase to a non-telomeric locus (Zhong et al. 2012; Schmidt et al. 2014). In particular, the acidic TEL-patch found on the surface of the OB-domain of TPP1 is both necessary and sufficient for telomerase recruitment (Nandakumar et al. 2012; Zhong et al. 2012; Nakashima et al. 2013; Sexton et al. 2014). Homozygous deletion of an acidic loop (Δ166-172) in the TEL-patch results in progressive telomere shortening and eventual apoptosis in human embryonic stem cells (Sexton et al. 2014). Importantly, homozygous deletion of hTERT phenocopies TPP Δ166-172, and the dual knock-out of TERT and the TEL-patch do not result in additive telomere-shortening or cell-death in epistasis analysis (Sexton et al. 2014). Thus, telomerase in humans is not recruited to telomeres through multiple redundant pathways. Rather, the TEL-patch mediates a direct interaction with the TEN-domain of hTERT, which will be described in chapter three (Schmidt et al. 2014).

Despite the clear differences in recruitment between humans and budding yeast, Est3 is a structural homolog of the TPP1-OB domain, as discussed above (Yu et al. 2008; Rao et al. 2014). Although Est3 in budding yeast is a bona fide component of the telomerase holoenzyme and not a telomere-capping protein, it contains an acidic loop that is analogous to the TEL-patch of TPP1 (Hughes et al. 2000; Lee et al. 2008; Rao et al. 2014). The interaction between the Est3 (including the TEL-patch) and the Est2 TEN-domain is important for telomere maintenance in vivo (Friedman et al. 2003; Talley et al. 2011; Yen et al. 2011; Tucey and Lundblad 2014). Similar to Est3, residues outside of the TEL-patch of TPP1 have been proposed to activate human telomerase (Rao et al. 2014; Sexton et al. 2014; Tucey and Lundblad 2014). Thus, although the apparent roles for TPP1 and Est3 are not conserved, the interaction
between an OB-domain and the TEN-domain of hTERT appears to be a mode of telomerase regulation shared between budding yeast and humans.

**Ccq1 is a bridging factor for telomerase recruitment in fission yeast**

The conservation of the TERT-TPP1/Est3 interaction in humans and budding yeast would suggest that Tpz1 and Trt1 (S. pombe TERT) should interact in fission yeast. However, the shelterin accessory factor Ccq1 is necessary to recruit telomerase in fission yeast (Miyoshi et al. 2008; Moser et al. 2011). Deletion or perturbation of Ccq1 results in i) moderate telomere shortening (consistent with reduced telomerase action), ii) Tpz1 fails to immuno-precipitate active telomerase and iii) telomerase recruitment *in vivo* is compromised (Miyoshi et al. 2008; Moser et al. 2011; Yamazaki et al. 2012). Furthermore, telomerase recruitment is mediated through an interaction between Ccq1 and Est1 in *S. pombe*, similar to recruitment in budding yeast although the exact mechanism appears to vary (Moser et al. 2011; Webb and Zakian 2012). Ccq1 may also to play a role in telomerase activation (Armstrong et al. 2014).

Intriguingly, recent reports indicate that analogous TEL-patch region of Tpz1 is required for telomerase activation, presumably through a direct interaction with Trt1 (Armstrong et al. 2014). In fission yeast, Tpz1 and Ccq1 are proposed to regulate the switch between the extendible and non-extendible states of the telomere (Jun et al. 2013). Consistent with both activation and regulation of telomere accessibility, recruitment of telomerase to the telomere is not sufficient for telomere maintenance in fission yeast (Jun et al. 2013). In summary, shelterin is conserved between humans and *S. pombe* and consequently, telomere end-protection in humans and fission yeasts shares many similarities (Nandakumar and Cech 2013). In contrast to the similarities shared between fission yeast and human telomeres, emerging similarities in recruitment can be noticed between budding and fission
yeast. Telomerase is recruited through a holoenzyme co-factor and, after recruitment, the TPP1 homologs activate or modulate telomerase activity (Moser et al. 2011; Webb and Zakian 2012; Armstrong et al. 2014). Elucidation of an interaction between Tpz1 and Trt1 would further support the conservation of OB-domain interactions with the TEN-domain of TERT.

**Cell-cycle regulation of telomerase recruitment in humans and fission yeasts**

Telomerase recruitment is also cell cycle-dependent in both humans and fission yeasts, although the timing of events and regulatory kinases are better understood in the latter. As in other organisms, the *S. pombe* ATM/ATR kinases mediate the DNA damage response, and they also play a vital role in telomerase recruitment. The ATM/ATR DNA-damage response kinases Tel1 and Rad3 redundantly regulate telomerase recruitment to the telomere in late S-phase between leading and lagging strand synthesis by DNA polymerases (Miyoshi et al. 2008; Moser et al. 2009; Moser et al. 2011; Chang et al. 2013). Phosphorylation of Thr93 on Ccq1 is predominantly carried out by Rad3 (ATR homolog); this modification makes Ccq1 competent for interaction with Est1 and thereby recruitment of telomerase (Miyoshi et al. 2008; Moser et al. 2011; Yamazaki et al. 2012). Est1 expression levels are not thought to participate in cell-cycle regulation as they do in budding yeast (Osterhage et al. 2006; Webb and Zakian 2012). The 14-3-3 zinc-finger-like domain of Est1 recognizes both phospho-Ccq1 and *S. pombe* TR, possibly in a mutually exclusive manner, suggesting a transient hand-off mechanism or activation event (Webb and Zakian 2012; Armstrong et al. 2014). Dephosphorylation of Thr93 is likely to prevent prolonged telomerase association at the telomere (Chang et al. 2013). Additional activation of telomerase is likely necessary, requiring both Ccq1 and the Tpz1 TEL-patch (Jun et al. 2013; Armstrong et al. 2014). Following telomerase extension, lagging strand synthesis is coordinated by shelterin components as well as Sten1/Ten1 (Chang et al. 2013). Distinct subsets of the shelterin components
impose a barrier to mistimed and uncontrolled phosphorylation of Ccq1 (Moser et al. 2011; Chang et al. 2013).

In comparison to yeast, relatively little is known about the master regulators of cell cycle-dependent telomerase recruitment in humans. Despite the striking role for ATM/ATR kinases in *S. pombe*, both ATM and ATR kinases are dispensable for telomerase recruitment and telomere extension in mice (Feldser et al. 2006; McNees et al. 2010). Analogous to the phosphorylation events described for the yeast recruitment factors Cdc13 and Ccq1, TPP1 is also proposed to undergo cell cycle-dependent phosphorylation events. TPP1 has a number of phosphorylation sites (Zhang et al. 2013). Cyclin-dependent kinase phosphorylation of Ser111 in TPP1 was proposed to be cell-cycle regulated (Zhang et al. 2013). Overexpression of Ser111 TPP1 mutants results in decreased telomerase recruitment and telomere shortening (Zhang et al. 2013). However, changes in telomere length were not observed after genomic editing of a human embryonic cell line to express endogenous levels of TPP1 phosphomutants in a TPP1 knockout background, suggesting that phosphorylation of Ser111 may not regulate cell cycle-dependent recruitment of telomerase (Sexton et al. 2014). Thus, elucidation of the phosphorylation sites, and the regulatory kinases that target these sites, presents an exciting avenue for future investigation of the regulation and timing of telomerase recruitment.

In addition to phosphorylation, a number of other mechanisms may also regulate the timing of telomerase action at telomeres. As mentioned previously, levels of hTERT mRNA were recently shown to increase during late S/G2; in contrast hTR levels remained constant. However, as hTR levels exceed hTERT levels, increased expression of hTERT may lead to increased levels of telomerase in a cell cycle-dependent manner (Xi and Cech 2014). Consistent with increased levels of RNP assembly, telomerase activity also peaks during S-phase (Zhu et al. 1996). Finally, the human CST complex has also been
proposed to halt telomerase action at the end of S-phase and coordinate DNA polymerase fill-in synthesis by competing with the shelterin complex (Chen et al. 2012).

**The role of the Cajal body in human telomerase recruitment**

Although the underlying mechanisms that regulate telomerase recruitment are not well understood, human telomerase localizes to telomeres during S-phase. Telomerase is not associated with telomeres throughout most of the cell cycle, instead being sequestered in Cajal bodies (Jady et al. 2006; Tomlinson et al. 2006). Telomerase is tethered to the Cajal body through the interaction of TCAB1 with the CAB box motif in hTR, as discussed above (Jady et al. 2004; Tycowski et al. 2009; Venteicher et al. 2009). Disruption of the hTR-TCAB1 interaction is not required for the production of active telomerase, suggesting that Cajal body is not necessary for telomerase assembly (Figure 1.11) (Cristofari et al. 2007; Stern et al. 2012). However, Cajal bodies may be important for the maturation of telomerase or association with other protein cofactors, such as Sm proteins and coilin which may also play roles in telomerase recruitment (Fu and Collins 2006; Stern et al. 2012). The association of telomerase with TCAB1 is crucial for telomere maintenance (Cristofari et al. 2007). Disruption of the TCAB1-hTR interaction results in mislocalization of telomeres to the nucleolus and loss of telomerase recruitment (Tycowski et al. 2009; Venteicher et al. 2009; Zhong et al. 2011). The dependence of telomerase localization to Cajal bodies in humans is similar to the mandatory nuclear localization of telomerase in budding yeasts.

In addition, TCAB1 may play a direct role in telomerase recruitment (Stern et al. 2012). Consistent with this, TCAB1 associates with telomeres in a telomerase-dependent manner during S-phase (Figure 1.11) (Stern et al. 2012). Although, Cajal bodies are not strictly required for TCAB1-dependent telomerase recruitment (Stern et al. 2012), they appear to localize to telomeres during S-
phase (Jady et al. 2006). Furthermore, overexpression of telomerase results in the formation of neo-Cajal bodies at telomeres (Nandakumar et al. 2012; Zhong et al. 2012). A number of possibilities for the importance of Cajal bodies in telomere maintenance exist. First, Cajal bodies may simply concentrate limited amounts of telomerase; however, telomerase overexpression alone cannot compensate for a compromised interaction between TCAB1 and hTR (Stern et al. 2012). Second, Cajal bodies might actively deliver telomerase to the telomere (Cristofari et al. 2007). Third, TCAB1 may directly participate in telomerase recruitment to telomeres as a redundant enhancer of the TPP1-hTERT TEN domain interaction. Enhancement of recruitment by TCAB1 is not sufficient to overcome mutation to the TEL-patch (Sexton et al. 2014), and TCAB1 does not mediate its role through a direct interaction with the TEN-domain of hTERT (Stern et al. 2012). Currently, it is difficult to distinguish between the aforementioned TCAB1 recruitment models, but TCAB1 is clearly a key player in the recruitment of telomerase from Cajal bodies to the telomere.

**Telomere-shortening diseases in humans**

Growing evidence suggests that a number of seemingly unrelated degenerative diseases may in fact be due to the unifying underlying cause of telomere dysfunction. These disorders are now recognized as telomere-shortening disorders or telomeropathies (Armanios and Blackburn 2012; Holohan et al. 2014). Prevalent examples include Dyskeratosis Congenita (DC) and some cases of Aplastic Anemia (AA) and Idiopathic Pulmonary Fibrosis (IPF). DC and AA are most often characterized by bone marrow failure, while IPF is characterized by lung fibrosis. The severity and symptoms can vary widely between different telomeropathies based on the penetrance of the disease allele as well the original telomere lengths that are inherited by the affected individual. Consequently, telomere disorders often display genetic anticipation (i.e. earlier onset and sometimes more severe disorders in subsequent generations of individuals who inherit mutations) (Vulliamy et al. 2004; Armanios and
Blackburn 2012; Guo et al. 2014). IPF is thought to affect 100,000 individuals in the United States alone (Armanios 2012). However, causative mutations are identified in only ~60-70% of DC cases (Armanios and Blackburn 2012; Kocak et al. 2014). In general, the prevalence and scope of the underlying causes for telomere shortening diseases are not fully understood.

A variety of mutations in telomere maintenance genes cause defects in telomerase activity, maturation, processing, and cellular localization and are implicated in a spectrum of telomere shortening disorders (Armanios and Blackburn 2012; Holohan et al. 2014). Several disease-associated mutations (e.g. in hTR or hTERT) result in compromised telomerase activity or processivity defects. However, other mutations in core telomerase components cause nominal reductions in telomerase activity and processivity (Robart and Collins 2010; Zaug et al. 2013), suggesting that mutations in telomerase and telomerase or telomeric co-factors may affect other telomere maintenance functions, such as holoenzyme maturation. Mutations in dyskerin and TCAB1 result in decreased hTR accumulation and decreased localization to the Cajal bodies, respectively (Mitchell et al. 1999b; Zhong et al. 2011). Mutations in TIN2 that prevent sister chromatid cohesion (Canudas et al. 2011; Sasa et al. 2012; Vulliamy et al. 2012), and mutations in CTC1 result in defects associated with telomere replication (Touzot et al. 2010; Anderson et al. 2012; Chen et al. 2013). A number of other telomere maintenance genes including NOP10, NHP2, Apollo, and RTEL1 have also been implicated in telomere shortening diseases (Holohan et al. 2014).

In addition to defects in telomerase activity and biogenesis, defects in telomerase recruitment also appear to predispose individuals to telomeropathies. The IPF-associated hTERT mutation V144M (Tsakiri et al. 2007) results in reduced TPP1-POT1 RAP stimulation of telomerase in direct extension assays and decreased recruitment of telomerase to telomeres in vivo (Zhong et al. 2012; Schmidt et al. 2014). Recently, mutations in the TPP1 TEL-patch were also shown to associate with AA and with
Hoyeraal-Hreidarsson syndrome (a severe form of DC) (Guo et al. 2014; Kocak et al. 2014), further implicating telomerase recruitment defects in telomere shortening syndromes. Thus, understanding the molecular nature and mechanisms of telomerase recruitment may play a vital role in understanding a class of telomere-shortening disorders as well as aide the development of novel therapeutics.

In summary, this chapter has introduced the molecular interactions between components of the telomerase RNP, between the protective protein components found at telomeres, and those which mediate telomerase recruitment to telomeres in budding yeasts and in humans. Although, the proteins associated with telomere maintenance in humans and yeast appear to be quite different in many cases, a number of recent studies have highlighted emerging similarities between these systems. This introduction provides background information for chapters 2-4 and for the Appendix. The remaining chapters in this thesis will further explore the specificity and nature of the RNA-protein and protein-protein interactions which are vital for proper telomere maintenance in budding yeasts and humans. Chapter 2 will discuss the elements of the Ku binding site RNA within TLC1 that are vital for association with the Ku heterodimer. Chapter 3 focuses on an analysis of the role of the TPP1 TEL-patch in telomerase recruitment and association with telomeric substrates. Chapter 4 investigates the direct interaction of the TEL-patch with the TEN-domain of hTERT. Finally, the Appendix investigates the requirements for telomerase extension of Ku-bound substrates by budding yeast telomerase.
Chapter II: RNA recognition by the DNA end-binding Ku heterodimer

Introduction

The goal of the work presented in this chapter was to determine the elements of the TLC1 Ku binding site (KBS) RNA that are necessary for association with the Ku heterodimer, thereby enabling the identification of additional Ku-binding RNAs in other eukaryotic TRs, or in the context of other cellular RNAs. Photo-crosslinking experiments were carried out by David Chen. KBS mutant RNA transcriptions, dissociation constant determination, and RNase V1 structure probing were carried out by Karen Goodrich. Jen Pfingsten initiated V1 structure probing and mutagenesis and designed KBS mutants 1-8, 12, and 16. I was conceptually involved in the design of all experiments, carried out all data analysis, designed the remaining KBS mutants, generated experimental reagents, and carried out chemical probing and phosphorothioate footprinting experiments.

Most nucleic acid-binding proteins selectively bind either DNA or RNA, but not both nucleic acids. The Saccharomyces cerevisiae Ku heterodimer is unusual in that it has two very different biologically relevant binding modes: (1) Ku is a sequence-nonspecific double-stranded DNA end-binding protein with prominent roles in nonhomologous end-joining and telomeric capping (Boulton and Jackson 1996; Dynan and Yoo 1998), and (2) Ku associates with a specific stem-loop in the TLC1 RNA of budding yeast telomerase (Peterson et al. 2001). TLC1 RNA-binding and dsDNA-binding are mutually exclusive, so they may be mediated by the same site on Ku (Pfingsten et al. 2012). The Ku-TLC1 association is necessary for proper nuclear localization of this ribonucleoprotein enzyme in S. cerevisiae (Gallardo et al. 2008; Pfingsten et al. 2012).

¹This chapter has been modified from the following publication: Dalby AB, Goodrich KJ, Pfingsten JS, Cech TR. 2013. RNA recognition by the DNA end-binding Ku heterodimer. RNA 19(6): 841-851.
In spite of the clear Ku-TLC1 association in *S. cerevisiae*, it is unclear whether Ku’s role as a protein co-factor for telomerase is conserved among other eukaryotes. Deletion of YKU70 in a *S. paradoxus* strain with long telomeres results in a telomere shortening phenotype, providing indirect evidence for a TLC1-Ku interaction (Liti et al. 2009b). KBSs are proposed to exist in both sensu stricto and sensu lato Saccharomycotina, and a putative KBS that varies in both hairpin size and sequence appears to be present in the *Candida glabrata* TER (Chappell and Lundblad 2004; Kachouri-Lafond et al. 2009). Knockout of YKU80 in *Kluyveromyces lactis* does not result in a telomere shortening phenotype, and structural modeling suggests that *K. lactis* TER does not contain a KBS (Brown et al. 2007; Carter et al. 2007; Kabaha et al. 2008). Outside of the Saccharomycotina clade, KBSs have not been detected in the TERs of the fission yeast *Schizosaccharomyces pombe* or the filamentous ascomycete *Neurospora crassa* (Webb and Zakian 2008; Qi et al. 2012a). Although the precise Ku binding sites have not been identified, Ku is reported to bind to both human TER and specific TER isoforms in *Arabidopsis thaliana* (Ting et al. 2005; Ting et al. 2009; Cifuentes-Rojas et al. 2012) see also (Chai et al. 2002).

Ku also appears to bind to a host of other cellular RNAs. Prior to DNA damage, human Ku resides in the nucleolus and associates with a host of RNA-binding proteins in an RNA-dependent manner; following DNA damage, Ku disperses throughout the nucleus and incorporates into DNA damage complexes (Adelmant et al. 2012). Immunoprecipitation experiments demonstrate that Ku interacts with a pool of RNAs in HeLa nuclear extracts (Zhang et al. 2004). Ku is reported to bind the HIV TAR element stem-loop with high affinity in vitro (Kaczmarski and Khan 1993). Thus, mounting evidence suggests that RNA binding may be a conserved function of the Ku heterodimer.

In order to better understand the RNA-binding function of Ku, we set out to characterize the interaction between Ku and the TLC1 KBS. We first identify the site of Ku binding on the TLC1 KBS with nucleotide resolution. We next provide evidence that the KBS secondary structure folds into an unusual
bulged hairpin. Through mutagenesis of the KBS, we identify the critical determinants for Ku binding and define a minimal KBS motif. Finally, we provide evidence that KBSs are likely retained in several Saccharomyces TERs.

**Results**

**Ku interacts with the terminal stem-loop of the template boundary arm**

To identify the site of Ku binding on the TLC1 RNA with nucleotide resolution, we carried out phosphorothioate footprinting experiments. Protein contacts on an RNA molecule can be identified by protection of phosphorothioate linkages from chemical cleavage by iodine (Schatz et al. 1991; Rudinger et al. 1992). *In vitro* transcribed KBS RNAs, with randomly incorporated phosphorothioate nucleotide analogs, were subjected to iodine-mediated cleavage in the presence or absence of the purified Ku heterodimer (Figure 2.1A). The cleavage of a number of phosphorothioate linkages was reduced in the presence of Ku (Figure 2.1B). The most stable protections were clustered in the terminal loop of the KBS RNA at nt C300, A302, A303, and U306. Protections in the terminal loop had roughly two-fold reductions in cleavage throughout the duration of the time course (Figure 2.1C). In contrast, less stable protections extended from the loop into the adjacent stems surrounding the AU bulge. For example, nt U290, A294, G295, and G311 all had greater than two-fold reductions in cleavage at ten seconds but not ten minutes (Figure 2.1C).
Figure 2.1. Phosphorothioate footprinting of the Ku-KBS RNA interaction. A. Coomassie-stained SDS-PAGE analysis of the Ku heterodimer purified from *S. cerevisiae*. B. Ku-dependent protection from iodine-mediated cleavage of a TLC1 KBS RNA construct transcribed in the presence of A/U or C/G phosphorothioate NTPs. Cleavage times are denoted above the gel and lasted for 10 s, 34 s, and 10 min. Seq indicates the sequencing lanes, and –I$_2$ is a no-iodine control. Protected nt are denoted by arrows. C. Heatmaps of the fold-change in band intensity on a log$_2$ scale are plotted on the secondary structure of the TLC1 KBS. KBS numbering corresponds to the numbering for full length TLC1 RNA.
To further test the interaction of Ku with the terminal stem-loop of the KBS, nuclease V1 footprinting was carried out on the Ku-KBS complex (Figure 2.1A). Nuclease V1 cleaves duplexed or structured RNAs in a sequence-nonspecific manner to release products with a 3’ hydroxyl and a 5’ phosphate (Ehresmann et al. 1987). The KBS was most strongly protected by Ku from V1 cleavage from nt 284 - 310 (Figure 2.2B). We note that V1 nuclease gives a bigger and less resolved footprint than iodine, as expected because an enzyme is large and much more subject to steric hindrance than a small-molecule probe. Given the overlap between the iodine and V1 nuclease footprinting, it is likely that the protections observed here are due to direct protein contacts. Nevertheless, it must be kept in mind that changes in iodine reactivity may be due to dynamics in the RNA-protein contacts or protein-dependent changes in the structure of the RNA (Rose and Weeks 2001; Webb et al. 2001) Our results agree with previous in vivo mutagenesis studies that identified the AU bulge and surrounding basepairs as important for the Ku-TLC1 interaction (Peterson et al. 2001) and show that the interaction extends into the terminal loop.
Figure 2.2. RNase V1 probing of Ku-KBS RNA interaction. A. Gel showing protection of a TLC1 KBS RNA construct from nuclease V1 cleavage in the presence of Ku. OH corresponds to a ladder generated by alkaline hydrolysis and T1 for a ladder generated by nuclease T1. Arrowheads denote position of select G’s as indicated by the T1 ladder. The bracket denotes the region of the KBS which is most strongly protected by Ku. B. Heatmap of the fold change in band intensity on a log₂ scale is plotted on the secondary structure of the TLC1 KBS.

The terminal loop of the TLC1 KBS contacts Ku70

The phosphorothioate footprinting suggested that the Ku heterodimer makes direct protein contacts with the terminal loop of the TLC1 KBS (Figure 2.1B-C). In order to verify the interaction between Ku and the KBS terminal loop, and to determine which Ku subunit or subunits contact the KBS, photo-cross-linking with halo-pyrimidine-substituted KBS RNAs was carried out. KBS RNAs bearing single 5-iodo-uracil (5IU) substitutions were complexed with saturating amounts of Ku heterodimer and then
subjected to irradiation at 312 nm. Substitution to 5IU at position 301 resulted in the formation of a halo-pyrimidine specific covalent cross-link to one subunit of Ku (Figure 2.3A, E). Cross-linking efficiency was found to be time dependent (data not shown). At longer irradiation times, lower efficiency cross-links were generated with unmodified RNAs as well as RNAs substituted at positions U290, U297, U313, suggesting that the cross-links were 5IU independent (Figure 2.2A). Although mid-range UV bulbs with peak emission at 312 nm were used in these experiments, shoulder emission from the bulbs may result in excitation at lower wavelengths (Meisenheimer et al. 2000).

![Figure 2.3. 5IU cross-linking of the KBS-Ku complex. A. Cross-linking of site-specific 5IU-modified RNAs in the presence (+) and absence (-) of the Ku heterodimer as visualized on an SDS-PAGE gel by phosphoimagery. Unmodified RNA (Unmod) or modification position denoted above the gel. Arrows indicate cross-linked Ku-KBS complex (X-linked KBS) or free KBS RNA. B. Electrophoretic mobility shift assay of KBS-Ku complexes under native cross-linking conditions,
labeling as described in A. C. The Ku-KBS cross-link is RNA dependent, as analyzed by RNase A treatment of cross-linked complexes prior to SDS-PAGE electrophoresis and phosphoimaging. The presence (+) or absence (–) of Ku, 5IU modification at position 301, or RNase A treatment indicated above gel. Arrows same as noted in A, except bottom arrow denotes digested KBS RNA. D. The KBS RNA cross-links to Ku70. Co-visualization of Coomassie staining (red) and autoradiograph (green) of the same SDS-PAGE gel used to analyze the mobility of cross-links between internally 32P-labeled randomly 5IU-substituted KBS following treatment with RNase T1 or Trypsin (+). (–) absence of 5IU substitution or treatment with RNase or Trypsin. Molecular weight in kDa indicated on right side of gel. Images shown in false color. E. Summary of site-specific 5IU substitutions that result in cross-links to Ku shown in red, while positions shown in blue resulted in lower-efficiency background cross-links. Grey nucleotides indicate non-native sequence in the TLC1-38 KBS construct, added to stabilize the folded structure.

A native gel-shift assay confirmed that all substituted RNAs used were fully bound by Ku, and that the 5IU modification did not interfere with the KBS-Ku interaction under cross-linking conditions (Figure 2.3B). Cross-links were found to be dependent on the presence of both Ku and the TLC1 KBS RNA, and treatment with either RNase or protease resulted in loss of the cross-link (Figure 2.3C-D). As sequence substitutions to the terminal loop of the KBS had little impact on Ku-KBS affinity, G299-5IU and A303-5IU mutant RNAs were tested for their ability to form 5IU-dependent crosslinks to Ku. Both substituted mutant KBS RNAs cross-linked to one subunit of Ku with similar efficiency (~5%) as the U301-5IU KBS construct (data not shown). Thus, the generation of site-specific halo-pyrimidine cross-links further supports the conclusion that at least one subunit of the Ku heterodimer makes protein contacts with the terminal loop of the KBS RNA.

In order to determine which of the two Ku subunits contacts the KBS, cross-linked complexes were subjected to a battery of chemical and proteolytic digestion methods. The use of chemical digestion methods proved difficult, as there was little to no recovery of cross-linked peptides following purification from digestion with formic acid, 2-iodosobenzoic acid, and N-chlorosuccinimide/urea. Proteolytic cleavage of the Ku heterodimer with furin or cathepsin L were unsuccessful and non-specific,
respectively. The Ku 80 subunit, which contains three thrombin cleavage sites, was selectively digested into multiple peptides. However, digestion with thrombin did not result in mobility changes of Ku-KBS cross-links as determined by radiography. It seemed possible that the cross-linked KBS interfered with thrombin cleavage, or that the KBS cross-linked to the Ku 70 subunit.

Given the difficulty of site-selective digestion, we resorted to a more qualitative method to identify the cross-linked Ku subunit. TLC1 KBS was transcribed in the presence of α-32P- ATP and 5-iodo-UTP to generate internally labeled, randomly substituted RNAs. Cross-linking with the substituted RNAs resulted in the presence of a cross-link to a single subunit of the Ku heterodimer. Overlay of the same SDS-PAGE gel, visualized by Coomassie staining and autoradiography, revealed that cross-linked species migrated just below the Ku 80 subunit on the gel (Figure 2.3D, lane 5). Given that cross-linking of RNA generally retards the migration of a protein, this implicated Ku 70 as the cross-linked subunit. Treatment of cross-linked complexes with RNase T1 under elevated temperatures resulted in a decrease in radioactive signal as well as a shift of the cross-linked species to co-migrate with the Ku 70 subunit (Figure 2.3D, compare lanes 5 and 6). Collectively, our data suggest that 5-ido-uracil substituted KBS RNAs cross-link to the Ku 70 subunit.

**The TLC1 KBS forms a hairpin with a bulge motif**

In order to experimentally test the previously predicted terminal KBS secondary structure (Peterson et al. 2001; Dandjinou et al. 2004; Zappulla and Cech 2004), chemical modification experiments were carried out to identify unpaired nucleotides. TLC1 KBS RNA was treated with either dimethyl sulfate (DMS) or 1-cyclohexyl-3-(2-morpholinoethy)carbodiimide metho-p-toulene sulfonate (CMCT). Accessible imino groups at position 1 on adenosine and position 3 on cytosine can be methylated by DMS, and accessible imino groups at position 3 of uracil and position 1 of guanosine can
be modified by CMCT (Ehresmann et al. 1987). Modifications can be detected as stops in a reverse transcription reaction one nucleotide before the site of modification (Inoue and Cech 1985).

Treatment of the KBS RNA with DMS resulted in a number of strong stops in the reverse transcription reaction. Modification occurred at adenosines 302-304 in the terminal loop of the KBS RNA (Figure 2.4A, Figure 2.4C). Likewise, treatment of the KBS with CMCT modified U301 and U293 (Figure 2.4 B, Figure 2.4 D). Nucleotides in the putative stem immediately adjacent to the terminal loop were not heavily modified upon treatment with CMCT or DMS, given that the band intensities at these positions were within one standard deviation of the mean band intensity. The modification pattern of bases in the penultimate stem (nt 288-291 and 309-312) showed more variability than the terminal stem, suggesting a more complex motif. Additionally, stops corresponding to weak modification (<1 standard deviation above the mean band intensity) of U301 and U287 were observed in the DMS data. DMS is not known to react with uracil, and U287 is not modified by CMCT, so the meaning of these data is unknown. Collectively, the strong modification of the terminal loop and low reactivity of the majority of nucleotides in the putative stems are consistent with the hypothesis that nt 288-312 form a stem-loop.
Figure 2.4. Testing the secondary structure model of the KBS by chemical modification. A. DMS and B. CMCT modification experiments. Modifications were detected as stops, one nt prior to the site of modification, by reverse transcription. DMS modifies the N-1 of A and N-3 of C if the bases are unpaired. CMCT modifies the N-3 of U and N-1 of G if the bases are unpaired. Sequencing lanes generated by reverse transcription of the unmodified RNA in the presence of di-deoxy NTPs, denoted above the lane. RNA was allowed to react with DMS for 2, 5, or 15 min prior to quenching, and CMCT modification was carried out for 5, 15, or 30 min prior to quenching. Pre-mix controls consisted of the addition of RNA to the quench solution prior to addition of either DMS or CMCT. Lanes labeled as Denature denote RNA modification at 85ºC for 30 s. Selected A’s and U’s are indicated by arrows. C. DMS modification at 15 min or D. CMCT modification at 30 min plotted on the KBS secondary structure as a heatmap of the Z-score of band intensity for each position.

As mentioned above, the modification pattern for nt 291-293 did not appear to be consistent with a simple two-base bulge structure. U293 showed very high reactivity towards CMCT (>3 standard
deviations above mean band intensity), while A292 showed virtually no reactivity towards DMS (below the mean band intensity). U291 had a moderate band intensity, greater than one standard deviation above the mean, while U290 and U308 were weakly reactive with band intensities that were above average (Figure 2.4C, D). The modification data suggest that this portion of the KBS may fold into a specific structural motif. Varying chemical reactivity has been observed in the two nt bulge of domain V in the group II intron (Costa et al. 1998), which can likely be explained by the formation of a base-triple including one of the nucleotides in the bulge (Keating et al. 2010). Factors such as ion-binding sites or water-mediated hydrogen bonds may contribute to the protection of single-stranded nucleotides as well (Leontis and Westhof 1998). These modification data are consistent with previous secondary structure predictions in which the TLC1 KBS forms a hairpin; additionally, our data suggest that the hairpin contains a non-canonical bulge motif.

**Ku from *S. cerevisiae* binds to the KBSs of non-cognate TERs**

An alignment of TLC1 sequences from 34 *S. cerevisiae* strains and 25 *S. paradoxus* strains, in addition to single TLC1 sequences from *S. kudriavzevii, S. cariocanus, S. bayanus, S. pastorinus, S. mikatae, S. arboricola,* and *S. castellii,* demonstrated that the KBS is highly conserved from nt 289-311 (*S. cerevisiae* numbering, Figure 2.5A,B). Variation between the *Saccharomyces* spp. occurred in the predicted terminal loop (e.g., larger loops for *S. bayanus* and *S. castellii*). The length of the stem preceding the AU bulge varies between three and four basepairs (e.g., *S. castellii* compared to *S. cerevisiae*), and one A-to-G transition can be observed in the presumptive AU bulge (c.f. *S. cerevisiae* and *S. arboricola*). Overall, the high degree of sequence conservation in the KBS relative to the weak conservation in other portions of this arm of TLC1 (Zappulla and Cech 2004) suggests that the RNA sequence is critical for structure and for recognition by Ku.
Figure 2.5. Alignment of the KBS for select Saccharomycotina. Yellow shading corresponds to invariant nucleotides in the KBS. Alignment modified from the telomerase database alignment (Podlevsky et al. 2008), using BioEdit v7.1.3.0.

To verify the importance of conserved sequence in the KBS, affinity measurements were made using Ku from S. cerevisiae and the putative non-cognate KBSs from selected sensu stricto strains, a sensu lato strain S. castellii, and the ascomycete K. lactis (Figure 2.6A, B). Phylogenomic analyses indicate that S. paradoxus and S. cerevisiae are very closely related organisms (Fitzpatrick et al. 2006); correspondingly, the KBSs from these species show 100% identity from nt 288 – 312. Consistent with the importance of the conservation of the terminal KBS, Ku from S. cerevisiae bound the non-cognate S. paradoxus KBS with an apparent Kd similar to that of the cognate interaction (Figure 2.6B). Conversely, K. lactis is an outgroup to both the sensu stricto and sensu lato yeasts and is not predicted to have a KBS (Fitzpatrick et al. 2006; Brown et al. 2007). As expected, Ku from S. cerevisiae had a severely diminished affinity for the distal stem-loop of the template boundary arm from K. lactis.
Figure 2.6. Binding analysis of *S. cerevisiae* Ku with non-cognate KBSs. A. Representative native gel-shift assays. Increasing concentrations of *S. cerevisiae* Ku were incubated with trace amounts of either the *S. cerevisiae* or *S. bayanus* KBS RNA. Ku-KBS complexes were resolved on a native gel to separate bound from free KBS. B. Proposed secondary structures for putative KBSs from other budding yeasts and dissociation constants of *S. cerevisiae* Ku for the putative non-cognate KBSs. Also shown are the % amino acid identities between Ku from *S. cerevisiae* and Ku from other budding yeasts.

Non-cognate KBSs with intermediate sequence conservation revealed additional determinants of Ku recognition. Apparent *K_d*'s from *S. cerevisiae*, *S. bayanus*, and *S. castellii* KBSs revealed that the
size of the terminal loop, but not the sequence, is important for recognition. In *S. castellii*, the terminal loop of the KBS increased from seven to eleven nt and changed in sequence, but had only a slight decrease in affinity for the *S. cerevisiae* Ku (Figure 2.6B). In contrast, the *S. bayanus* KBS terminal loop increased from seven to twenty-four nt and had an approximately 50-fold reduction in affinity (Figure 2.6A, B). The length of the penultimate stem is either three or four basepairs and appears to have a modest effect on affinity. In the case of the *S. arboricola* KBS, there is an A-to-G transition at position 292 in the bulge and a shortening of the stem preceding the bulge (Figure 2.6). Both changes resulted in a moderate reduction in affinity for *S. cerevisiae* Ku. Collectively, the binding studies with TLC1 KBS homologs demonstrate that the size of the terminal loop and conservation of the AU bulge and flanking helices are important for recognition by Ku from *S. cerevisiae*. These data also provide evidence that the KBS may be a conserved feature of the TERs in both *sensu stricto* and *sensu lato* yeasts.

**Terminal stem length and bulge motif are critical for Ku recognition**

Binding assays with mutant *S. cerevisiae* KBS constructs were carried out to further elucidate the elements critical for Ku binding (Table 2.1, Figure 2.7A). The complete set of mutant KBS constructs is shown in Figure 2.8. Due to the high number of uridines in the conserved region of the KBS, we reasoned that mutants would have a high propensity to improperly fold and thereby artificially increase the *Kd*. To try to identify potentially misfolded mutants and hence exclude them from our study, RNA secondary structures were analyzed by mFold. Then to experimentally confirm the mFold predictions and verify that sequence changes did not cause RNAs to adopt unanticipated conformations, we carried out V1 nuclease digests of selected mutants (Figure 2.9A). We found that when the lowest free energy structure for a mutant was misfolded (i.e. did not maintain the mFold-predicted 4 basepair stems on either side of the bulge), the mutant RNA typically misfolded as judged by V1 nuclease digests (Figures 2.9B, C). Although our chemical modification data suggest that the native KBS may contain a more
complex bulge motif than is represented by the predicted mFold secondary structure, we found mFold a useful tool for eliminating mutants prone to misfolding.

**Table 2.1. Binding data for mutant KBS RNAs**

<table>
<thead>
<tr>
<th>Mutant Number</th>
<th>Region of Hairpin</th>
<th>Original Basepair or Sequence</th>
<th>Mutated Basepair or Sequence</th>
<th>Nuclotides Changed</th>
<th>$K_d$ nM</th>
<th>$\Delta\Delta G^\circ$ kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reverse complement terminal loop</td>
<td>GGCUAAC</td>
<td>CCAGUU</td>
<td>-</td>
<td>1.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Reverse complement portion of terminal loop</td>
<td>GUAAC</td>
<td>GAUUU</td>
<td>-</td>
<td>3.0 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Compensatory double mutant</td>
<td>A:U, U:G</td>
<td>C:G, C:G</td>
<td>-</td>
<td>2.4 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Combined changes from mutants 2&amp;3</td>
<td>A:U, U:G, CAAA</td>
<td>C:G, C:G, GAUUU</td>
<td>-</td>
<td>5 ± 1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>AU bulge deletion</td>
<td>AU</td>
<td>No sequence change</td>
<td>-</td>
<td>292-293</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Internal loop deletion</td>
<td>AU, UUA</td>
<td>No sequence change</td>
<td>-</td>
<td>286-287, 312-314</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>GUG bulge deletion</td>
<td>GUG</td>
<td>No sequence change</td>
<td>-</td>
<td>280-282</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AU bulge sequence inversion</td>
<td>AU</td>
<td>UA</td>
<td>-</td>
<td>292-293</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Compensatory mutant</td>
<td>U:A</td>
<td>G:C</td>
<td>-</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Terminal loop to GNRA tetraloop</td>
<td>GGCUAAC</td>
<td>GAAAC</td>
<td>-</td>
<td>289-291</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Wobble pair to Watson-Crick pair</td>
<td>U:G</td>
<td>U:A</td>
<td>-</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Compensatory mutant</td>
<td>U:G</td>
<td>G:U</td>
<td>-</td>
<td>35 ± 8</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Watson-Crick pair to wobble pair</td>
<td>U:A</td>
<td>U:G</td>
<td>-</td>
<td>2.11</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Compensatory mutant</td>
<td>U:A</td>
<td>U:G</td>
<td>-</td>
<td>294</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Compensatory mutant</td>
<td>G:C</td>
<td>A:U</td>
<td>-</td>
<td>295, 307</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Compensatory mutant</td>
<td>A:U</td>
<td>G:C</td>
<td>-</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Lengthening of terminal stem</td>
<td>AGAUACUC</td>
<td>AGAGAUACUC</td>
<td>Ins 293-294, 308-309</td>
<td>164 ± 17</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Lengthening of terminal stem</td>
<td>AGAUACUC</td>
<td>AGAGAUACUC</td>
<td>Ins 293-294, 308-309</td>
<td>&gt;270 ± 2.91</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Compensatory mutant</td>
<td>U:A</td>
<td>G:C</td>
<td>-</td>
<td>1.17 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Compensatory mutant</td>
<td>G:C</td>
<td>C:G</td>
<td>-</td>
<td>35 ± 8</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Compensatory mutant</td>
<td>A:U</td>
<td>U:G</td>
<td>-</td>
<td>288, 312</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Compensatory mutant</td>
<td>U:A</td>
<td>G:C</td>
<td>-</td>
<td>305, 306</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>AU bulge sequence change</td>
<td>AU</td>
<td>G</td>
<td>-</td>
<td>292-293</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>AU bulge sequence change</td>
<td>AU</td>
<td>AA</td>
<td>-</td>
<td>293</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>AU bulge sequence change and insertion</td>
<td>AU</td>
<td>AAA</td>
<td>-</td>
<td>292-293, 293</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>AU bulge sequence change and insertion</td>
<td>AU</td>
<td>AAA</td>
<td>-</td>
<td>292-293, 293</td>
<td></td>
</tr>
</tbody>
</table>

*Compensatory mutations correspond to changes between Watson-Crick basepairs in the paired regions of the stem. *For mutants 5-7 nucleotides were deleted however the remaining sequence was not changed. *Ins designates an insertion between the bases listed. *$K_d$ values reported represent an average of three determinations normalized to the wildtype $K_d$ from that set of experiments. *$\Delta\Delta G^\circ = \Delta G^\circ$ (mutant) - $\Delta G^\circ$ (wildtype) = $RT \ln$ (mutant $K_d$/wildtype $K_d$), calculated from the mean $K_d$ values, measurements made under the conditions described in materials and methods. Mutants 9-11 and 13-15 were suspected of misfolding and therefore not included in the analysis for this study.
Figure 2.7. Determining the minimum requirements for Ku-KBS RNA binding by mutagenesis.

A. Binding analysis of selected RNA mutants. Trace amounts of TLC1 KBS constructs were complexed with increasing concentrations of purified Ku heterodimer and run on a native gels. The fraction of KBS bound was quantitated and plotted as a function of the Ku concentration to determine the dissociation constant for each of the mutants. Representative curves reflect the data normalized to the wild-type measurements for that set of replicates as described in materials and methods. B. A minimum consensus for a KBS RNA. Nucleotides shown have a greater than five-fold impact on binding affinity, while circles represent nucleotides that can be mutated with little or no effect on binding. R indicates that a purine is tolerated at position 292. Nucleotides or circles strongly protected by Ku at the 10 s time point are colored red, and nucleotides that were not protected are shown as open circles.
Figure 2.8. Secondary structures for the RNA constructs used in this study. Black nucleotides represent native sequence, green nucleotides indicate mutations, and red nucleotides indicate non-native anchor sequences. The single stranded extension on the TLC1-38 primer extension construct serves as a primer binding site. Note, wild-type TLC1-96 corresponds to the previously predicted secondary structure (Zappulla and Cech 2004), and the wild-type TLC1-56 corresponds to another predicted secondary structure (Dandjinou et al. 2004).
Figure 2.9. Assessing mutant RNA structure. A. Gel showing conservation of the folding pattern for the TLC1 KBS RNA construct for selected mutants as assessed by nuclease V1 cleavage. Note the conservation of the cleavage pattern from positions 287-297 for wild type and mutants 1, 6 (red boxes). Mutants that do not impact affinity are protected from cleavage in the presence of Ku. Mutant 5 does not retain an AU bulge, has a reduced affinity for Ku and is not protected from V1 cleavage which is strong in the vicinity of the deleted bulge. Mutants 19 and 28 have V1 cleavage patterns that are similar to wild-type but have reductions in affinity for Ku and thus are not protected by Ku from V1 cleavage (c.f. red boxes to blue boxes). OH corresponds to a ladder generated by alkaline hydrolysis and T1 for a ladder generated by nuclease T1. The positions of select Gs are indicated by the T1 ladder. B. Example of the intended and mFold predicted lowest energy secondary structure for a mutant KBS. Green bases represent changes from wild type. C. V1 digests to test predicted folds for mutant shown in 3B. Notice the strong V1 cleavage that
extends from G295 (top of bracket) to U289 in lane 5 indicating that the RNA is likely double stranded in this region.

Previous models of the *S. cerevisiae* KBS secondary structure differ slightly in the conformation of the helices formed by nt 276 – 287 and 313-323 (Peterson et al. 2001; Dandjinou et al. 2004; Zappulla and Cech 2004). We tested two KBS constructs predicted to fold into these secondary structures (Figure 2.8; c.f. Zappulla: TLC1-96 and Dandjinou: TLC1-56). We found that Ku bound both constructs with the same affinity (Figure 2.6 and Table 2.1). The construct with the predicted Zappulla fold (TLC1-96) bound Ku with a $K_d$ of $1.3 \pm 0.5$ nM, and the construct with the predicted Dandjinou (TLC1-56) fold bound with a $K_d$ of $1.13 \pm 0.02$ nM. In addition, both the sequence and predicted folds for the non-cognate *S. paradoxus* and *S. castellii* KBSs differed from that of *S. cerevisiae* KBS, yet both non-cognate KBSs bound *S. cerevisiae* Ku with only slightly diminished affinities. In sum, these data support the conclusion that the specific sequence or structure of the template boundary arm preceding the terminal hairpin is not a factor in Ku recognition, but that the terminal conserved region of the KBS (nt 289-311) is the critical Ku recognition element.

Considering the terminal hairpin loop, KBSs with sequence changes in the terminal loop bound Ku with near wild-type affinity (mutants 1 and 2; Table 2.1, Figure 2.7A). In contrast, mutant 16, which shortened the terminal loop to four bases, had a moderate five-fold reduction in affinity. These data were consistent with the findings of the non-cognate binding studies, which indicated that the size of the terminal loop was important for high affinity interactions with Ku. Deletions of internal loops and bulges outside of the conserved region of the KBS (nt 289-311) had minor effects on Ku recognition (mutants 6 and 7).
Our structure-probing data suggested that the conserved portion of the KBS formed a hairpin with a non-canonical bulge motif. In order to test this prediction, we made a series of mutants to produce compensatory basepair substitutions within the helix. We reasoned that compensatory mutations in the helix should have little or no impact, provided that both the KBS structure and potential KBS-Ku contacts were preserved. Basepair substitutions at three of the four basepairs in the terminal helix and at basepair A288:312U in the penultimate helix had very little impact on KBS affinity for Ku (mutants 3, 21, 22, and 26) (Table 2.1, Figure 2.7A, and Figure 2.8). In contrast, mutations of basepairs surrounding the bulge at positions U290:310A, U291:309G, and A294:308U had moderate to severe reductions in affinity (~5-60 fold, see mutants 12, 17-20, 25). To test whether the length of the helix between the AU bulge and terminal loop is important, the stem was lengthened by two or four basepairs while preserving the native KBS sequence immediately adjacent to the bulge. Lengthening of the stem resulted in increasingly drastic reductions in Ku affinity (mutants 23 and 24).

To determine the importance of the bulge motif for Ku association, a series of mutants were made to probe the size and sequence requirements within the bulge. Deletion of both bulge nucleotides resulted in a severe reduction in affinity (>230 fold, see mutant 5), indicating that this bulge is essential for proper Ku binding (Table 2.1, Figure 2.7A, Figure 2.9A). Other mutations within the bulge had variable effects. For example, reduction of the AU bulge to one nucleotide also resulted in a severe binding defect (mutant 28). On the other hand, mutations that altered the sequence at positions 292 or 293 resulted in much less severe binding defects. Inversion of nt 292 and 293 resulted in a six-fold reduction in affinity, while the transversion U293A had no impact on KBS-Ku binding (mutants 8 and 29, respectively). Finally, increasing the size of the bulge by the addition of adenosines resulted in moderate reductions in affinity (mutants 30 and 31), indicating that increases in bulge size were tolerated more than decreases in bulge size. We conclude that the terminal helix length and the
nucleotides in and surrounding the non-canonical bulge motif (nt 290-294, 308-310) are crucial in mediating the KBS-Ku interaction.

Based on the results of the mutagenesis studies, non-cognate binding assays, and footprinting experiments, we propose a minimum consensus for the KBS (Figure 2.7B). Mutations resulting in greater than five-fold reductions in affinity are identified as positions in the stem-loop that are critical for Ku binding. The sequence of the two basepairs preceding the bulge, and the first basepair following the bulge, is important for recognition by Ku or the overall fold of the hairpin. The ability to substitute but not delete bulged nucleotides argues for a specific fold being important for Ku binding, but sequence-dependent contacts within the bulge cannot be entirely dismissed. The nucleotide identity at position 292 appears to be marginally important while position 293 can be mutated without significantly impacting affinity (mutant 29). Based strictly on our mutagenesis, an adenosine appears to be important for Ku recognition; however, previous mutagenesis studies indicate that a guanosine is tolerated much better than a cytidine at this position in vivo (Peterson et al. 2001). With the exception of the compensatory mutations listed above, mutations that preserved helical structure, changed loop sequence, or disrupted internal loops had little effect on affinity and were deemed not to be critical determinants of Ku binding. In summary, we can now more precisely define the KBS for yeast Ku as a hairpin containing a non-canonical bulge motif that is capped by a four basepair stem and terminal loop with seven to eleven nt.

**Discussion**

Previous studies identified a 48 nt hairpin as important for the TLC1-Ku interaction in vivo in *S. cerevisiae* (Peterson et al. 2001; Stellwagen et al. 2003). Here we extend these studies with footprinting, which indicates where Ku physically interacts with the KBS RNA, and with mutational analysis, which
quantifies the contributions of individual RNA structural features to the binding energy. We identify a non-canonical bulge motif within the highly conserved portion of the KBS, and show that spacing between the bulge and terminal loop is critical for Ku recognition. Additionally, we provide direct evidence that *S. cerevisiae* Ku is able to recognize several non-cognate KBSs. We suggest that the KBS is a conserved element within both *sensu stricto* and *sensu lato* *Saccharomyces* species and discuss the possibility that Ku recognizes additional cellular RNAs.

**Structural features of the TLC1 KBS that enable recognition by the Ku heterodimer**

Phosphorothioate footprinting shows that the Ku interaction is restricted to 25 nt in the KBS and identifies putative contacts at nucleotide resolution. The contacts are in the terminal loop and stems on either side of the AU bulge. The footprint encompasses the portion of the KBS that is highly conserved between putative non-cognate KBSs, and the non-canonical bulge motif that is particularly susceptible to mutation. The time-dependent contacts in the stem may be the result of transient KBS-protein interactions or Ku-dependent changes in the KBS structure (Rose and Weeks 2001; Webb et al. 2001). Although we cannot definitively distinguish between these possibilities, the complex was equilibrated prior to footprinting and any Ku-induced changes in local KBS structure to promote binding likely occurred prior to iodine-mediated cleavage.

In addition to our footprinting data, the generation of cross-links with halo-pyrimidine substituted KBS RNAs strongly suggests that the KBS terminal loop is intimately associated with the Ku-heterodimer. 5IU is short-range crosslinking agent which predominantly crosslinks with aromatic amino acids through a mechanism which is thought to involve π-stacking between the halo-pyrimidine and aromatic amino acids (Meisenheimer et al. 2000). Furthermore, the co-migration of Ku 70 and cross-linked species suggests that the KBS terminal loop makes contacts with Ku70. The observation that the
KBS crosslinks to only one subunit of the Ku heterodimer does not preclude the possibility that the KBS contacts both subunits. This conclusion is supported by the stability of the cross-linked species upon treatment with thrombin, a Ku80 specific protease. We favor a model in which Ku directly interacts with the terminal loop as well as the stems on either side of the bulge, as supported by the phosphorothioate, cross-linking, and V1 nuclease protection data.

Our footprinting data show that Ku interacts with the highly conserved portion of the KBS (nt 289-311). Covariations supporting the RNA secondary structure model are not present among currently identified yeast KBSs. Therefore, we utilized chemical probing experiments, which provided support for the proposed bulged hairpin structure. In addition, the modification pattern suggested that the KBS adopts a non-Watson-Crick helical fold from positions 290-293. The structure of this bulge motif cannot be predicted solely from the modification pattern. Some possibilities for the protection of A292, but not U293, include the formation of a base-triple including A292, intercalation of bases into the helical stack, ion-coordination, or water-mediated hydrogen bonding (Costa et al. 1998; Leontis and Westhof 1998). Further investigation will be necessary to distinguish between these possibilities. The importance of this bulge is underscored by the detrimental effect that mutations have in this region of the RNA, as discussed below.

Our direct binding data are consistent with previous in vivo studies, which show that disruption of the proposed KBS hairpin secondary structure perturbs the Ku-TLC1 genetic interaction (Peterson et al. 2001). Mutations in and surrounding the bulge region have the greatest impact on KBS function in vivo (Peterson et al. 2001). Because mutations in the terminal loop have a small impact on KBS function (Peterson et al. 2001) and binding (mutants 1, 2, and 4, this study), contacts between Ku and the terminal loop of the KBS are likely to be sequence non-specific and may involve the RNA backbone. Mutations in and directly adjacent to the bulge have a strong impact both in vivo (Peterson et al. 2001)
and in vitro (positions 291 and 292, this study). Our data suggest that mutations to the two terminal basepairs adjacent to the loop do not significantly affect the ability of the KBS to bind Ku (mutant 3); in agreement, mutations at these positions do not seem to perturb the KBS-Ku interaction in vivo. In contrast, our data show that binding is not compromised in mutant 21 (G295A:C307U); however, there appears to be a minor impact on KBS function for this mutant in vivo (Peterson et al. 2001). Finally, we show a moderate binding defect when making compensatory mutations at the U290:310A base pair. In vivo, mutations at this position do not have a detrimental impact. The differences in the impact of mutations to the KBS may be a reflection of the different assays used to monitor the Ku-KBS interaction. The in vivo analysis measures the ability of the mutant KBS to disrupt telomeric silencing in the context of over-expression, while we measure the impact on Ku-KBS binding equilibria in vitro.

RNA binding may be a conserved function of the Ku heterodimer

The identification of KBSs competent for specific association with Ku in TERs of several Saccharomyces species adds to the growing evidence that RNA binding is a conserved function of the Ku heterodimer. Ku associates with cellular RNAs in human cells (Zhang et al. 2004; Ting et al. 2009; Adelmant et al. 2012). Ku interacts with non-coding RNAs such as TERRA and Y-RNAs (Zhang et al. 2011; Pfeiffer and Lingner 2012). RNA aptamers have been selected to bind human Ku with high affinity (Yoo and Dynan 1998). The association of human Ku with HIV-1 TAR RNA provides a specific example of another hairpin that is competent for binding Ku (Kaczmarski and Khan 1993). The TAR hairpin shows little or no sequence similarity with the TLC1 KBS; however, they share similar secondary structures and both are able to compete with double-stranded DNA for Ku binding (Kaczmarski and Khan 1993; Pfingsten et al. 2012). The TAR hairpin has a six nt terminal loop that is separated from a three nucleotide bulge by an intervening four basepair helix. The lack of sequence similarity between these RNAs may reflect differences between the human and S. cerevisiae Ku heterodimers; however, the
conserved bulge hairpin with specific spacing requirements suggests that Ku recognizes RNAs with a conserved fold. We therefore favor a model in which Ku recognizes the hairpin structure rather than making sequence-specific contacts. One possibility is that the KBS has the ability to mimic a B-form DNA helix and thereby associate with Ku’s preformed ring; B-DNA mimicry by RNAs to associate with proteins has been previously observed (Reiter et al. 2008; Bullock et al. 2010). The identification of additional Ku-binding RNAs and an increased understanding of how Ku recognizes these hairpins will certainly shed light on how this nucleic acid binding protein mediates a myriad of distinct cellular functions.

Materials and Methods

KBS constructs and in vitro transcription of RNAs

TER sequences and alignments for representative Saccharomycotina were obtained from the Telomerase Database (Podlevsky et al. 2008); TER sequences from additional Saccharomyces strains and species were manually added to the alignment using the program BioEdit V7.1.3.0. S. cerevisiae and S. paradoxus sequences were obtained by BLAST from the Saccharomyces genome re-sequencing project (Liti et al. 2009a). The S. arboricola putative TER sequence was recovered by BLAST from the whole genome assembly (GCA_000292725.1) using the S. mikatae TER as a query sequence.

For a complete list of RNA constructs used see Figure 2.8. RNAs were designed and checked for misfolding using mFold version 2.3; RNAs were folded at 30°C (Zuker 2003). Mutant RNA constructs were prepared by quick-change mutagenesis (Agilent) of the previously reported TLC1-96 KBS construct (Pfingsten et al. 2012). The TLC1 KBS constructs used for phosphorothioate footprinting and mutagenesis experiments were prepared by T7 in vitro transcription from PCR products, previously described (Pfingsten et al. 2012). The TLC1 KBS construct used for the chemical modification experiments, as well as putative Ku arms from other Saccharomyces TERs, were made by annealing and
cloning complementary oligos containing the putative Ku arm sequence, a T7 promoter, and FokI site. The RNAs were prepared by T7 polymerase run off transcription from the FokI linearized plasmid. All in vitro transcribed RNAs were purified on a denaturing 10% polyacrylamide gel. Prior to $^{32}$P-labeling RNAs, calf intestinal alkaline phosphatase (Roche) was used to remove the 5′ phosphate. RNAs were then 5′ labeled with $\gamma^{32}$P- ATP (Perkin Elmer) and T4 polynucleotide kinase (NEB), according to the manufacturer’s protocol. 5′ labeled RNAs were then re-purified on a denaturing 10% polyacrylamide gel.

**Purification of the Ku heterodimer**

Protein constructs and purification were previously described (Pfingsten et al. 2012), or had the following modifications. Yeast cells were grown to a final density of 1 to 4 x $10^7$ cells per mL. Lysate was passed over a 5 mL HisTrap HP column (GE Healthcare). Bound protein was eluted first in shallow gradient ranging from 20 to 80 mM imidazole over ten column volumes (CV), and second in a steep gradient from 80 to 500 mM imidazole over five CV. The elution buffer also contained 50 mM Tris pH 8.0, and 500 mM NaCl. Fractions containing Ku were dialyzed overnight into a buffer containing 50 mM Tris pH 8.0, 50 mM NaCl, and 2 mM DTT. Ku was then exchanged over a HiTrap Q HP column (GE Healthcare) under the same buffer conditions used for dialysis. Bound protein was eluted from the Q column in a shallow gradient ranging from 50 mM to 1 M NaCl over ten CV, and then in a steep gradient ranging from 1 to 2 M NaCl over five CV. Fractions containing Ku were then re-captured on a 1 mL HisTrap HP column. The nickel column was then directly connected to a Superdex 200 (GE Healthcare), and protein was injected from the nickel column onto the sizing column using 4 mL of 500 mM imidazole. Running buffer contained 50 mM Tris pH 8.0, 500 mM NaCl, and 2 mM DTT. Fractions containing Ku were then combined, dialyzed, and frozen as previously described (Pfingsten et al. 2012).
Phosphorothioate footprinting assays

A ratio of 1 mM NTPs to 0.05 mM ATP [αS] and UTP [αS] or CTP [αS] and GTP [αS] (Sp diastereomers, Glen Research) was used to generate phosphorothioate substituted RNAs. The nucleotide analogs were randomly incorporated into the TLC1-96 RNA construct using T7 polymerase. The RNAs were end-labeled and purified as described above. Prior to the footprinting reaction, Ku protein was buffer exchanged into a storage buffer lacking DTT (25 mM Tris pH 7.0, 20% glycerol, 200 mM NaCl, 0.5 mM EDTA, and 5 mM MgCl₂). This storage buffer was also used as a 2X stock to achieve the final buffer and salt conditions for the footprinting reactions.

Final footprinting reactions contained 5'-32P labeled phosphorothioate substituted TLC1 KBS RNA, 1X storage buffer, 0.05 μg yeast tRNA (Sigma), 135 nM Ku, and 0.2 mM I₂ (dissolved in 100% ethanol). RNAs were annealed in the presence of all reaction components, with the exception of Ku and I₂, by heating to 85ºC for one min, followed by snap cooling on ice. Annealed RNAs were then split between tubes containing Ku or an equal volume of storage buffer and complexed at room temperature for 20 min. Footprinting reactions were initiated by the addition of I₂, and reactions were subsequently quenched at various time points in an equal volume of 100 mM β-Mercaptoethanol. The no-I₂ controls were treated identically but the RNA was removed prior I₂ addition. Sequencing reactions were prepared by carrying out I₂-mediated cleavage of the substituted RNAs at 65ºC for 2 min. Reactions were precipitated by the addition of 2 volumes of RNase inactivation/precipitation buffer (Life Technologies), precipitated overnight and washed one time with 70% ethanol. RNAs were then suspended in equal volumes of H₂O and 2X formamide loading dye (0.5X TBE, 93.5% formamide, 30 mM EDTA, 0.5% xylene cyanol and bromophenol blue). Samples were heated to 85ºC for 1 min, cooled on
ice, and equal numbers of scintillation counts for each sample were loaded onto a 10% polyacrylamide sequencing gel. Gels were dried and visualized by phosphoimagery (GE Healthcare). The computer program SAFA was used to align the gel and calculate band intensities in order to determine the level of protection (Das et al. 2005). SAFA-calculated band intensities were normalized to the total number of counts per lane. The fold-change in band intensity upon addition of Ku was calculated with the normalized values. Intensities were plotted onto the secondary structure on a log₂ scale.

**Nuclease V1 footprinting assays**

RNAs for footprinting reactions were prepared by *in vitro* transcription as described above. Final footprinting reactions contained 5′-³²P labeled TLC1 KBS RNA, V1 footprinting buffer (15 mM Tris pH 8.0, 12% glycerol, 130 mM NaCl, 5 mM MgCl₂, 1.6 mM DTT), 0.05 µg yeast tRNA (Sigma), 1.08 uM Ku, and 0.005 or 0.0013 units of RNase V1 (Life Technologies) diluted in Ku storage buffer (25 mM Tris pH 8.0, 20% glycerol, 200 mM NaCl, 0.5 mM EDTA, and 2 mM DTT) prior to initiation of the reaction. RNAs were annealed as described for the footprinting reactions, and Ku-KBS complexes were reconstituted for 30 min at room temperature prior to the addition of nuclease V1. Digestions were carried out at room temperature for 10 min and then quenched in RNase inactivation/precipitation buffer (Life Technologies). RNAs were precipitated and processed as described above for the phosphorothioate footprinting reactions. The alkaline hydrolysis ladder was prepared by incubating 5′-³²P labeled TLC1 KBS RNA and 2 µg yeast tRNA (Sigma) in 50 mM sodium bicarbonate (pH 9.2) at 85°C for 4 min. The RNase T1 ladder reactions contained 5′-³²P labeled TLC1 KBS RNA, 2 µg yeast tRNA (Sigma), 1X RNA sequencing buffer (Life Technologies), and 0.1 units of RNase T1 (Life Technologies). RNAs were heated to 55°C for 1 min prior to the addition of RNase T1, and then incubated at 55°C for 3 min. RNase T1 and hydrolysis ladders were quenched in 1X formamide loading dye as described above. Samples were prepared, run on sequencing gels, and quantitated as described for phosphorothioate footprinting.
**Photo-cross-linking assays**

TLC1-38 KBS 5IU-substituted RNAs (Figure 2.3E) were either modified at single sites and chemically synthesized at Dharmaco (GE Healthcare), or prepared by *in vitro* transcription. Final transcription NTP concentrations were 0.03 mM ATPs, 0.3 mM α-32P- ATP (Perkin Elmer), 2.5 mM 5-iodo-UTP (TriLink), 2.5 mM UTP, and 5 mM CTP and GTP to generate internally radiolabeled and randomly 5IU-substituted KBS RNAs. Synthesized RNAs were 5´ labeled as described above. To form complexes for cross-linking, modified KBS RNA was bound by excess Ku heterodimer in a buffer containing 21 mM HEPES, pH 7.5, 6 mM Tris, pH 8, 150 mM NaCl, 16% glycerol, 5 mM MgCl2, 1 mM EDTA, 25 μg/ml tRNA, 0.1 mg/ml BSA, and 1 mM DTT. Cross-linking was carried out in a CL-1000 Ultraviolet Crosslinker (UVP) using lamps with maximum emission at 312 nm. Samples were irradiated on ice under maximum power for 2 hours. Chemical digestions were carried out as previously described (Walker 2002). Furin, thrombin, and cathepsin L digestions were carried out under manufacturers’ suggested protocols. Cross-linked complexes were trypsin digested with 0.4 μg of trypsin at room temperature for 2.5 min. Prior to RNase T1 digestion, cross-linked complexes were heated to 85°C for 5 min, cooled to 50°C and then digested for 30 min with 5U RNase T1 (Life Technologies). Samples were heated to 85°C for 2 min in 4X LDS loading dye prior to electrophoresis on a NuPage 4-12% Bis-Tris SDS PAGE gel (Life Technologies) for 35 min at 180 volts constant. Gels were dried and visualized by phosphoimager as described for footprinting assays. In some cases gel were Coomassie stained with SimplyBlue SafeStain (Life Technologies) prior to drying and phosphoimaging.
Chemical modification assays

Chemical modification and subsequent reverse transcription experiments were carried out as previously described (Zaug and Cech 1995; Brunel and Romby 2000), with minor modifications. Final reactions contained approximately 1 μg of TLC1-38 KBS primer extension RNA (2.7 μM), 150 mM NaCl, 25 mM HEPES pH 8.0, and 5 mM MgCl₂, and either 90 mM DMS (Sigma, diluted in 100% ethanol) or 18 mg/mL CMCT (Sigma, dissolved in H₂O). RNAs were annealed as described for the footprinting experiments in the absence of DMS or CMCT. Modification reactions were initiated by the addition of either DMS or CMCT and reactions were quenched at various time points. For the pre-mix negative control reactions, annealed RNA was added to the appropriate quench solution prior to adding DMS or CMCT. Denature controls consisted of modifying the RNA with the appropriate chemical reagent for 30 s at 85°C. DMS modification was quenched by addition of 9 volumes of DMS quench buffer consisting of 90 mM β-mercaptoethanol, 333 mM NaOAc pH 5.2, and 20 μg of glycogen (Roche). The CMCT quench buffer was identical to the DMS quench buffer except that it lacked β-mercaptoethanol. Quenched reactions were then precipitated by the addition of 2.5 volumes of ice-cold ethanol. RNAs were washed one time with 70% ethanol and then resuspended in H₂O.

Final reverse transcription reactions contained approximately 0.2 μg of modified RNA, trace amounts of ³²P-labeled primer, 4 mM dNTPs, AMV reverse transcriptase (NEB), and 1X AMV buffer (NEB). Primers were annealed to RNA in 1X AMV buffer by heating to 85°C for 2 min followed by snap cooling on ice. Reverse transcription reactions were initiated by the addition of an equal volume of mix containing the dNTPs, 1X AMV buffer, and the reverse transcriptase. Primer extension reactions were allowed to proceed at 37°C for 40 min. Sequencing reactions were carried out with the same reverse transcription conditions described above and the addition of a single ddNTP to a final concentration of 0.5 mM. Reverse transcription reactions were quenched by the addition of 2X formamide loading dye.
and heating to 95°C for 5 min. Reactions were loaded onto a 10% sequencing gel, and the gel was dried and band intensities were calculated as described for the phosphorothioate footprinting experiments. Modification levels were calculated by normalizing the SAFA calculated band intensities to the total counts per lane. The mean and standard deviation of the band intensities in a lane were used to calculate a z-score for each band in the lane.

**Determination of dissociation constants**

Dissociation constants for the mutant and additional putative yeast KBS RNAs were measured as previously described (Pfingsten et al. 2012). Briefly, trace amounts of KBS RNA were incubated with Ku at room temperature for 1 hour in a buffer containing 21 mM HEPES, pH 7.5, 6 mM Tris, pH 8, 150 mM NaCl, 16% glycerol, 5 mM MgCl₂, 1 mM EDTA, 25 μg/ml tRNA, 0.1 mg/ml BSA, and 1 mM DTT. Ku-bound KBS RNAs were separated from free KBS RNA on a native 4-20% acrylamide TBE gel (Invitrogen); gel boxes were placed in ice for the electrophoretic run. All measurements represent the average of three independent replicates. Mutant Kₐ values were normalized to the wild-type Kₐ for a given set of experimental replicates by multiplying the measured mutant Kₐ by the ratio of the measured wild-type Kₐ over the average wild-type Kₐ for all experiments. All measured wild-type KBS Kₐ values were within 2.5 fold of the average wild-type Kₐ reported in table 2.1.
Chapter III: Contributions of the TEL-patch amino acid cluster on TPP1 to telomeric DNA synthesis by human telomerase

Introduction

The work presented in chapter two investigated the structure of the budding yeast TLC1 KBS to determine the critical RNA elements for Ku binding. This chapter examines the recruitment process and catalytic cycle of human telomerase. Specifically, the goal of this work described herein was to develop an in vitro telomerase recruitment assay to investigate the contributions of the TPP1 TEL-patch to telomerase recruitment, translocation, and substrate dissociation. The experiments presented in this chapter are the result of collaboration with Ctirad Hofr, a visiting professor from Masaryk University. Ctirad and I worked together on experimental design and generation of experimental materials. Ctirad carried out a number of optimization experiments, determined the dissociation constants of wild-type and TEL-patch mutant TPP1-POT1 for substrate DNA, aided in curve fitting and plot generation for figures 3.1 and 3.3, and designed figure 3.1. I carried out the remaining experiments, analyzed the data, generated figures, and wrote the manuscript with input from Ctirad.

Telomere maintenance is a highly coordinated process, and its misregulation is linked to cancer as well as telomere-shortening syndromes (Stewart and Weinberg 2006; Armanios and Blackburn 2012). Recent studies have shown that the TEL-patch – a cluster of amino acids on the surface of the shelterin component TPP1 – is necessary for the recruitment of telomerase to the telomere in human cells (Nandakumar et al. 2012; Zhong et al. 2012; Nakashima et al. 2013; Sexton et al. 2014). Furthermore, the TEL-patch was shown to participate in the stimulation of telomerase repeat addition processivity (RAP) in direct telomerase extension assays (Nandakumar et al. 2012; Guo et al. 2014; Schmidt et al. 2014).
The observation that the TEL-patch contributes to both telomerase recruitment and RAP stimulation suggests that these phenomenon are manifestations of the same direct molecular interaction between TPP1 and telomerase (Nandakumar et al. 2012; Schmidt et al. 2014). Although the *in vivo* role for the TEL-patch is well established, there has been only basic biochemical analysis of the role of TPP1 TEL-patch in the telomerase recruitment process. It is not known whether TPP1-POT1 bound substrates are preferentially recruited to and extended by telomerase *in vitro*. In addition, TPP1 was previously shown to increase the apparent rate constant and efficiency of translocation and to decrease the rate of substrate dissociation (Latrick and Cech 2010). However, the contributions of TEL-patch during the telomerase catalytic cycle have not been examined.

To better understand the contributions of the TEL-patch to telomerase recruitment, we have developed a novel *in vitro* substrate competition assay. Using this assay, we show that the TEL-patch participates in the preferential binding and extension of TPP1-POT1 bound substrates and that mutation of the TEL-patch results in less efficient substrate usage by telomerase *in vitro*. In addition, we show that mutation to the TEL-patch reduces the apparent translocation rate, decreases the efficiency of translocation, and increases the rate of primer DNA dissociation from actively synthesizing telomerase.

**Results**

**The TEL-patch on TPP1 promotes the translocation of human telomerase**

Mutations in the TEL-patch impact the ability of TPP1 to stimulate RAP by telomerase *in vitro* (Nandakumar et al. 2012), suggesting that the TEL-patch interacts with telomerase during catalysis. To understand TEL-patch contributions in stimulating telomerase RAP, we compared wild-type TPP1 and a previously described TPP1 TEL-patch mutant E169A;E171A (EE mutant) (Nandakumar et al. 2012) in a
number of in vitro telomerase assays. Assays were used to query various steps in the telomerase catalytic cycle (Figure 3.1A).

**Figure 3.1. Mutations in the TEL-patch adversely impact telomerase translocation.** A. (Left) the human telomerase catalytic cycle. i) Telomerase is a ribonucleoprotein complex that contains an internal template Telomerase RNA (TER) which is utilized by Telomerase Reverse Transcriptase (TERT) to synthesize telomeric repeats ii) upon substrate binding. iii) Nucleotides are sequentially added to the 3’ end of the substrate until the end of the internal RNA template is reached. iv) The primer is next repositioned on the RNA template (translocation) and a subsequent round of repeat addition ensues. Additionally, the substrate or product can dissociate from the enzyme during any step of the cycle, although dissociation coincides most often with the translocation step in vitro. (Right) POT1 complexed with TPP1 binds the DNA primer; wild-type TPP1 with an intact TEL-patch engages with TERT. B. Translocation rate assay for primer alone, primer bound by wild-type TPP1-POT1, or primer bound by E169A;E171A (EE mutant) TPP1-POT1. Reaction time (min) noted above gel, and +2 and +4 products denoted on the side of the gel correspond to products shown in A. Precipitation and loading control shown below each translocation panel. C. Fraction translocated was calculated as the sum of counts in the +3 and
+4 products divided by the total counts and plotted as a function of time for primer alone (open squares), wild-type TPP1 (closed circles), and E169A;E171A TPP1 (closed triangles). TP signifies TPP1–POT1. Translocation data were fit to the equation $y = A(1-e^{-kt})$ where $k$ (or $k^{app}$) represents the rate constant for a single round of translocation plus subsequent nucleotide incorporation and $A$ (the horizontal limit) represents the maximum efficiency. The efficiency and rate constants for each condition are noted. The averaged data were plotted ± standard deviation (n = 2). D. Equilibrium binding of POT1 (closed circles) and complexes wild-type TPP1–POT1 (open circles) and E169A;E171A mutant TPP1–POT (triangles) to the primer a5TT. Native gel-shift assay data were fit to a one-site binding equation by non-linear regression to obtain equilibrium dissociation constants ($K_d$). One replicate was carried out as our values matched previous measurements. Figure layout, artistic renderings, and dissociation measurements contributed by C. Hofr.

Wild-type TPP1 was previously shown to impact both the apparent rate of translocation as well as the efficiency of translocation (Latrick and Cech 2010). We hypothesized that mutations in the TEL-patch would decrease RAP stimulation by impacting translocation, and we tested this with a single-turnover translocation experiment (Latrick and Cech 2010; Qi et al. 2012b; D'Souza et al. 2013). Wild-type TPP1–POT1 or EE mutant TPP1–POT1 was complexed with primer and pre-bound to telomerase. The apparent rate of translocation was measured by initiating telomerase extension by adding only dATP and dGTP (dTTP was omitted) and monitoring the fraction of product formation before (+2 products) and after translocation (+3,+4 products) (Figure 3.1B). We use “apparent rate of translocation” to indicate that the assay relied on translocation as well as nucleotide incorporation to determine the fraction translocated, yielding a complex rate constant. A single translocation event (Figure 3.1A; steps iii and iv) was observed because dTTP was absent and an excess of chase primer was added simultaneously with the dNTPs to prevent dissociated substrates from rebinding telomerase.

TPP1–POT1 increased both the apparent translocation rate and efficiency of translocation compared to primer alone. The apparent rate constant for primer alone was $0.09 \pm 0.01 \text{ min}^{-1}$, in agreement with previous measurement (Latrick and Cech 2010). Having wild-type TPP1–POT1 bound to the primer increased the apparent rate constant to $0.15 \pm 0.01 \text{ min}^{-1}$, while the TEL-Patch mutant TPP1–POT1 retained partial activity ($0.11 \pm 0.01 \text{ min}^{-1}$) (Figure 3.1C). In addition, the overall efficiency of
translocation differed between the samples. In the case of primer alone, only 80% of the incorporated dGTP was present in the +4 product, while 20% remained un-translocated. Addition of TEL-patch or wild-type POT-TPP1 resulted in incremental increases in efficiency to 84% and 94%, respectively (Figure 3.1C). We verified that differences observed in the translocation assay result in increased telomerase RAP, using a full extension assay with dTTP (Figure 3.2A,B). The full extension assay was carried out under similar conditions; however, the concentration of Mg\(^{2+}\) was titrated to account for overall higher dNTP concentration. Collectively these results confirm that the TEL-patch contributes to the apparent translocation rate and increases the efficiency of translocation.

![Figure 3.2. Magnesium ion dependence of RAP stimulation by WT TPP1-POT1. A. In vitro telomerase extension assays carried out under experimental conditions used in translocation assay. In order to observe processive telomerase extension under translocation conditions, 500 μM dTTP was included in the reaction. Telomerase appeared to stall more frequently under the translocation conditions (see first lane of each panel, 0.1 mM MgCl\(_2\)),](image-url)
presumably due to the increased dNTP concentration which resulted in additional chelation of free Mg$^{2+}$. To account for the increased dNTP concentration, a MgCl$_2$ titration was carried out in the presence (+) and absence (−) of wild-type TPP1-POT1. MgCl$_2$ concentrations noted above gel. Number of telomeric repeats indicated on the left side of gel. Precipitation and loading control (LC) shown below telomerase gel. B. Plot of processivity ($R_{1/2}$) values as a function of the natural log of MgCl$_2$ concentration, ranging from 0.2 and 0.8 mM, for primer with no TPP1-POT1 (No TP; open squares) and primer bound by wild-type TPP1-POT1 (WT TP, closed circles). $R_{1/2}$ corresponds to the repeat number at which 50% of the primer has dissociated from telomerase for a given reaction, values were calculated as previously described (Latrick and Cech 2010). Note that the data were corrected for the number of radiolabeled G residues in each band, which otherwise makes the reactions appear more processive than they are.

To ensure that our TEL-patch EE mutant TPP1 was active in interacting with POT1 and correctly folded, we determined the $K_d$ of both wild-type and mutant TPP1-POT1 complexes for DNA by electrophoretic mobility shift assay. TPP1 is known to enhance the affinity of POT1 for DNA (Wang et al. 2007), and mutation to the TEL-patch should not disrupt POT1 binding (Nandakumar et al. 2012). POT1 alone bound the DNA with a $K_d$ of 50 nM, and the addition of either wild-type or mutant TPP1 resulted in increased affinity to 7 and 9 nM, respectively (Figure 3.1D), consistent with previous results (Wang et al. 2007; Nandakumar et al. 2012). These results indicate that the TEL-patch EE mutant TPP1 is competent to form a complex with POT1 and thereby increase its affinity for DNA.

**Developing an in vitro assay for telomerase recruitment to a telomere**

The TEL-patch of TPP1 was previously shown to interact with the TEN-domain of TERT, and it is crucial for telomerase recruitment to telomeres in vivo (Nandakumar et al. 2012; Zhong et al. 2012; Schmidt et al. 2014). We postulated that telomerase should be preferentially recruited (i.e., binding and extension) to TPP1-POT1-bound substrates with a wild-type TEL-patch. To test this hypothesis, we developed a competition assay with two competing substrates (Figure 3.3A). Two primers with slightly different lengths (38 and 44 nt) and the same molar concentration were used in the competition. One primer was complexed with wild-type TPP1-POT1 and the other with the EE mutant TPP1-POT1 (Figure 3.4A). The competition was initiated when the two substrates were simultaneously added to a low
concentration of telomerase in buffer containing dATP and dGTP (dTTP was again omitted). In the competition, the two substrates act as competitive inhibitors of one another (Fersht 1977) – i.e. a single active site can only accommodate and catalyze the product formation of one substrate at a time. Thus, ratio of the initial velocities of product formation for the two substrates gives the ratio of their specificity constants \( \frac{k_{\text{cat}}}{K_m} \text{substrate}_1 / \frac{k_{\text{cat}}}{K_m} \text{substrate}_2 \) (Fersht 1977).
Figure 3.3. TPP1-POT1-bound primer competition experiment. 

A. Schematic for substrate competition experiment with two primers: one bound with wild-type TPP1-POT1 and the other bound by TEL-patch mutant TPP1-POT1. TPP1-POT1-bound primers are simultaneously added to free telomerase and act as competitive inhibitors of one another. Bound primers are extended in the absence of dTTP, thus only nucleotide addition and a single translocation step are observed. 

B. Primer competition for primer 1 bound to wild-type (WT) TPP1-POT1 and primer 2 bound by E169A;E171A (EE mut) TPP1-POT1. Time (min) denoted above gels, and +2 and +4 products indicated on the right side of the gels. Precipitation and loading control (LC) shown below each competition panel. 

C. Initial velocity plot for competition assay shown in b. Product formation was calculated as described in Figure 3.4D-F legend. \((k_{cat}/K_m)\) ratio noted in plot. Error bars represent the standard deviation of the replicates \((n = 2)\). 

\[ k_{cat}/K_m \text{ ratios calculated using the equation: } \frac{k_{cat}/K_m}{\text{primer2}}(k_{cat}/K_m)_{\text{primer1}} = \left(\frac{v_{\text{primer2}}}{v_{\text{primer1}}}\right)\times\left(\frac{[\text{primer1}]}{[\text{primer2}]}\right). \]
D. Flow chart of TPP1 equilibrium experiment. Primer 1 was bound to TEL-patch mutant TPP1-POT1 (EE mut) and in a separate tube primer 2 was bound by wild-type TPP-POT1. The two primers were mixed for varying amounts of time to allow TPP1, or TPP1-POT1 to exchange between the primers (pre-equilibration). Primers were then extended in a competition experiment for 10 min. E. TPP1 exchange equilibrium experiment. Primer 1 was bound to TEL-patch mutant TPP1-POT1 (EE mut) and primer 2 was bound by wild-type TPP-POT1, as noted on left side of gel. No T-P, control experiment consisting of a competition between primer 1 and primer 2 with no TPP1-POT1 present. TPP1 Pre-mix, control experiment in which primers 1 and 2 were first mixed and then added to a mixture of wild-type TPP1-POT1 and E169A;E171A TPP1-POT1. Primer pre-equilibration time above gel, note that products visualized on gel were extended by telomerase for 10 min in addition to the time noted above the gel. Asterisk above 2 min pre-equilibration time corresponds to the final time point (12 min) in the initial velocity competitions shown in Figure 3.4 (i.e. 2 min of TPP1 pre-equilibration + 10 min telomerase extension = 12 min of TPP1 exchange). Products corresponding to +2 and +4 additions indicated on the right side of the gels. Precipitation and loading control (LC) shown below competition panel. F. Plot of the product ratio versus TPP1 pre-equilibration time in minutes. Product ratios were calculated by summing the +2, +3, +4 products for each primer and then dividing the total counts of primer 1 by the total counts of primer 2. The ratio of the TPP1-premix control is shown as a horizontal line at the top of the plot, and the pre-equilibration time at which TPP1 has undergone 50% exchange is denoted with a dashed line, assuming the starting product ratio of 15% and the pre-mix control ratio of 91% as the point at which equilibrium is reached.
Figure 3.4. The TEL-patch contributes to substrate recruitment and extension by telomerase. In vitro telomerase competition assays between two primers. 

**A.** Substrate recruitment experiment with two primers: a 38 nt primer bound with wild-type TPP1-POT1 and a 44 nt primer bound by TEL-patch mutant TPP1-POT1, cartoon depicts competition shown in panel E. 

**B.** Competition between primer 1 and primer 2 each pre-bound to wild-type (WT) TPP1-POT1. 

**C.** Initial velocity plot for the competition shown in B. Solid line denotes primer 1 and dashed line primer 2. Blue circles indicate wild-type TPP1-POT1. 

**D.** Primer 1 and primer 2 each pre-bound to E169A;E171A (EE
mut) TPP1-POT1. **E.** Initial velocity plot for the competition shown in D. Solid line denotes primer 1 and dashed line primer 2. Red triangles indicate E169A;E171A TEL-patch mutant TPP1-POT1. **F.** Primer 1 pre-bound to EE mut TPP1-POT1 and primer 2 pre-bound to WT mut TPP1-POT1. **G.** Initial velocity plot for competition shown in F, colors and symbols are the same as C,D. Time (min) after simultaneously mixing telomerase and both substrates denoted above gels, and +2 and +4 products indicated indicated on the right side of the gels. Precipitation and loading control (LC) shown below each competition panel. Product formation was calculated by summing the counts of the +2, +3, and +4 products, normalized to the loading control and expressed as a fraction of the total counts incorporated for primer bound by WT TPP1-POT1 at 12 min for each replicate. The average normalized product formation for each competition was plotted as a function of time, fit by linear regression, and the initial velocity of product formation was determined by the slope of the line. Error bars represent the standard deviation in normalized product formation at each time point for the replicates (n = 2). $k_{cat}/K_m$ ratios calculated using the equation: $(k_{cat}/K_m)_{primer2}/(k_{cat}/K_m)_{primer1} = ([v_{primer2}]/[v_{primer1}])^*([primer1]/[primer2])$. Final concentration of each primer was 100 nM.

We first verified that measured differences in the initial velocity of product formation between two TPP1-POT1-bound substrates were due to mutations in the TEL-patch rather than other intrinsic differences (e.g., primer length or secondary structure). Both primers were bound either to wild-type TPP1-POT1 or to EE mutant TPP1-POT1 and used in telomerase competitions (Figure 3.4B-E). The difference in initial velocity of product formation was negligible when both primers were bound by the same TPP1-POT1 complex (Figure 3.4C,E). Competing primers bound to wild-type TPP1-POT1 had a specificity constant ratio of 1.00 \(\pm\) 0.18 and primers bound by EE mutant TPP1-POT1 had a ratio of 1.11 \(\pm\) 0.36, consistent with equivalent primers having an expected ratio of 1. Collectively, these data demonstrate that primers of slightly different lengths bind to and are extended by telomerase with equivalent efficiency when associated with the same TPP1-POT1 complex. Additionally, the total velocity of product formation (i.e. $v_{primer1} + v_{primer2}$) increased when both primers were bound to wild-type TPP1-POT1 compared to both primers bound to TEL-patch mutant TPP1-POT1 (Figure 3.4C, E).

Next we carried out a competition between a primer bound to wild-type TPP1-POT1 and a second primer bound to EE mutant TPP1-POT1 (Figure 3.4F,G). The initial velocity and specificity constant ratio for the wild-type over mutant TPP1-POT1-primer was 2.59 \(\pm\) 0.07. As a control, a second competition in which the opposite primer was bound to TEL-patch mutant TPP1-POT1 gave a specificity
ratio of 1.52 ± 0.03 (Figure 3.3B,C). Although the reason for the difference between these two values is unknown, the main conclusion is that primer complexed with wild-type TPP1-POT1 was preferentially bound and extended by telomerase in both cases. For analysis of how this assay relates to telomerase recruitment, see the Discussion.

Our experimental design would underestimate the difference between mutant and wild-type TPP1 if the proteins exchanged between the primers during the course of the experiment. We therefore tested how TPP1 exchange impacted our competition experiment. One primer bound to wild-type TPP1-POT1 and a second primer bound to EE mutant TPP1-POT1 were pre-mixed for varying amounts of time prior to a fixed length of competition extension by telomerase (Figure 3.3D,E). Plotting the product ratio versus time revealed that approximately 50% of the TPP1 exchanged after 30 min (note: 20 min of pre-equilibration followed by 10 min of extension). The experiment also revealed that after 12 min (the last time point in our competition assay) approximately 6% of the TPP1 appeared to have exchanged (Figure 3.3F). Our data demonstrate that the TPP1-POT1 complexes bind stably enough to primers to allow performance of the competition assay.

**Mutations in the TEL-patch of TPP1 induce faster dissociation of cycling telomerase from DNA substrates**

Wild-type TPP1-POT1 was previously shown to slow the rate of primer dissociation from actively cycling telomerase (Latrick and Cech 2010). We hypothesized that mutations in the TEL-patch may destabilize the interaction between TPP1 and telomerase, and thereby increase primer dissociation. To examine this hypothesis, we measured the primer dissociation rate from telomerase engaged in telomeric repeat synthesis (Latrick and Cech 2010). In this experiment, primers were pre-bound to telomerase and repeat synthesis was initiated by addition of cold dATP, dGTP, and dTTP. At time zero, a
large excess of 3'-phosphorylated chase primer was added to the reaction to prevent telomerase from re-associating with active primers. Telomerase extension was carried out for varying lengths of time in the presence of cold nucleotides, and products were pulse labeled with $\alpha^{32}$P-dGTP prior to reaction termination.

We measured the dissociation rates for primer alone, primer bound by wild-type TPP1-POT1, and primer bound by EE mutant TPP1-POT1. Primers without TPP1-POT1 underwent biphasic dissociation from telomerase - an initial rapid dissociation followed by slower dissociation (Figure 3.5A,B), consistent with previous observations (Latrick and Cech 2010). In the absence of TPP1-POT1, primers dissociated with an estimated $t_{1/2}^{\text{Apparent}} \approx 1$ min. In contrast, primers bound with wild-type TPP1-POT1 dissociated at a much slower rate, with $t_{1/2}^{\text{Apparent}}$ of $\approx 6.5$ min (Figure 3.5A,B). The 6.5-fold decrease in the dissociation rate in the presence of wild-type TPP1-POT1 is consistent with a previously reported 4-fold decrease (Latrick and Cech 2010). When primers were bound by EE mutant TPP1-POT1, the dissociation rate increased compared to primers bound by wild-type TPP1-POT1; the $t_{1/2}^{\text{Apparent}}$ was $\approx 3.5$ min. These results indicate the following mechanism for TEL-patch-dependent RAP stimulation: the TEL-patch stabilizes the interaction between primer-POT1-TPP1 and telomerase during active repeat synthesis, and thus increases the efficiency of telomerase translocation.
Figure 3.5. Mutations in the TEL-patch increase primer dissociation from extending telomerase. A. Cycling telomerase off-rate assays for free primer (left panel, No TPP1-POT1), primer bound by wild-type TPP1-POT1, and primer bound by EE Mut TPP1-POT1.
(middle panel), or primer bound by E169A;E171A (right panel, EE Mut TPP1-POT1). “Pre-Ch” indicates pre-chase control samples in which 3´-phosphorylated primer was added to the telomerase prior to addition of the substrate primer. Time (min) of the chase denoted above gel, number of telomeric repeats indicated on left side of gel. Precipitation and loading control (LC) shown below each off-rate panel. B. The total counts (TC) incorporated in each lane at time n were expressed as fraction of counts incorporated at time zero TC(t = n)/TC(t = 0) and plotted versus time. The data were fit to a double exponential. Values of $t_{1/2}$ were estimated by determining the time at which half of the primer dissociated, represented by the dashed line. Fits for free primer (No TP, open square), wild-type TPP1-POT1 (WT TP, closed circle), and E169A/E171A mutant TPP1-POT1 (EE TP, closed triangle). Averaged data were plotted ± standard deviation (n = 2).

**Discussion**

Telomerase-telomere interactions are vital to homeostatic telomere maintenance. Here we demonstrate that the TEL-patch contributes to telomerase-substrate interaction at multiple points during the telomerase catalytic cycle including increased binding and extension for the first repeat synthesized, increased translocation, and decreased dissociation. We present a first step towards reconstituting an in vitro telomerase recruitment assay, and validate the dependence of single-repeat synthesis on the TEL-patch of TPP1.

**Telomerase recruitment can be measured independently of RAP stimulation in vitro**

The crucial role for the TEL-patch in telomerase recruitment has been well established by a variety of cell-based experimental approaches (Nandakumar et al. 2012; Zhong et al. 2012; Schmidt et al. 2014; Sexton et al. 2014). Evidence for TEL-patch-dependent telomerase recruitment in vitro is less direct, and has been inferred from measurements of TPP1-POT1 RAP stimulation of telomerase or TPP1-dependent telomerase pull-down (Nandakumar et al. 2012; Sexton et al. 2012; Schmidt et al. 2014). The relationship between the RAP stimulation assay and telomerase recruitment is unclear, because the interaction being measured could occur after telomerase binds to the primer. The pull-down assay provides some measure of the efficiency of telomerase recruitment in vitro, but the rate constants or
equilibrium constants that determine the pull-down efficiency are unknown. Our *in vitro* competition assay utilizes substrates that simultaneously compete for the telomerase active site and subsequent extension, so it provides the ratio of specificity constants ($k_{cat}/K_m$) for the two substrates. Our data indicate that the TEL-patch contributes to the synthesis of the first repeat of TPP1-POT1-bound primers, with the E169A;E171A mutation within TPP1 resulting in an approximately two-fold decrease in the initial velocity and specificity constant of telomerase for mutant TPP1. We note that our assay does not rely on the measurement of TPP1-POT1 stimulation of telomerase RAP. Furthermore, any enzyme turnover in this assay is distributive rather than processive (i.e. after the +4 incorporation telomerase could in theory dissociate and act on another substrate), as an unextendable chase primer is not added to the competition assay as it was in the translocation assay. Thus, the impact of the TEL-patch in binding and extension of a TPP1 bound substrate is not dependent upon repeat addition processivity in our *in vitro* recruitment assay. Finally, our competition assay strengthens the evidence that the same molecular interaction between the TEL-patch of TPP1 and hTERT mediates both RAP stimulation and recruitment.

One advantage of using the telomerase reaction rather than binding to measure recruitment is that only productive enzyme-TP-primer complexes are counted. A disadvantage, however, is that the assay does not separate binding from the nucleotide addition steps. We acknowledge that the measurement of initial velocity in our competition experiment includes nucleotide additions +1 to +4 and the translocation step, resulting in the measurement of a complex rate constant. One possibility is that both wild-type and EE mutant TPP1-POT1-bound primers associate with telomerase equally well, and the TEL-patch slows dissociation during some other step that is rate-limiting (such as a nucleotide addition), dictating the difference in specificity constants (Figure 3.3). For example, dGTP incorporation may be rate-limiting in our experiment as its concentration is below $K_m$ (Xi and Cech 2014) (although the
dGTP concentration used in our experiments is roughly physiological (Traut 1994; Mathews 2006)). Future work will be required to measure the individual rate constants of each step in the synthesis of a single repeat to definitively determine whether the TEL-patch contributes to substrate binding in a manner ultimately resulting in an increased efficiency of +1 nucleotide addition.

The two-fold stimulating effect of the TEL-patch on first repeat addition measured in vitro seems insufficient to explain the essentially on-off switch in telomerase recruitment seen upon TEL-patch mutation in vivo (Nandakumar et al. 2012; Zhong et al. 2012; Schmidt et al. 2014; Sexton et al. 2014). One possible explanation is that telomerase may need to interact with multiple telomere-bound TPP1 proteins in order to locate the 3' overhang. In this case, a small deficiency in binding would be realized during each association resulting in a large additive recruitment defect. It is also likely that recruitment is further enhanced by additional factors in vivo. Possibilities include post-translational modification of TPP1 and/or TERT, trafficking of telomerase to the telomere by other proteins, or further stabilization of an initial TPP1-TERT interaction by additional components. The in vitro competition assay developed here should be useful in validating candidate telomerase recruitment factors in the future.

The TEL-patch stabilizes the telomerase-TPP1 interaction during processive telomeric synthesis

Processive repeat synthesis by telomerase requires translocation. During translocation, the telomeric DNA substrate must dissociate and subsequently reposition on the complementary RNA template (Greider 1991). Repeat addition is a dynamic process, and several factors contribute to telomerase-substrate association, translocation, and processivity. Interactions between the telomeric substrate and the TEN-domain provide stability throughout the catalytic cycle (Jurczyluk et al. 2011; Robart and Collins 2011; Wu and Collins 2014). Processive synthesis is facilitated by multiple portions of the RT domain on hTERT, including the insertion in the fingers domain, motif 3, and the CTE (Huard et al.
2003; Xie et al. 2010). hTR, the telomerase RNA subunit, also makes direct contributions to translocation and processivity (Miller and Collins 2002; Berman et al. 2011). However, despite the evolutionary tuning of telomerase to synthesize multiple repeats processively, translocation is an inefficient step and primers frequently dissociate (Greider 1991).

A number of studies indicate that TPP1 is a processivity factor for human telomerase during active telomeric synthesis (Wang et al. 2007; Latrick and Cech 2010; Zaug et al. 2010; Nandakumar and Cech 2013). TPP1 mediates its role as a processivity factor at least in part by reducing the telomerase-substrate off-rate (Latrick and Cech 2010). Our data demonstrate that the TEL-patch of TPP1 contributes to enzyme-substrate stability during processive telomeric synthesis, resulting in longer products. Moreover, the TEL-patch of TPP1 decreases the dissociation of telomeric DNA from telomerase by approximately two-fold (Figure 3.5). Increased RAP was previously shown to correlate with decreased primer dissociation in a gain-of-function Tetrahymena TERT mutant (Bryan et al. 2000). In addition, we show that the interaction between telomerase and the TEL-patch of TPP1 stimulates the increase of both the apparent rate of translocation and the overall translocation efficiency. Translocation efficiency was shown to correlate with RAP for a panel of hTERT mutants (Qi et al. 2012b). Our results demonstrate that TPP1-POT1-dependent increases in translocation efficiency also correlate with increased telomerase RAP stimulation.

There are a number of explanations for how TPP1-POT1 might influence telomerase-substrate interaction to act as a processivity factor. First, TPP1-POT1 might contribute to the interaction solely by influencing the single-stranded DNA conformation in order to make it more accessible to telomerase; but both our data and previous studies argue against this. Heterologous telomerase-TPP1-POT1 mixing experiments showed that non-cognate TPP1-POT1 complexes do not stimulate processivity (i.e. human TPP1-POT1 does not stimulate mouse or medaka telomerases all of which have the same telomeric
repeat sequence), indicating the specificity of the TPP1-POT1 complex for its cognate TERT (Zaug et al. 2010). Furthermore, our data indicate that EE mutant and wild-type TPP1 interact with POT1-DNA equally well (Figure 3.1C), but binding and extension, translocation, and dissociation are compromised in the presence of TEL-patch mutant TPP1 (Figures 3.3-3.5). Second, TPP1 may act as an allosteric regulator or activator of telomerase by inducing conformational changes in telomerase making it more competent for processive elongation. Finally, TPP1 may directly interact with telomerase to stabilize the primer during key steps of the catalytic cycle, acting as an anchor point to provide an additional contact between telomerase and the telomere (Latrick and Cech 2010). Currently our data do not distinguish between the allosteric and anchor mechanisms for TEL-patch stimulation of processive telomerase synthesis. However, dissociation rate experiments suggest that TPP1-POT1 actively interacts with telomerase during processive synthesis, not transiently at the initiation of synthesis (this study and (Latrick and Cech 2010)).

In our current recruitment model, the TEN-domain of hTERT directly engages the TEL-patch of TPP1 to scan along the ~ 50-100 TPP1-POT1 complexes on a telomere (Takai et al. 2010) to locate the 3´ overhang. *In vivo* experiments suggest that the interaction between the TEL-patch and telomerase is necessary and sufficient for telomerase recruitment (Zhong et al. 2012; Nakashima et al. 2013; Nandakumar and Cech 2013; Schmidt et al. 2014; Sexton et al. 2014). Our competition data confirm that the TEL-patch-telomerase interaction contributes to the synthesis of the first telomeric repeat. Once telomerase engages in synthesis, the TEL-patch of TPP1 directly contacts the TEN-domain of hTERT to either induce a conformational change in telomerase or merely stabilize telomerase-telomere association, resulting in processive synthesis. Previous works suggest that multiple surfaces on TPP1 may contact telomerase (Nandakumar et al. 2012; Sexton et al. 2012; Sexton et al. 2014), and TPP1 may make TEL-patch independent contributions to telomerase activation or processivity (Sexton et al. 2014).
TPP1 may also directly interact with the primer, when complexed with POT1, to increase processivity (Hwang et al. 2012; Rajavel et al. 2014). As mutation to the TEL-patch did not completely abrogate the stimulatory effects of TPP1 in our experiments, it is possible that TPP1 may contribute to translocation efficiency and substrate dissociation in additional manners. Therefore, our current model does not discount the possibility of multiple TPP1 contributions to the activation, processivity enhancement, or regulation of telomerase. Further elucidation of the contributions and mechanism by which TPP1 contributes to telomerase action is merited.

**Deficiencies in telomerase recruitment lead to telomere-shortening disorders**

Mutations in hTERT that result in telomerase recruitment defects are thought to cause telomere shortening diseases. The IPF-associated mutation in hTERT V144M (Tsakiri et al. 2007) has deficiencies in RAP stimulation by wild-type TPP1-POT1 (Schmidt et al. 2014), as well as in vivo telomere localization (Zhong et al. 2012). Recently a mutation in the TEL-patch of TPP1 was shown to associate with aplastic anemia and with Hoyeraal-Hreidarsson syndrome (a severe form of DC) (Guo et al. 2014; Kocak et al. 2014). Patients with ΔK170 TPP1 allele had significantly reduced telomere lengths; furthermore, ΔK170 TPP1 reduced telomerase recruitment in cell-based assays and reduced RAP stimulation in direct telomerase extension assays (Guo et al. 2014; Kocak et al. 2014). These studies strongly suggest that telomerase recruitment is specifically compromised by deletion of K170 in the TEL-patch. K170 is immediately adjacent to the mutated residues in TPP1 used in this study. Based on our data, it is tempting to speculate that K170 disrupts the TPP1-hTERT interface to increase telomerase dissociation during synthesis, in addition to the previously shown recruitment defects (Guo et al. 2014; Kocak et al. 2014).
In summary, we have shown that the TEL-patch amino acid cluster of TPP1 stabilizes telomerase on telomeric DNA throughout the catalytic cycle. We have developed a novel telomerase competition assay and demonstrate that the telomerase interacts directly with the TEL-patch during synthesis of the first repeat on telomeric DNA substrates. Our *in vitro* competition assay presents a facile way to directly test the impacts of new TPP1 mutants or additional recruitment factors. We also show that the TEL-patch increases the rate of product formation, improves translocation efficiency, and reduces telomeric DNA dissociation during active telomerase synthesis. Thus, TPP1 makes multiple contributions to telomerase. Quantitative studies of the TEL-patch-dependent recruitment of telomerase will contribute to further understanding of molecular origins of disease-associated alleles and might be applied in development of new molecular therapies.

**Materials and Methods**

**Super telomerase extract preparation**

In order to overexpress and assemble human telomerase, plasmids encoding hTR (pBS-U1-hTR) and N-terminal HA-tagged hTERT (pVan145) were co-transfected into HEK 293T cells. The wild-type telomerase plasmids were a generous gift of J. Lingner (EPFL, Lausanne) (Sauerwald et al. 2013). Mutant hTERT plasmids were generated by quick-change mutagenesis (Agilent). Whole cell extracts were prepared after two days of transient transfection using a CHAPS lysis buffer (Cristofari and Lingner 2006); extracts were snap frozen in liquid nitrogen and stored at -80°C.

Telomerase was isolated from whole cell extracts with slight modifications to a previous purification protocol (Sauerwald et al. 2013). In short, 200 IgG Sepharose 6 Fastflow beads (GE Healthcare) were equilibrated in 30 mL of buffer A lacking 1 mM DTT. Buffer A was comprised of 20 mM HEPES-KOH pH 7.9, 2 mM MgCl₂, 300 mM KCl, 10 % glycerol (v/v), 1 mM DTT, 1 mM EDTA, 0.1 % Triton
X-100 (v/v), 1 mM PMSF. The ionic strength of the extract was adjusted by diluting the extract in an equal volume of 2X buffer A. Telomerase was captured by nutating at 4ºC for approximately 4 h. The Sepharose beads were then washed in 50 mL of buffer A. Telomerase was cleaved from the IgG beads with 50 U of AcTEV protease (Life Technologies), in the presence of 200 U of RNasin Plus (Promega), overnight while nutating at 4ºC. Following elution, telomerase was frozen in liquid nitrogen and stored at -80ºC for future use.

**Protein purification**

Recombinant human wild-type and mutant TPP1-N constructs (TPP1N is comprised of the OB and POT1 binding domain) were overexpressed in BL21-DE3 cells and purified as previously described (Wang et al. 2007; Nandakumar et al. 2012). Recombinant human full length POT1 was overexpressed and purified from insect cells as previously described (Lei et al. 2004). Protein concentrations were determined by absorbance at 280 nm, and corrections for % active protein were made.

**Native gel shift assays**

Binding assays were performed as described earlier (Lei et al. 2004). POT1, wild-type TPP1-POT1 or E169A;E171A TPP1-POT1 protein complexes were added to 5'-32P-labelled DNA primer a5TT (5´TTAGGGTTAGCGTTAGGGTT 3´) and incubated for 30 min at room temperature. Binding was carried out in buffer containing 50 mM Tris–HCl pH 8.0, 100 mM NaCl, and 1 mM DTT. Reactions were then loaded onto a native 1X TBE 8–20% polyacrylamide gradient gel (Life Technologies), run in the cold room (4°C) at 200 V for 1 h and dried.
Telomerase full extension assay

Telomerase extension assays were carried out with minor modifications (Zaug et al. 2010). Reactions were carried out in 1X reaction buffer: 50 mM Tris pH 8.0, 30 mM KCl, 0.1 to 1 mM MgCl₂, 1 mM spermidine, and 5 mM β-mercaptoethanol. Final nucleotide concentrations in the assay were 500 μM dATP, dTTP, and 3.23 μM dGTP. The final dGTP concentration included 0.33 μM α-³²P-dGTP (Perkin Elmer). Reactions were initiated by addition of S4A5TT primer (5′(TTTGGC)₄TTAGGGTTAGCGTTAGGGTT 3′, 100 nM final), and extension was allowed to proceed for 120 min at 4ºC. Reactions were stopped in five volumes of a solution containing 3.6 M ammonium acetate, 20 μg of glycogen, and a 5′-³²P-labeled oligo loading control. Reactions were then precipitated with 0.5 mL of 100% ethanol and then washed one time with 1 mL 70% ethanol. The reaction products were then resuspended in equal parts H₂O and 2X formamide loading dye (0.5X TBE, 93.5% Formamide, 30 mM EDTA, 0.5% xylene cyanol and bromophenol blue). Telomerase extension products were electrophoresed on a 10% agarose denaturing gel (1X TBE, 7 M urea) at a constant power of 90 watts for approximately 1.5 h. Gels were visualized by phosphoimagery and then quantitated using ImageQuant TL (GE Healthcare).

Translocation assays

Translocation assays were carried out as previously described (Latrick and Cech 2010), with minor modifications. POT1 and TPP1 were incubated with 100 nM S4A5TT primer for 30 min at room temperature. TPP1-POT1 bound primers were then bound to telomerase in 1X reaction buffer, lacking dNTPs, for 20 min at 4ºC. At time zero, the reactions were initiated by the simultaneous addition of dNTPs and a competitive 3′ phosphorylated chase primer at a final concentration of 1 μM. The chase primer was included to prevent telomerase from re-engaging after substrate dissociation (Latrick and Cech 2010). Reactions contained 100 nM primer, 250 nM POT1, 125 nM TPP1, 50 mM Tris pH 8.0, 30
mM KCl, 0.1 mM MgCl₂, 1 mM spermidine, and 5 mM β-mercaptoethanol. Final nucleotide concentrations in the assay were 500 µM dATP and 3.23 µM dGTP; dTTP was omitted from the reaction. The final dGTP concentration included 0.33 µM α-32P-dGTP. Reactions were carried out at 4°C. Reactions were stopped by removing aliquots of the reaction at various time points and quenching in stop solution described above for full extension assays. The fraction translocated was calculated by summing the counts (C) in the +3 and +4 bands, and dividing by the total counts; i.e., fraction translocated = (C₃ + C₄)/(C₂ + C₃ + C₄). The fraction of translocated product was plotted as a function of time and fit to a single exponential using SigmaPlot (Systat).

**Competition assays**

Competition assays were performed by simultaneously initiating a telomerase reaction with two substrates, in this case two primers of different length. Primer 1 (S4A5TT) in 1X reaction buffer was incubated in with POT1 and TPP1 (either wild-type or mutant TPP1) for 30 min at room temperature, followed by 20 min at 4°C. Primer 2 (S3A5TT = 5’-(TTTGGC)₃TTAGGTTAGCGTTAGGGTT-3’) was incubated under identical conditions in a separate tube with POT1 and either mutant or wild-type TPP1. While primers were complexed with TPP1-POT1, reactions containing telomerase, buffer, and dNTPs were assembled and cooled to 4°C for 20 min. Reactions were initiated by simultaneously adding primer 1 and primer 2 to the telomerase in buffer and nucleotides at 4°C. Competition reactions were stopped by removing aliquots of the reaction at various time points and quenching in the stop solution described above for full extension assays. Competition assays were carried under the buffer and nucleotide conditions as described for the translocation assay, the final concentration of each primer was 100 nM, 500 nM POT1, and 250 nM wild-type TPP1 and 250 nM mutant TPP1. Total counts (TC), at each time point, for both primers were normalized to the loading control and expressed as fractions of the product formed for primer complexed with wild-type TPP1-POT1 at the final time point - e.g., Normalized
product formation at time \( n \) for primer with EE TPP1 = \((\frac{TC_{\text{primerEE}}(t = n)}{C_{\text{control}}})\)\((\frac{TC_{\text{primerWT}}(t = 12 \text{ min})}{C_{\text{load control}}})\). The initial velocities were calculated by plotting the normalized product formation versus time, and determining the slope by linear regression. The error values reported in the text were calculated using the equation: reported error = \( \mu (v_{\text{primerWT}}/v_{\text{primerEE}})((\sigma v_{\text{primerWT}}/\mu v_{\text{primerWT}})^2 + (\sigma v_{\text{primerEE}}/\mu v_{\text{primerEE}})^2)^{1/2} \).

**Extending primer dissociation rate assays**

Dissociation rate assays were carried with slight modifications from those previously described (Latrick and Cech 2010). Primer and 3’-phosphorylated chase primer in 1X buffer were complexed with excess TPP1-POT1 at room temperature in separate tubes. Complexed primer was then allowed to equilibrate with telomerase for five min; next cold dATP, dTTP, and dGTP were added to initiate the telomerase reaction. The reaction proceeded for five min, and at time zero a large-excess of 3’ phosphorylated chase primer was added to prevent substrate primer re-association. After chase addition, aliquots of the reaction were removed at varying time points and pulse labeled with \(^{32}\)P-\( \alpha \)-dGTP for five min. Reactions stopped as described for full extension assays. Reactions were carried out at 25ºC. Final reaction concentrations were 50 nM A5 primer (5’ TTAGGGTTAGCGTTAGGG 3’), 1 \( \mu \)M 3’ phosphorylated chase primer, 1.2 \( \mu \)M POT1, 1.2 \( \mu \)M TPP1, 50 mM Tris pH 8.0, 150 mM KCl, 1 mM MgCl\(_2\), 1 mM spermidine, and 5 mM \( \beta \)-mercaptoethanol. Final nucleotide concentrations in the assay were 500 \( \mu \)M each of dATP and dTTP, and 3.23 \( \mu \)M dGTP. The final dGTP concentration included 0.33 \( \mu \)M \( \alpha \)-\(^{32}\)P-dGTP. The total counts (TC) for each lane were expressed as a fraction of counts at time zero and plotted as a function of time (i.e. fraction bound = \((\frac{TC(t = n)}{TC(t = 0)})\)), as previously described (Latrick and Cech 2010). Data were fit to a double exponential and \( t_{1/2}^{\text{Apparent}} \) was taken as the time required for 50% dissociation.
Chapter IV: Identification of human TERT elements necessary for telomerase recruitment to telomeres

Introduction

The work presented in chapter three illustrated the importance of the TPP1 TEL-patch in telomerase recruitment. The goal of the work presented in this chapter was to determine whether the TPP1 TEL-patch physically interacted with the TEN-domain of hTERT and to gain understanding about the nature of the interaction. This work was the result of collaboration with a post-doc in the lab Jens Schmidt. Jens and I contributed equally to the conceptual framework of these experiments, generation of experimental materials, and authorship of the paper. Specifically, I carried out the biochemical experiments, while Jens was responsible for the cell-based experiments. Analysis and authorship pertaining to the in vivo work presented in this chapter are ascribed to Jens; his contributions are included to enhance the continuity of the chapter. Conversely, I analyzed and authored the sections pertaining to the in vitro biochemical work presented below. In addition, I composed the discussion of all experiments with input from Jens, and we are listed as co-first authors on the manuscript.

As previously discussed, shelterin is the protein complex that associates with telomeric DNA to prevent recognition by the DNA damage machinery, inhibit telomere dysfunction, and recruit telomerase (Palm and de Lange 2008; Nandakumar and Cech 2013). The shelterin component TPP1 interacts with telomerase; however, the site and nature of the interaction is poorly understood from the telomerase side. In vitro, the POT1-TPP1 complex acts as a processivity factor for telomerase, stimulating repeat addition processivity (RAP) (see chapter three, (Wang et al. 2007). Recent work has demonstrated that the TEL-patch, a cluster of amino acids on the TPP1 OB-domain, is necessary for RAP

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1This chapter has been modified from the following publication: Schmidt JC, Dalby AB, Cech TR. 2014. Identification of human TERT elements necessary for telomerase recruitment to telomeres. eLife 3.
stimulation as well as telomerase recruitment in vivo (Nandakumar et al. 2012; Sexton et al. 2012; Zhong et al. 2012). Although the TEL-patch participates in telomerase recruitment and RAP stimulation, it is unclear whether TPP1 physically interacts with telomerase or requires a bridging factor. For example, Ccq1 mediates the interaction between Tpz1 (TPP1 homologue) and telomerase in Schizosaccharomyces pombe (Miyoshi et al. 2008; Moser et al. 2011). A homologue of Ccq1 has not been identified in humans (Nandakumar and Cech 2013).

Furthermore, the interaction site on telomerase has not been elucidated. One possibility is that the TEN-domain of hTERT directly interacts with TPP1. Multiple studies have implicated the N-DAT region of the TEN-domain in telomerase recruitment, using cell based assays (Armbruster et al. 2001; Zhong et al. 2012). The mutation G100V, in the TEN-domain of hTERT, drastically reduces RAP stimulation of telomerase by POT1-TPP1 in vitro and recruitment in vivo (Zaug et al. 2010; Zhong et al. 2012). However, G100V telomerase also has enzymatic activity defects which obscure conclusions regarding a direct interaction between TPP1 and the TEN-domain.

In this study we screened and identified separation-of-function mutants which retain high enzymatic activity but are defective for RAP stimulation by TPP1-POT1 in direct telomerase extension assays. The separation of function TEN-domain mutant telomeres are sequestered in Cajal bodies and consequently are prevented from maintaining telomeres in vivo. We also report a compensatory mutation in the TEL-patch of TPP1 that rescues RAP stimulation by POT1-TPP1 in vitro, telomerase-TPP1 interactions, and telomere maintenance in an hTERT TEN-domain mutant. We conclude that the TEN-domain of hTERT directly interacts with the TEL-patch of TPP1 to promote telomerase recruitment and processivity.
Results

TEN-domain hTERT mutants defective for POT1-TPP1-mediated processivity enhancement

Conserved acidic amino acids on the surface of the TPP1 OB-domain (TEL-patch) are necessary for the recruitment of telomerase to telomeres (Nandakumar et al. 2012; Sexton et al. 2012; Zhong et al. 2012). We hypothesized that a corresponding region of basic amino acids would exist on the surface of hTERT, which directly associates with the TEL-patch via charge-charge and other interactions to recruit telomerase to telomeres (Figure 4.1A).
Figure 4.1. Identification of mutants in the TEN-domain of hTERT that affect the interaction with POT1-TPP1 in vitro but not enzymatic activity. A. Domain structures of hTERT and TPP1 proteins. Question mark, proposed interaction between the TEL-patch on TPP1 and the N-DAT region of the TEN-domain of hTERT. In TPP1, the OB-domain (OB) is followed by the POT1-binding domain (PBD), and finally the TIN2-binding domain (TIN2 BD). For hTERT, the Telomerase Essential N-terminal (TEN) domain is adjacent to the RNA binding domain (RBD), which precedes the reverse transcriptase domain (RT) and finally the C-terminal extension (CTE). B. An alignment of the N-DAT region of the TEN-domain of TERT proteins from selected species. Basic residues conserved in greater than five of the seven species are highlighted blue. The conserved residue G100 is highlighted in orange above the alignment. C. Western blot, probed for hTERT, showing the immuno-purification of telomerase over-expressed in HEK 293T cells. To monitor relative quantities of hTERT, equal fractions of lysate, flow through (FT), IgG bead capture (capture), and cleaved eluate (elution), were analyzed by SDS-PAGE. D. Western blot of the relative quantities of wild-type and mutant hTERTs after immuno-purification of the telomerases. E. Direct telomerase activity assays in the absence and presence of the POT1-TPP1 heterodimer for wild-type (WT) and mutant
telomerases. LC, loading control. +4, oligonucleotide marker corresponding to the addition of the first four nucleotides to primer. Numbers on left, telomeric repeats added. F-H, Bar graphs representing the quantification of activity, RAP, and RAP stimulation (decay method) by wild-type POT1-TPP1. Values are normalized to WT telomerase, and to WT telomerase with WT POT1-TPP1 for RAP stimulation (n = 3, Mean ± SD).

To identify candidate residues on the TEN-domain of hTERT that might interact with the TPP1 TEL-patch, we performed a multiple sequence alignment of TERT from higher eukaryotes (Figure 4.1B). The alignment revealed a number of conserved basic amino acids; we tested these, as well as some non-conserved basic amino acids in close proximity in the primary sequence. We generated a panel of hTERT mutants, replacing individual or combinations of basic amino acids on hTERT with the acidic amino acid aspartate. Mutant hTERTs and hTR were over-expressed in HEK293T cells and the telomerase were immuno-purified (Figure 4.1C). The hTERT variants were expressed at levels similar to those of wild-type telomerase (Figure 4.1D).

To identify separation-of-function hTERT mutants that do not interact with POT1-TPP1 but retain near wild-type telomerase activity in vitro, we carried out direct telomerase extension assays. (In these assays, dATP, dTTP, and 32P-dGTP are added to the 3’ end of a DNA oligonucleotide by immuno-purified telomerase and products are separated on a polyacrylamide sequencing gel and visualized by autoradiography. The enzymatic activity of each telomerase is determined by quantifying the total amount of 32P-dGTP incorporated into reaction products, and RAP is measured by analyzing the distribution of product lengths). To measure physical interaction between TPP1 and telomerase, we carried out direct telomerase assays with and without a previously described minimal POT1-TPP1 complex bound to substrate oligonucleotides (Wang et al. 2007) and calculated “RAP stimulation by PT” (equation given in Materials and Methods). RAP stimulation by PT depends on the association of telomerase with POT1-TPP1 and therefore provides a biochemical readout for this interaction.
In the hTERT mutant screen, some telomerases had defects in either activity (R72E, R143E, R142E;R143E), RAP (R87E;R91E;K94E, R120E), or had no effect (R142E) (Table 4.1, Figure 4.1, Figure 4.2). However, hTERT K78E retained wild-type activity and processivity (92% and 100% respectively), but RAP stimulation by POT1-TPP1 was reduced to 68% that of wild-type telomerase (Figure 4.1E-H, Figure 4.3). Additionally, the R132D mutant was reported to have wild-type activity but failed to localize to telomeres in vivo (Stern et al. 2012). We generated a R132E mutant hTERT, which retained moderate activity (73%) but was defective in RAP stimulation by POT1-TPP1 (24% of wild-type hTERT, Figure 4.1E-H and supplement 2). Furthermore, the K78E;R132E double mutant retained 63% activity but further reduced RAP stimulation by POT1-TPP1 to 18% of wild-type hTERT (Figure 4.1E-H). Two different methods of quantitating RAP gave comparable results (Figure 4.3).

### Table 4.1. Telomerase TEN domain mutant activity, processivity, and RAP stimulation by wild-type TPP1

<table>
<thead>
<tr>
<th>TEN Domain Mutant</th>
<th>Activity % of WT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Processivity % of WT</th>
<th>RAP Stimulation by PT&lt;sup&gt;b&lt;/sup&gt; % of WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>R72E</td>
<td>48 ± 3</td>
<td>99 ± 1</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>K78A</td>
<td>19 ± 2</td>
<td>101 ± 7</td>
<td>88 ± 2</td>
</tr>
<tr>
<td>R78E</td>
<td>92 ± 1</td>
<td>100 ± 1</td>
<td>68 ± 5</td>
</tr>
<tr>
<td>R87E;R91E;K94E</td>
<td>74 ± 6</td>
<td>75 ± 0.3</td>
<td>61 ± 4</td>
</tr>
<tr>
<td>R120E</td>
<td>99</td>
<td>96</td>
<td>55</td>
</tr>
<tr>
<td>K78E;R120E</td>
<td>82 ± 18</td>
<td>87 ± 3</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>R132E</td>
<td>73 ± 2</td>
<td>92 ± 3</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>R132E;K78E</td>
<td>63 ± 10</td>
<td>94 ± 8</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>R142E</td>
<td>97 ± 7</td>
<td>111 ± 10</td>
<td>103 ± 4</td>
</tr>
<tr>
<td>R143E</td>
<td>16 ± 12</td>
<td>81 ± 3</td>
<td>N.D.</td>
</tr>
<tr>
<td>R142E;R143E</td>
<td>4 ± 1</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>V144M</td>
<td>48 ± 6</td>
<td>95 ± 5</td>
<td>44 ± 7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percentage of wild-type telomerase activity, processivity, or RAP stimulation. Activity values normalized to hTERT levels, loading control, ± standard deviation for 2 or more replicates.

<sup>b</sup>Repeat addition processivity (RAP) stimulation upon addition of WT POT1-TPP1, values relative to WT telomerase with WT POT1-TPP1 (i.e. RAP stimulation by PT % of WT = (WT PT RAP stimulation of telomerase mutant)/(WT PT RAP stimulation WT telomerase)) * 100.

<sup>c</sup>Not determined (N.D.) due to low telomerase activity.
Figure 4.2. Additional TEN-domain mutants tested in this study. A. Direct telomerase assays of additional hTERT TEN-domain mutants (all with WT hTR) without (−) and with (+) WT POT1-TPP1. LC and +4 marker as in Figure 4.1E. Quantification in Table 4.1. B. Western blot of the relative quantities of wild-type and mutant hTERTs following immuno-purification.

Figure 4.3. Comparison of methods for quantifying RAP stimulation by POT1-TPP1. A. Example of linear regression of data from one replicate that was used to calculate RAP stimulation by POT1-TPP1 in figure 4.1H, using the decay method. All four assays included wild type POT1-TPP1. R² values and slope of regression are shown. B. Comparison of RAP stimulation by POT1-TPP1 for data calculated using the decay (shown in figure 4.1H) and fraction methods as described in Materials and Methods.
An alignment of the human TEN-domain with the *T. thermophila* TEN-domain in combination with a secondary structure prediction shows that K78 and R132 are in close proximity to each other, on a surface distinct from the proposed DNA-binding region of the TEN-domain (Figure 4.4, Figure 4.5).

**Figure 4.4.** Conserved basic residues K78 and R132 are found in close proximity on the surface of the TEN-domain. Cartoon representation of the TEN-domain crystal structure from *Tetrahymena thermophila* (PDB: 2B2A). Amino acid numbers are from *Tetrahymena* (human counterparts in parentheses). Anchor site (DNA binding) residues are highlighted in blue. The positions of the residues corresponding to human K78 and R132 are highlighted in red, and F143 corresponding to human V144 is highlighted in green. Figure courtesy of Jens Schmidt.

**Figure 4.5.** Alignment and secondary structure prediction for human TEN-domain. Secondary structure prediction for hTERT TEN domain and alignment to selected eukaryotic TERT TEN domains. Human secondary structure prediction made with JPRED3 (Cole et al. 2008), while *T. thermophila* secondary structure based on crystal structure (PDB: 2B2A). The *T. thermophila* and *S. cerevisiae* TEN-domains were aligned to the human TEN-domain using Psi-blast, followed by manual alignment of the remaining species. Similar amino acids are shaded by color.
These results demonstrate that K78 and R132 within the TEN-domain of hTERT make a much larger contribution to RAP stimulation by POT1-TPP1 than to enzymatic activity in vitro. RAP stimulation defects in K78E and R132E mutants are most likely due to the failure of telomerase to interact with TPP1. Thus, this surface of hTERT is a candidate TPP1-interacting element.

**TEN-domain mutants fail to localize to telomeres in vivo**

If mutations of K78 and R132 in hTERT affect the interaction of telomerase with TPP1, they should disrupt telomerase localization to telomeres in vivo. To test whether the mutant telomerases are recruited to telomeres, we transiently over-expressed mCherry-tagged hTERT variants and hTR in HeLa cells and determined the subcellular localization of telomerase by immuno-fluorescence (IF). Under all conditions hTERT and hTR were expressed at similar levels, indicating that mutations in the TEN-domain did not affect hTERT or hTR stability (Figure 4.6A-B). As previously described (Zhong et al. 2012), wild-type telomerase localized to telomeres and promoted the formation of neo-Cajal bodies at most telomeres, as shown by the co-localization of TRF2, hTERT and coilin foci (Figure 4.6C). In contrast, all three hTERT mutants tested (K78E, R132E, K78E;R132E) localized to bona fide Cajal bodies, forming ~1-3 large foci per cell that co-localized with coilin but did not localize to telomeres marked by TRF2 (Figure 4.6C). These results demonstrate that TEN-domain mutants that do not interact with POT1-TPP1 in vitro also fail to localize to telomeres in vivo. Furthermore, because Cajal body localization of hTERT requires its association with hTR, our observations indicate that telomerase assembly is unaffected by mutations in the TEN-domain.
Figure 4.6. TEN-domain mutations disrupt telomere localization of telomerase. A. Western blots of lysates of HeLa cells transfected with expression plasmids for various hTERT alleles and hTR, probed with an antibody against hTERT. Actin was used as a loading control. B. Northern blots of RNA isolated from HeLa cells transfected with expression plasmids for various hTERT alleles and hTR, using probes for hTR. In vitro transcribed hTR (500 pg) was used as positive control. Blots were probed for RNase P RNA as loading control. C. Immuno-fluorescence (IF) analysis of HeLa cells transiently transfected with mCherry-hTERT and hTR plasmids. Cells were fixed and probed with antibodies against mCherry, collin, and TRF2 to visualize telomerase, Cajal bodies and telomeres, respectively. Images were deconvolved. Numbers indicate the fraction of cells analyzed showing the displayed phenotype (scale bar = 5 μm). Figure courtesy of Jens Schmidt.

TEN-domain mutants are defective in telomere maintenance in vivo

To determine the effects of the mutant telomerases on telomere maintenance, we generated cell lines stably expressing mCherry-tagged hTERT variants by retroviral transduction. Overexpression of hTERT alone leads to a moderate increase in telomerase activity per cell, more closely resembling endogenous telomerase levels than those obtained by overexpression of both hTERT and hTR (Cristofari et al. 2007; Xi and Cech 2014). Thus, while the absolute expression level of mCherry-hTERT varied between the stable cell lines (Figure 4.7A), telomerase immuno-purified from the cell lines had
comparable activities per cell, and these activity levels were 3-4 fold higher than levels in untransfected control cells (Figure 4.7B). This result confirmed that mutations in the TEN-domain did not strongly reduce telomerase activity. The exogenous hTERT was overexpressed relative to endogenous hTERT, which was not detectable by western blot (Figure 4.7A). Therefore the majority of endogenous hTR should be assembled into telomerase RNPs containing the mutant hTERT protein (Figure 4.7A).

Figure 4.7. TEN-domain mutants that do not localize to telomeres fail to elongate telomeres in vivo. A. Western blot probed for hTERT and for Actin as a loading control, showing hTERT expression in lysates of parental HeLa cells and cell lines stably expressing mCherry-hTERT variants. hTR was not ectopically expressed. B. Direct enzyme assay of telomerase immuno-purified from lysates generated using equal number of cells, from parental HeLa or cell lines stably expressing mCherry-hTERT variants. Activity per cell relative to WT hTERT overexpressing cells (n = 4, Mean ± SD, p < 0.05). C. Telomeric restriction fragment Southern blot of cell lines stably expressing mCherry-hTERT variants over the time course of 8 weeks. Figure courtesy of Jens Schmidt.
To analyze the effect of mutant hTERT expression on telomere length, DNA isolated from stable cell lines over the course of 8 weeks after viral transduction was subjected to telomeric restriction fragment (TRF) analysis by Southern blot. The telomere length of the parental HeLa cell line was stable over the course of the experiment at ~6.6 kb (Figure 4.7C). Expression of wild-type hTERT caused a progressive increase in telomere length to ~14.8 kb (Figure 4.7C). In contrast, TEN domain mutants K78E and R132E failed to elongate telomeres, despite the fact that telomerase enzymatic activity per cell was higher than in the parental HeLa cell line (Figure 4.7B, C). In addition the TEN domain mutant K78E;R132E also failed to elongate telomeres, but due to its reduced activity (Figure 4.1F), we cannot conclude that this is exclusively due to a localization defect. These observations reinforce the conclusion that telomerase with K78E or R132E mutations in the TEN-domain fails to elongate telomeres due to an inability to localize to telomeres, not an activity defect.

**A compensatory mutation in TPP1 rescues the mutant hTERT – TPP1 interaction in vitro**

Taken together, our experiments demonstrate that mutations in the TEN-domain of hTERT recapitulate the effects of mutations in the TEL-patch of TPP1 (i.e. loss of telomerase recruitment to telomeres *in vivo*, and reduced POT1-TPP1 RAP stimulation *in vitro*). If the stimulation of telomerase RAP and telomerase recruitment to telomeres are the manifestation of a direct interaction between charged residues in the TEN-domain of hTERT and the OB-fold of TPP1, a charge-swap mutation in the OB-fold of TPP1 could rescue the TEN-domain mutation (Figure 4.8A). On the other hand, if a bridging factor were necessary for the interaction of TPP1 with hTERT, individually deleterious mutations in both TPP1 and the TEN-domain should have an additive negative impact on the TEN-TPP1 interaction when combined (Figure 4.8A).
Figure 4.8. A compensatory mutation in the TEL-patch of TPP1 rescues RAP stimulation of TEN-domain mutant telomerase in vitro. A. Schematic of the charge-swap experiment to test the interaction between specific amino acids on the TEL-patch on TPP1 and the N-DAT region of the TEN-domain of hTERT. Predicted experimental outcomes are illustrated for two competing models: A direct TPP1 telomerase interaction and an interaction bridged by a yet-unidentified factor (BF?). B. Coomassie-stained gel of WT, E169K, and E215K TPP1 co-purified with wild-type POT1. C. Overlays of the Superdex 200 16/60 sizing column chromatograms for wild-type and mutant TPP1/POT1 complexes. D. Direct telomerase activity assay to test the rescue of various telomerase containing WT, K78E, R132E, and K78E;R132E mutant hTERTs in the absence of POT1-TPP1 (No PT) or WT, E169K, and E215K TPP1 in complex with wild-type POT1. LC and +4 marker as in Figure 4.1E. E. Quantification (decay method) of the RAP stimulation by POT1-TPP1 (PT) relative to the stimulation of wild-type telomerase with wild-type POT1-TPP1 (n = 3, Mean ± SD, * p < 0.05, ** p < 0.01, Student’s t-test).
To test this hypothesis, we generated a number of mutant TPP1N proteins, changing individual conserved negatively charged residues in the TEL-patch to the basic amino acid lysine. Mutant TPP1 proteins were co-purified with POT1 to apparent homogeneity (Figure 4.8B). All mutant POT1-TPP1 complexes had identical elution profiles from the sizing column (Figure 4.8C). The presence of a single elution peak indicated that all TEL-patch mutant TPP1 proteins were not globally misfolded and formed heterodimers with POT1 (Figure 4.8C).

To test the ability of the mutant TPP1 proteins to bind TEN-domain mutants, we used direct telomerase enzyme assays to measure RAP stimulation by POT1-TPP1. Strikingly, TPP1 E215K fully rescued the processivity defect of hTERT K78E; processivity increased from 68% to 98% relative to wild-type telomerase and wild-type TPP1 (Figure 4.8D,E). Importantly, the non-cognate combinations of (i) wild-type telomerase and E215K TPP1 and (ii) K78E telomerase with wild-type TPP1 showed a similar reduction of RAP stimulation, to 78% and 68% of the full level, respectively (Figure 4.8D,E). TPP1 E169K gave dramatic reductions in RAP and did not stimulate any of the tested telomerases above 24% of wild type (Figure 4.8D,E). hTERT mutant R132E and the double mutant K78E;R132E were stimulated at levels less than 23% of wild type by all TPP1 proteins tested (Figure 4.8D,E). An alternative method for quantification of RAP stimulation by POT1-TPP1 gave similar results (Figure 4.9). Furthermore, to rule out the possibility of a bridging factor that co-purifies with telomerase from HEK293 T cells, we carried out the charge-swap experiment with telomerase purified from rabbit reticulocyte lysates (RRLs). The charge-swap rescue was statistically significant when using telomerase from RRLs, although the absolute PT stimulation of RRL telomerase was diminished (Figure 4.10).
Figure 4.9. The charge-swap is statistically significant by alternative quantitation methods. RAP stimulation by PT calculated using the fraction method to quantitate data presented in Figure 4.8D (n = 3, Mean ± SD, * p < 0.05, ** p < 0.01, Student's t-test).

Figure 4.10. E215K TPP1 rescues RAP stimulation of K78E mutant telomerase produced in RRLs. A. Western-blot for hTERT produced in RRLs. Also shown is a serial dilution of overexpressed telomerase immuno-purified from HEK293T cells. 1X signifies amount of telomerase used in direct extension assays. B. Direct telomerase activity
assay to test the rescue of WT and K78E telomeres produced in RRLs, in the absence of POT1-TPP1 (No PT) or WT, E169K, and E215K TPP1 in complex with wild-type POT1. LC as in Figure 4.1E. Rescue is apparent by looking at the long extension products (>12 repeats), which decrease in intensity from lane 2 to lane 4 for WT hTERT, but then increase from lane 2 to lane 4 for K78E hTERT. C. Quantification (decay method) of the RAP stimulation by POT1-TPP1 (PT) relative to the stimulation of wild-type telomerase with wild-type POT1-TPP1 (n = 3, Mean ± SD, * p < 0.05).

To further illustrate the specificity of the charge-rescue, a disease allele V144M found in some cases of idiopathic pulmonary fibrosis (IPF) (Tsakiri et al. 2007) was tested for interaction with POT1-TPP1. V144M telomerase retained moderate telomerase activity and high processivity (48% and 95% of wild-type telomerase, respectively) but POT1-TPP1 RAP stimulation was significantly decreased to 61% when compared to wild-type telomerase (Table 4.1, Figure 4.11). Furthermore, TEL patch mutants E169K and E215K each resulted in an additive loss of RAP stimulation when tested with disease mutant V144M, to 28% and 37% respectively (Figure 4.11). Thus, the specific and compensatory nature of the hTERT K78E and TPP1 E215K mutant combination strongly suggests that K78 on the TEN-domain of hTERT directly interacts with E215 on the TEL-patch of TPP1 to stimulate RAP in vitro.
Figure 4.11. The IPF allele V144M is deficient in RAP stimulation by TPP1. A. Direct telomerase assays of wild type and V144M hTERT telomerases in the presence of WT, E169K, or E215K POT1-TPP1. LC and +4 marker as in Figure 4.1E. B. Western blot of the relative quantities of wild-type and V144M hTERTs following immuno-purification. (Note: activity is proportional to hTERT, but RAP is independent of the amount of hTERT in these assays). C. Quantification of the RAP stimulation by POT1-TPP1 (PT) relative to the stimulation of wild-type telomerase with wild-type POT1-TPP1 (n = 6, Mean ± SD, ** p < 0.01, Student’s t-test).
A compensatory mutation in the TEL-patch of TPP1 restores the telomerase TPP1-OB interaction at a non-telomeric locus \textit{in vivo}

To analyze mutant hTERT-TPP1-OB-fold interactions \textit{in vivo}, we utilized a Lac-repressor (LacI) assay previously described (Zhong et al. 2012). The TPP1-OB-fold domain was inserted between GFP and the LacI protein (Figure 4.12A). GFP-TPP1-OB-LacI was expressed alongside mCherry-tagged hTERT and hTR in the U2OS 2-6-3 cell line, which contains a single Lac-operator DNA array on chromosome 1 (Janicki et al. 2004), thereby tethering the OB-fold domain of TPP1 to a non-telomeric chromosomal locus (Figure 4.12A). The interaction between telomerase and the TPP1-OB-fold domain was assessed by co-localization of GFP- and mCherry foci in cell nuclei. Wild-type telomerase co-localized with the TPP1-OB-fold domain in \sim 90\% of nuclei. In contrast, in cells expressing K78E, R132E, or K78E;R132E telomerase, the fraction of TPP1-OB-fold domain foci showing telomerase signal was reduced to \sim 55\%, \sim 30\%, and 0\%, respectively (Figure 4.12B,C, additional examples in Figure 4.13). The reduction in co-localization demonstrated that mutation of the hTERT TEN-domain interferes with the interaction between telomerase and the TPP1-OB-fold domain \textit{in vivo}. 
Figure 4.12. A compensatory mutation in the TEL-patch of TPP1 rescues TEN-domain mutant telomerase binding to the TPP1 OB-domain in vivo. A. Model showing the experimental design. Fusion of the OB-domain of TPP1 to the lac repressor (LacI) recruits the OB-domain to a single non-telomeric chromosomal locus (LacO array), allowing the interaction between telomerase and the TPP1 OB-domain to be assessed by co-localization of GFP (TPP1 OB) and mCherry (hTERT) in cell nuclei. B. Fluorescence images showing the localization of GFP-TPP1-OB-LacI and mCherry-hTERT fusion proteins in cell nuclei stained with DAPI. Cells were fixed, permeabilized and stained with DAPI. The intrinsic GFP- and mCherry fluorescence was used to detect GFP-TPP1-OB-LacI and mCherry-hTERT (scale bar = 5 μm). C. Quantification of the experiments shown in (B), showing the fraction of nuclei with co-localization of GFP- and mCherry-foci (n = 3, 117-176 nuclei total, Mean ± SD, * p < 0.05, ** p < 0.01, Student’s t-test). Figure courtesy of Jens Schmidt.
Figure 4.13. Additional examples of U2OS 2-6-3 cells expressing GFP-TPP1 OB-LacI and mCherry-telomerase. Fluorescence images showing the localization of GFP-TPP1-OB-LacI and mCherry-hTERT fusion proteins in cell nuclei stained by DAPI. Cells were fixed, permeabilized and stained with DAPI. The intrinsic GFP- and mCherry fluorescence was used to detect GFP-TPP1-OB-LacI and mCherry-hTERT (scale bar = 5 μm). Figure courtesy of Jens Schmidt.

To determine whether mutations introduced in the OB-fold domain of TPP1 can compensate for the mutations introduced in hTERT in vivo, we carried out the LacI-assay with E215K TPP1-OB. As shown in Figure 4.12B and C, the co-localization of wild-type telomerase with the E215K TPP1-OB-fold domain was greatly reduced; instead, wild-type telomerase localized to many foci distinct from the LacI site, presumably telomeres. In contrast, introduction of the E215K mutation significantly increased the co-localization of K78E telomerase with the TPP1-OB-fold domain (P < 0.05). Thus the E215K mutation in
the TPP1-OB-fold compensates for the presence of the K78E mutation in hTERT in vivo. Importantly, E215K TPP1 had no effect on the localization of R132E and K78E;R132E telomerases, demonstrating that E215K specifically compensates for the presence of the K78E mutation in hTERT (Figure 4.12B,C). If another protein were bridging telomerase and TPP1, any combination of two defective mutations should be even more defective. Thus, finding a compensatory double mutant demonstrates that telomerase is recruited to telomeres by a direct interaction between the TEN-domain of telomerase and the TPP1-OB-fold domain in part by a contact formed between K78 in the TEN-domain and E215 in the TPP1-OB-fold domain.

**TPP1 E215K rescues telomere maintenance in cells expressing K78E hTERT**

To address whether TPP1 E215K could rescue recruitment of hTERT K78E to telomeres, we transduced cell lines stably expressing WT and K78E mCherry-hTERT with retrovirus expressing wild-type (WT), E169K, and E215K TPP1-FLAG alleles. The resulting cell lines expressed both TPP1-FLAG and mCherry-hTERT proteins (Figure 4.14A-B). Importantly, retroviral transduction was carried out after substantial telomere erosion had occurred due to the expression of K78E hTERT (Figure 4.7C, transduction carried out after 8 weeks).
Figure 4.14. TPP1 E215K rescues telomere maintenance in cells expressing hTERT K78E. A. Western blot probed for TPP1-FLAG and for Actin as a loading control, showing TPP1 expression in lysates of parental HeLa cells and cell lines stably expressing TPP1-FLAG and mCherry-hTERT variants. B. Western blot probed for hTERT and for Actin as a loading control, showing hTERT expression in lysates of cell lines stably expressing TPP1-FLAG and mCherry-hTERT variants. C. Telomeric restriction fragment Southern blot of cell lines stably expressing K78E mCherry-hTERT and TPP1-FLAG variants over the time course of 6 weeks. Figure courtesy of Jens Schmidt.

To test whether the expression of E215K TPP1 rescued telomere maintenance in cells expressing K78E hTERT, we carried out TRF analysis by Southern blot (Figure 4.14C). Telomere length in the cell line expressing only K78E hTERT was constant at ~3.4 kb during the 6-week experiment. Expression of WT TPP1 led to an increase of telomere length to ~6.2 kb. Importantly, expression of E215K TPP1 triggered a more pronounced increase in telomere length to ~7.7 kb over the same time period, while E169K gave lead to a subtle increase to ~4.9 kb. These observations demonstrate that expression of E215K TPP1 in
cells expressing K78E hTERT rescues telomere length maintenance, most likely by driving telomerase recruitment to telomeres.

Discussion

Telomerase recruitment to telomeres is a crucial step in telomere maintenance. Here, we demonstrate that K78 and R132 within the TEN-domain of hTERT are critical residues that mediate the direct interaction of telomerase with the TEL-patch of TPP1, recruiting telomerase to telomeres (Figure 4.15). Mutation of either interaction partner results in sequestration of telomerase in Cajal bodies and failure to elongate telomeres in vivo. Our observations provide a molecular mechanism explaining the failure of previously described N-DAT mutants to function in vivo. Additionally, the identification of the interface between telomerase and the telomere will allow more targeted approaches to alter telomerase recruitment as a potential therapeutic approach for human diseases.
Specific hTERT TEN-domain amino acids are necessary for telomerase recruitment to telomeres

During the S-phase of the cell cycle, telomerase moves from Cajal bodies to telomeres to counteract the progressive shortening of the telomere that occurs during DNA replication (Jady et al. 2006; Tomlinson et al. 2006; Cristofari et al. 2007; Stern et al. 2012). The regulatory mechanisms underlying this process are not well understood, in part because the necessary molecular interfaces are not fully defined. On the telomere side, TPP1 is well established as being necessary for the recruitment of telomerase (Abreu et al. 2010; Nandakumar et al. 2012; Sexton et al. 2012; Zhong et al. 2012).
Localization of telomerase to an artificial non-telomeric focus by the TPP1 OB-domain (Zhong et al. 2012) suggests that the TPP1 OB-domain is the minimal sufficient telomerase recruitment module of the shelterin complex. The TEL-patch provides a molecular surface that mediates telomerase binding, as single mutations in the TEL-patch result in loss of telomere recruitment and of RAP stimulation of telomerase by TPP1 (Nandakumar et al. 2012; Sexton et al. 2012; Zhong et al. 2012).

While the telomeric half of the interface required for telomerase recruitment to telomeres is well understood, the molecular determinants on telomerase required for telomere localization were thus far ill defined. Several lines of evidence, including naturally occurring disease-associated alleles of hTERT (V144M) (Tsakiri et al. 2007) and synthetic mutations (G100V, R132D, N-DAT), have implicated the TEN-domain of hTERT in telomerase recruitment to telomeres (Armbruster et al. 2001; Zaug et al. 2010; Stern et al. 2012). Unfortunately, due to the involvement of the TEN-domain in catalysis and RAP, many of these mutations also alter the enzymatic activity of telomerase. Thus, TEN-domain mutants can have pleotropic effects and may not specifically disrupt the telomere-telomerase interface (Zaug et al. 2010; Zaug et al. 2013). Our results demonstrate that a region in the TEN-domain including K78, R132, and V144 is required for telomerase recruitment in vivo and POT1-TPP1 mediated RAP stimulation in vitro, albeit to varying degrees. The differences in RAP stimulation may reflect the importance of the respective residue in an interaction with TPP1. The recruitment defects and failure to maintain telomeres in vivo occur in the context of wild-type levels of activity, suggesting that mutants described in this study are separation-of-function alleles that dissociate enzymatic activity from recruitment. Importantly, the recruitment defects of K78E and R132E telomerases recapitulate those of TEL-patch mutant TPP1 proteins (Zhong et al. 2012; Nandakumar and Cech 2013). The interaction between the TEL-patch of TPP1 and the TEN-domain of hTERT provides a molecular mechanism to explain the failure of N-DAT mutations to immortalize cells.
Telomerase recruitment to telomeres in human and other organisms

Our observation that charge-swap mutations on the TEN-domain of hTERT and the TEL-patch of TPP1 restore telomerase recruitment indicates that these elements interact directly; for other examples see (Jucovic and Hartley 1996; Tansey and Herr 1997; Pennock et al. 2001). If instead there were an “adapter” protein bridging the two elements, the combination of two individually-deleterious mutations would lead to an additive loss of function (Figure 4.8A). We note that hTERT K78E and TPP1 E215K lead to similar reductions in RAP stimulation when combined with wild-type TPP1 and wild-type hTERT, respectively. This observation adds additional support to the model that hTERT K78E and TPP1 E215K eliminate reciprocal residues of the same molecular interaction. In contrast, the V144M mutation in hTERT and the E215K mutation in the TEL-patch of TPP1 display an additive reduction in RAP stimulation, indicating that they affect separate components of the telomerase-TPP1 interface.

In other organisms, however, the association of telomerase with the telomere occurs through different mechanisms. For example, a bridging factor Ccq1 appears to be necessary for recruitment of telomerase to the telomeric protein Tpz1 (TPP1 homolog) in *Schizosaccharomyces pombe* (Miyoshi et al. 2008; Moser et al. 2011). A homologue of Ccq1 has not been identified in humans (Nandakumar and Cech 2013). Another variation occurs in *Saccharomyces cerevisiae*, where Est3 shares structural conservation with TPP1 (Yu et al. 2008; Rao et al. 2014). Est3 is part of the telomerase holoenzyme instead of the telomeric cap (Hughes et al. 2000). Est3 is thought to make direct contacts with the TEN-domain of Est2, the yeast TERT (Friedman et al. 2003; Talley et al. 2011; Yen et al. 2011). However, Est3 appears to stimulate telomerase activity rather than act as a recruitment factor (Talley et al. 2011). Recruitment is instead mediated by the interaction of the Est1 subunit of the telomerase holoenzyme and Cdc13 at the telomere (Evans and Lundblad 1999), and charge-swap mutations indicate that this interaction is direct (Pennock et al. 2001). Thus, in budding yeast the interaction between a TPP1-like
OB-domain protein (Est3) and the TEN-domain of TERT (Est2) is conserved, but the recruitment mechanism differs significantly from that in humans.

In addition to the interaction between TPP1 and hTERT in humans, other factors contribute to the productive recruitment of telomerase to the telomere. Depletion of TIN2 from shelterin (Abreu et al. 2010) and perturbation of the TCAB1-hTR interaction (Stern et al. 2012) both result in a reduction of telomerase association with the telomere. In spite of the aforementioned defects, these proteins likely play important but indirect roles in telomeric recruitment. TCAB1 is necessary for telomerase maturation and trafficking to Cajal bodies (Venteicher et al. 2009), while TIN2 is required for TPP1 localization to telomeres (Abreu et al. 2010).

Previous work also suggested that the C-DAT region of the CTE of hTERT contributed to telomerase recruitment (Banik et al. 2002; Zhong et al. 2012). However, synthetic C-DAT mutants have severe activity defects (Huard et al. 2003; Jurczyluk et al. 2011). In addition, disease-associated mutations in and near C-DAT have varying effects on activity and cellular localization of telomerase. E1117X has ~10% of wild type activity and appears to be sequestered to Cajal bodies in vivo (Tsakiri et al. 2007; Zhong et al. 2012); in contrast, F1127L has reasonable activity (~70%) and localizes to telomeres, and it is stimulated by POT1-TPP1 (Zhong et al. 2012; Zaug et al. 2013). Thus, CTE mutants do not have clear recruitment defects dissociated from activity loss, despite their importance based on disease association. In contrast, the observation that a mutant TPP1-OB-domain increases the localization of a mutant hTERT to a non-telomeric locus in vivo, while it strongly decreases the association with wild-type hTERT, demonstrates that a direct TEN-domain-TEL-patch interaction is necessary and may be sufficient for telomerase recruitment to telomeres in humans.
The interface of telomerase recruitment as a therapeutic target

The identification of a direct protein-protein interaction surface between telomerase and the telomere is key to identifying modulators of telomerase recruitment as potential therapeutic agents. Multiple disease mutations associated with IPF -- P33S, L55Q, Pro112ProfsX16, and V144M -- cluster in TEN-domain of hTERT. Mutation carriers have significantly shorter telomeres than non-carrier relatives (Armanios et al. 2007; Tsakiri et al. 2007). These mutations have variable impacts on telomerase enzymatic activity (Armanios et al. 2007; Tsakiri et al. 2007; Zaug et al. 2013). Importantly, V144M is defective in TPP1 mediated RAP stimulation in vitro (this study) and fails to localize to telomeres in vivo (Zhong et al. 2012). Given the propensity of both synthetic and IPF- associated mutations in the TEN-domain of hTERT to disrupt telomere localization, it is likely that some cases of IPF are directly caused by a decrease in telomerase recruitment to telomeres. Agonists of the TEL-patch-TEN-domain interaction that compensated for these mutations and attenuated telomere shortening associated with the disease might be pharmaceutically important.

In contrast to the loss of telomerase function associated with IPF, reactivation of telomerase is a hallmark of most human cancers. Inhibitors that disrupt telomerase recruitment to telomeres would provide an additional approach to target telomerase activity in cancer cells. In support of this idea, disruption of the TEL-patch diminishes cell growth and triggers apoptosis in HeLa cells, and this effect is exacerbated in combination with a small molecule inhibitor of telomerase activity (Nakashima et al. 2013). Our finding that the TEN-domain of hTERT interacts directly with the TEL-patch of TPP1 to bring telomerase to the telomere defines a key interface and provides a direct target for the design of novel therapeutic inhibitors of telomerase action.
Materials and Methods

Telomerase purification

Telomerase was overexpressed in HEK293T cells and purified as previously described (Sauerwald et al. 2013). Briefly, 1 ml whole cell lysates of 50-60 x 10^6 HEK293T cells overexpressing ZZ-TEV-3xFlag-hTERT variants and hTR were incubated with ~350 µl IgG-Sepharose at 4°C for 3-4 hours. Following a wash with 50 ml wash buffer (20 mM HEPES-KOH pH 7.9, 300 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1% Triton X-100, 10% glycerol), telomerase was eluted from with beads by cleavage with TEV-protease. The telomerase bound to IgG-Sepharose was incubated with ~500 µl of elution buffer (20 mM HEPES-KOH pH 7.9, 150 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1% Triton X-100, 10% glycerol) supplemented with 5 µl of Act-TEV (Life technologies) and 5 µl RNAsin+ (Promega) overnight at 4°C. Telomerase-containing eluate was aliquoted, snap frozen in liquid nitrogen, and stored at -80°C until use.

To purify endogenous telomerase from HeLa cells and HeLa cell lines overexpressing mCherry-hTERT variants, telomerase was immuno-purified using a sheep polyclonal antibody (a kind gift from S. Cohen) as previously described (Cohen and Reddel 2008). Briefly, cell lysates of 1x10^6 cells were incubated with 40 µg of anti-hTERT antibody, which was captured using protein-G agarose. Telomerase was eluted using the peptide antigen used to raise the antibody. Eluates were used for direct telomerase extension assays.

Telomerase was produced in RRLs as previously described (Zaug et al. 2013) using ProA-tagged hTERT and purified using the same protocol as telomerase from HEK293T cells described above.
**Western Blot**

Western blots were carried out using 4-12% polyacrylamide Bis Tris Glycine gels (Life Technologies) and antibodies against hTERT (Abcam, Ab32020, 1:1000), beta-Actin (Sigma, A5441, 1:5000) and an HRP-conjugated FLAG-antibody (Sigma, A8592, 1:1000). Secondary antibodies (Jackson) were used at 1:5000. Detection was carried out using SuperSignal Western Pico Chemiluminescence substrate (Thermo Scientific).

**POT1-TPP1N purification**

Cell pellets from insect cells overexpressing GST-POT1 and *E. coli* cells overexpressing 6xHis-SUMO-TPP1N were lysed by sonication in lysis buffer (PBS supplemented with 250 mM KCl, 1 protease inhibitor tablet (Thermo), 1 mM PMSF). Lysates were cleared by centrifugation at 40000xg for 35 min at 4°C. Equal volumes of the insect-cell lysates expressing GST-POT1 and bacterial lysates expressing 6xHis-SUMO-TPP1N were combined and incubated with 1 ml of glutathione-sepharose resin for 1 hour at 4°C. After washing three times with 50 ml of GST-wash buffer (PBS supplemented with 250 mM KCl, 1 mM DTT, and 1 mM PMSF), the POT1-TPP1N complex was eluted with 3 ml of GST-elution buffer (50 mM Tris pH 8.1, 75 mM KCl, 10 mM glutathione). The elutions were supplemented with 0.5% w/w PreScission protease and SUMO-protease and incubated on ice for 0.5-1 hour, followed by size-exclusion chromatography on a Superdex 200 16/60 column (GE Healthcare) in gel filtration-buffer (50 mM Tris pH 7.0, 150 mM NaCl, 1 mM DTT). Size-exclusion fractions were pooled, concentrated, supplemented with 10% glycerol v/v, snap-frozen in liquid nitrogen and stored at -80°C until use.

**Direct telomerase activity assay**

Direct telomerase activity assays were carried out as previously described (Zaug et al. 2013), with slight modifications. The final 20 µl reactions contained 2 µl eluted telomerase extract, 500 nM
primer, 500 nM POT1-TPP1 complex, and 150 mM KCl. Direct telomerase assays were quantified as previously described (Zaug et al. 2013), except the summed counts incorporated into extension products were normalized to both the hTERT levels (determined by western blot) and the loading control and then normalized to WT telomerase. Processivity was measured using the decay method previously described (Latrick and Cech 2010). Briefly, counts for repeats 1-20 were corrected for the number of dGTP nucleotides incorporated. The natural log of the counts left behind in each repeat was graphed versus repeat number and fit by linear regression. The slopes of the linear regressions were compared and normalized to WT telomerase. RAP stimulation by PT, for direct extension assays with POT-TPP1, was measured in the same manner as processivity except values were normalized to WT telomerase with WT POT1-TPP1 (i.e. RAP stimulation by PT = processivity of mutant telomerase with TPP1/processivity of WT telomerase with WT POT1-TPP1). The processivity in the absence of POT1-TPP1 was not included the calculation, because (1) it did not vary greatly among the mutants (Table 4.1), and (2) it was so small relative to the processivity in the presence of POT1-TPP1 that dividing by this distorted the calculations and led to irreproducibility. An alternative fraction method of quantitation was also used to determine RAP stimulation by PT, calculated as the fraction of counts in bands 9 and above divided by the total sum of counts incorporated, normalized as described for the decay method. Telomerase from stable cell lines was immuno-purified from identical number of cells and was subjected to direct telomerase assays as previously described (Cohen and Reddel 2008). Activity was determined by dividing the total counts by the loading control and normalized to the activity level of cells overexpressing WT hTERT. Telomerase purified from RRLs was assayed using 10 µl of eluate under identical conditions as telomerase from HEK293T cells.
Northern Blot

Northern blots were carried out as previously described, using 5 separate probes for hTR and 2 probes for RNase P (Xi and Cech 2014).

Molecular cloning

The mCherry-hTERT vector was generated by restriction cloning of the hTERT gene into a modified pBabe-puro vector (a kind gift from Iain Cheeseman) containing the N-terminal mCherry-tag using Sall/EcoRI. GFP-TPP1-OB-LacI was cloned by restriction cloning of the TPP1-OB-fold domain into a modified pBabe-Blas vector (a kind gift from Iain Cheeseman) containing the GFP and LacI sequences using Xhol/EcoRI. All point mutations in hTERT and TPP1 were introduced using the Quickchange II mutagenesis kit (Agilent). The presence of the mutations was verified by Sanger sequencing.

Cell culture

All human cell lines were cultured at 37ºC, 5% CO₂ in growth medium (Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM glutamax (Life Technologies)), 100 units/ml penicillin, and 100 µg/ml streptomycin. The growth medium for the U2OS 2-6-3 cell line (a kind gift from David Spector) was additionally supplemented with 100 µg/ml hygromycin B to maintain the LacO array.

Transient transfection of human cells

All transient transfections of human cells were carried out using Lipofectamine 2000 (Life Technologies) according to the instructions of the manufacturer.
Generation of stable cell lines

Retroviruses were generated by transfection of pBABE vectors encoding mCherry-hTERT variants and a puromycin resistance gene along with a vector encoding the coat protein VSV-G into the packaging cell line 293-GP. Virus-containing supernatants were harvested 72 hours post-transfection, filtered through a 0.22 µm filter, and supplemented with 8 µg/ml polybrene. HeLa cells were incubated with virus-containing supernatant for 16 hours. After infection, the growth medium was replaced with fresh medium containing 1 µg/ml puromycin for mCherry-hTERT constructs and 1 µg/ml blasticidin for TPP-FLAG constructs to select for transduced cells. To ensure maintenance of the transgenes, cell lines were kept under selection with 1 µg/ml puromycin and 1 µg/ml blasticidin (TPP1 rescue only) for the duration of the experiments.

Immunofluorescence and fluorescence

IF experiments were carried out as previously described (Nandakumar et al. 2012), using the following antibodies: mouse monoclonal anti-TRF2 (Imgenex; IMG-124A, 1:500), rabbit polyclonal anti-coilin (Santa Cruz; sc-32860, 1:100), rat monoclonal anti-mCherry (Life Technologies; M11217, 1:1000), and rabbit polyclonal anti-RAP1 (Novus Biologicals, NB-100-292, 1:500). Secondary antibodies (Life Technologies, Abcam) were pre-absorbed to prevent cross-reactivity between rat and mouse antibodies.

Telomere length analysis

Telomeric restriction fragment length analysis by Southern blot was carried out as previously described (Nandakumar et al. 2012). Briefly, 1.5 µg of genomic DNA extracted from human cell lines was digested with RsaI/HinfI and separated by gel electrophoresis using a 0.8% 1xTBE agarose gel for a total of 1100 V-hours. DNA was transferred onto a Hybond N+ membrane (GE) and telomeric restriction fragments were detected using a radiolabeled (TTAGGG)$_4$ probe. To determine mean telomere length,
lane intensity profiles were extracted using ImageQuant TL and fit to a Gaussian using Kaleidagraph. The mean of the Gaussian distribution was used as telomere length of the respective sample.

**LacI recruitment assay**

Plasmids encoding mCherry-hTERT, hTR, and GFP-TPP1-OB-LacI were co-transfected into U2OS 2-6-3 cells grown on coverslips as described above. Cells were fixed for 5 min in PBS supplemented with 0.4% formaldehyde followed by permeabilization with PBS supplemented with 0.2% (v/v) Triton X-100 for 5 min. Finally, coverslips were embedded in DAPI containing mounting media (Vectashield). Imaging was carried out visualizing the intrinsic fluorescence of the mCherry- and GFP-fusion proteins without additional staining.

**Microscopy**

All images were acquired on a Deltavision Core deconvolution microscope (Applied Precision) using a 60x 1.42NA PlanApo N (Olympus) or 100x UPLanSApo 1.4NA (Olympus) objective and a sCMOS camera. 20 Z-sections with 0.2 µm spacing were acquired for each image with identical exposure conditions within each experiment. For analysis, maximum intensity projections were generated and scaled identically. For presentation in figures, representative images were deconvolved (where indicated), followed by generation of maximum intensity projections of 10 Z-sections, which were scaled identically for all experimental conditions.
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Appendix A: TEV protease elution of Mini T telomerase

My initial project in the lab was to test the Ku recruitment hypothesis for yeast telomerase in vitro. Because Ku was thought to simultaneously bind TLC1 RNA and duplex telomeric DNA, Ku might help recruit yeast telomerase to telomeres (Peterson et al. 2001; Stellwagen et al. 2003; Fisher et al. 2004). I was specifically testing whether Ku-bound duplex telomeric DNA substrates were preferentially recruited to yeast telomerase compared to single-stranded DNA substrates. Ultimately, the hypothesis that Ku serves as a simple bridging factor between the telomere and telomerase turned out to be unlikely. Namely, Ku can bind to both RNA and DNA but it does so in a competitive and mutually exclusive manner suggesting that Ku is unable to recruit telomerase simply by simultaneously bridging telomerase and the telomere (Pfingsten et al. 2012). However, during my investigation a number of other interesting developments and findings were made. Highlights of the Mini T telomerase-Ku project are discussed in appendices A-D.

The large size of the TLC1 RNA (~1.1 kb) prompted David Zappulla to design a minimal TLC1 RNA that supported telomere maintenance in vivo and that could also be transcribed and reconstituted with in vitro translated Est2 to generate an active “Mini T” telomerase (Zappulla et al. 2005). I used Mini T telomerase for the majority of yeast direct extension assays. One drawback of the original Est2-Protein A tagged construct was that active telomerase could not be eluted from the beads following immunopurification. Extension assays had to be carried out with telomerase bound to IgG beads, making reproducible quantitative measurements difficult. Due to the variability associated with pipetting beads between experimental samples, I set out to produce an elutable Mini T telomerase construct.
To generate a cleavable Est2 construct, the plasmid encoding a cleavable hTERT construct (pVan145) (Sauerwald et al. 2013) was PCR amplified to generate a DNA fragment encoding a protease-cleavable 3X Flag-Tag linker for insertion. The PCR product from the linker amplification was sub-cloned at BamHI and Pst1 sites, between the Pro-A Tag and Est2, in a derivative of pKF409 (Friedman and Cech 1999). Prior to insertion of the linker, the DNA encoding the first ten amino acids of Est2 was removed from the N-terminal end of the Pro-A tag by quick-change mutagenesis (Agilent). An attempt was made to introduce the 3X Flag-TEV cleavable linker by quick-change mutagenesis; however, this proved unsuccessful. Sub-cloning of the linker PCR products re-introduced DNA encoding the first ten amino acids of Est2 to the N-terminal end of Est2, following the TEV cleavage site. The cloning strategy also introduced a non-native 8 amino acid linker between the 10 and 11th amino acids of Est2, which could be removed, but its removal was not necessary to complete my experiments.

*In vitro* translation and reconstitution of Mini T telomerase were carried out as previously described (Zappulla et al. 2005), with minor modifications. Mini T telomerase RNA was prepared by T7 polymerase run-off transcription from a FokI-linearized plasmid, and purified on a denaturing 10% polyacrylamide gel. *In vitro* translations were carried out using the TNT coupled reticulocyte lysate systems (Promega), according to the manufacturer’s suggested protocol. Translations were carried out at 30°C for 2 hours with final concentrations of 50 nM Mini T RNA and 50 ng/μl Est2 plasmid. Note that a 50 μl translation generated enough telomerase for one extension assay. 50 μl translations were captured on 10 μl of IgG Sepharose 6 Fastflow beads (GE Healthcare); beads were pre-equilibrated in 20 volumes of TMG 150 (10 mM Tris (pH 8.0), 1 mM MgCl2, 150 mM NaCl, 5% (v/v) glycerol, 0.1% Triton-X 100), and Mini T telomerase was captured by nutating at 4°C for 4 hours. Following capture, beads were washed in batch four times with 15 volumes of TMG 150, and beads were pelleted at 800 x g prior to removing washes. Beads were resuspended in 15 μl of TMG 150 per 50 μl translation, supplemented
with DTT at a final concentration of 1 mM. Mini T telomerase was cleaved overnight at 4°C with 3 U of AcTEV protease per translation (Life Technologies). Soluble Mini T telomerase was recovered by filtration through a 0.2 μM sodium acetate spin filter (ThermoFisher) at 1000 x g for 1 min. Cleaved Mini T telomerase was aliquoted, snap frozen in liquid nitrogen and stored at -80°C. The higher monovalent salt concentration was necessary for the recovery of soluble Mini T telomerase. Efficient cleavage of the Mini T telomerase was not obtained in less than 100 mM NaCl (data not shown); cleavage could likely also be carried out in KCl as it is for human telomerase. Mini T activity decreases at increased monovalent salt concentrations (approximately 6 fold between 50 and 200 mM monovalent). TEV-cleaved Mini T telomerase appears to have roughly the same activity in both NaCl and KCl at a given concentration (data not shown).

Mini T Telomerase extension reactions were carried out in 1X reaction buffer: 80 mM Tris pH 8.0, 75 mM NaCl, 5 mM MgCl₂, 1% glycerol, 1 mM spermidine, and 1 mM DTT; note monovalent salt contributed by telomerase lysate as Mini T telomerase was cleaved in 150 mM NaCl and diluted two-fold in the final reaction to a concentration of 75 mM. Other final reaction conditions were not significantly impacted by the lysate. Final nucleotide concentrations in the assay were 10 μM dATP, dCTP, dTTP, and 0.54 μM α-32P-dGTP (Perkin Elmer). 15 μl of Mini T telomerase eluate was used per reaction, final reaction volumes were 30 μl. Duplex primers were pre-annealed in 1X telomerase buffer, and a slight excess (5-20%) of the non-substrate strand was added to ensure that the substrate strand was duplexed. Reactions were initiated by addition of 200 nM primer, and extension was allowed to proceed for 20 min at 30°C, unless otherwise noted in figure legends. Reactions were stopped in 10 μl of 3.6 M sodium acetate (pH 5.2), 20 μg of glycogen. Reaction volumes were adjusted to 100 μl with H₂O and precipitated with 0.3 mL of 100% ethanol and then washed one time with 0.4 mL 70% ethanol. The reaction products were resuspended in equal parts H₂O and 2X formamide loading dye (0.5X TBE,
93.5% Formamide, 30 mM EDTA, 0.5% xylene cyanol and bromophenol blue). Telomerase extension products were electrophoresed on a 10% agarose denaturing gel (1X TBE, 7 M urea) at a constant power of 90 watts for approximately 1.5 h. Gels were visualized by phosphoimagery and then quantitated using ImageQuant TL (GE Healthcare).

In order to identify initial velocity conditions for Mini T telomerase, a time course at varying Mini T telomerase concentrations was carried out. Product formation increased as a function of both enzyme concentration and time (Figure A1A). To ensure that reactions were carried out in conditions with excess primer, a primer titration at a fixed time point was carried out. Product formation appeared to plateau between ~30-130 nM primer indicating that 200 nM primer is dependably saturating under the experimental conditions used for Mini T extension assays (Figure A1B, data not shown). Collectively, these experiments demonstrate that TEV-eluted Mini T telomerase is an active and well behaved enzyme where product formation increases with time, enzyme, and substrate concentration. In theory, this specific TEV elution should increase the purity of Mini T telomerase and be useful in applications to make quantitative measurement of enzymatic parameters. Finally, it should be possible to generate yeast strains harboring genomic copies of cleavable Est2, allowing for a more robust purification of endogenous budding yeast telomerase.
Figure A1. Product formation by TEV-eluted Mini T telomerase increases with time, enzyme concentration, and substrate concentration. A. Mini T telomerase reaction time course at three concentrations of enzyme corresponding to 15 (1X), 7.5 (0.5X) and 3.75 μl (0.25X) of Mini T lysate. Time in sec above gel. Primer +2 indicates product corresponding to +2 nt addition. Corresponding loading control shown in panel below gel. Time course was carried out at 4ºC. B. Primer titration at two concentrations of Mini T telomerase as in A. Reaction also carried out at 4ºC for 25 min. Primer concentration denoted above gel, Primer +2 and Loading Control same as A.
Appendix B: The minimal 3’ overhang length is 6-8 nucleotides for extension of telomeric substrates by Mini T yeast telomerase

Ku is predominantly known as a double-stranded DNA end-binding protein (Dynan and Yoo 1998; Walker et al. 2001). In order to test the impact of Ku-bound substrates on Mini T telomerase action, suitable duplex substrates first had to be identified. Many of the previous studies of direct yeast telomerase extension utilized single-stranded DNA oligos, and the overhang length requirements for S. cerevisiae telomerase have not been tested to our knowledge. To determine the minimal 3’ overhang length for a duplex telomeric DNA, a substrate primer was annealed to a series of complementary oligos of increasing length and subjected to direct extension by Mini T telomerase (Figure B1A-B). In this experiment, the annealed oligos formed a three-way junction near the 5’ end of the substrate strand (Figure B1A). The purpose of this three-way junction was to constrain the position of Ku on duplex substrates in subsequent experiments, although Ku was not used in this experiment. The sequence and structure for the duplex portion of the oligo bound to the human Ku heterodimer was previously described (Walker et al. 2001).
Figure B1. Minimum DNA 3’ overhang length requirement for Mini T telomerase. A. Schematic of the secondary structure of primers containing the three-way junction used in the single-stranded overhang experiment. Black circles represent the non-telomeric sequences of the duplex crystalized with human Ku (Walker et al. 2001) or C-strand telomeric sequence, while red
circles represent the G-strand 14 nt telomeric sequence (5’ GTGTGGTGTGGG 3’) which is a substrate for telomerase. B. Mini T telomerase direct extension assay to test the minimal 3’ overhang length for duplex substrates. Labels above gel correspond to substrates represented in A. Primer +2 indicates product corresponding to +2 nt addition. Loading control denoted by arrow at bottom of gel. C. Total product formation normalized to the loading control and amount of product formation for the duplex primer with a 14 nt 3’ overhang. Averaged data plotted ± standard deviation (n = 4).

Preparation of TEV-cleaved Mini T telomerase and direct extension assay conditions were the same as the conditions as described in appendix A. Product formation for substrates with 3’ overhangs ranging from 8 to 12 nt was increased compared to substrates with 14 nt overhangs (Figure B1B,C). Substrates with overhangs of 8-12 nt were utilized with similar efficiency. However, product formation steadily decreased for substrates with overhangs of 6 and 4 nt and was virtually undetectable for duplexes with shorter overhangs (Figure B1B,C). As expected, fully duplexed oligos are not extended by Mini T telomerase. The roughly two-fold decrease in product formation between substrates with 8 and 6 single-stranded tails suggests that substrates must have minimal 3’ overhang lengths of 6-8 nt to be efficiently extended by S. cerevisiae telomerase. The decreased efficiency in product formation for the single-stranded and 14 nt primers may reflect the propensity of substrates with greater lengths of single-stranded DNA to form intramolecular secondary structures, which in turn lower the efficiency of hybridization to the telomerase template. In addition to verify that the three-way junction of the primers used in this study did not influence the experimental results, a similar experiment was carried out with primers lacking a three-way junction. An approximately six-fold decrease (93% to 16%) in normalized product formation was noted between duplex substrates (lacking a three-way junction) with overhang lengths of 8 and 5 nt, respectively (data not shown). Thus, the presence of the three-way junction did not impact my conclusion regarding the minimal overhang length requirement.
Minimal 3´ overhang length requirements have been determined for other telomeres. Human telomerase requires a single-stranded tail of 6 nucleotides (Rivera and Blackburn 2004). *Euplotes aediculatus* and *Tetrahymena thermophila* telomerases have minimal 3´ overhang length requirements of 4-6 nt and ~20 nt respectively (Lingner and Cech 1996; Wang and Blackburn 1997). Thus, the overhang requirement of 6-8 nt for *S. cerevisiae* telomerase is quite similar to the requirement in other organisms. This similarity suggests that eukaryotic telomerases recognize their substrates in a conserved manner. The single-stranded telomeric overhang in *S. cerevisiae* is 12-15 nt during G1 and 30-100 nt during late S/G2 (Wellinger and Zakian 2012) My in vitro data suggest that telomeric DNA in yeast cells is inherently competent for extension throughout the cell cycle. In agreement, deletion of Rif1 or Rif2 result in in appropriate telomere extension during G1 (Gallardo et al. 2011). Therefore, my data are consistent with a model in which telomeric capping proteins act as negative regulators of telomerase and prevent unrestricted extension of telomeres throughout the cell cycle.
Appendix C: Length of the 3’ overhang dictates the efficiency of telomerase extension for Ku-bound substrates

As mentioned in the introduction to appendix A, the original goal was to test whether Ku-bound DNA substrates were preferentially recruited to and extended by Mini T telomerase. In preliminary experiments with Ku-bound substrates I found that Ku did not appear to impact telomerase action (data not shown). There are a number of explanations why Ku did not impact telomerase action on a duplex primer. One possibility is that Ku is displaced from the duplex upon telomerase binding (e.g., slides off of the other end of the duplex). Another possibility is each duplex DNA contains multiple binding sites: one on each end of the duplex and possibly more if Ku slides inward on the duplex. Thus an impact on telomerase action may be obscured by multiple binding modes. In order to circumvent these issues, I switched to a duplex primer that contained a three-way junction on one end of the duplex to generate a substrate primer with one Ku binding site at the single-stranded to double-stranded DNA transition, as discussed in appendix B (Walker et al. 2001).

In order to test the impact of Ku on telomerase action, excess Ku was complexed with a number of primers with differing duplex and single-stranded overhang lengths (Figure C1). For these experiments the Ku heterodimer was purified as described in the materials and methods section for Chapter 2. This experiment was carried out using the experimental conditions described in appendix A, except primers were complexed with a three-fold molar excess of Ku (600 nM Ku). Buffer conditions for the samples lacking Ku were adjusted by the addition of an equal volume of Ku storage buffer (30 mM Tris (pH 8.0), 200 mM NaCl, 20% glycerol, 1 mM EDTA, 2 mM DTT). Ku was complexed with primers for 1 hour at room temperature.
Figure C1. Ku-bound substrates must have a sufficiently long 3' overhang to be efficiently extended by Mini T telomerase. A. Mini T telomerase direct extension assay testing for the extension of Ku-bound duplex DNA substrates. Substrate primer length, primer number corresponding to substrate schematics in B, and addition (+) or absence (-) of Ku denoted above gel. Primer +2 indicates product corresponding to +2 nt addition. Loading control denoted by arrow at bottom of gel. B. Schematic of the secondary structure of primers containing the three-way junction used in the single-stranded overhang experiment. Black circles represent the non-telomeric sequences of the duplex crystalized with human Ku (Walker et al. 2001) or C-strand telomeric sequence, while red circles represent the 14 nt G-strand telomeric sequence (5' GTGTGGTGTGTGGG 3') which is a substrate for telomerase. Note primers 3-5 have two tandem repeats of the telomeric repeat sequence.

Single-stranded substrates of either 35 or 49 nt were efficiently extended by the presence of telomerase both in the absence and presence of Ku (lanes 1,2 and 5,6, Figure C1A). Telomerase also extended duplex DNA substrates with 3' single-single stranded overhangs in the absence of Ku (lanes 3,
7, 9, Figure C1A), consistent with the minimal overhang length requirements presented in appendix B. In contrast, productive telomerase extension decreased when duplex substrates with shorter 14 nt overhangs were complexed with Ku (lanes 4, 8 Figure C1A). There was some visible extension of primer 4 in the presence of Ku suggesting that the longer duplex of primer 4 compared to primer 2 may relieve some Ku-dependent inhibition of telomerase extension. In addition, some lower molecular weight products were apparent when Ku was added to the reaction. The appearance of spurious products may be due to trace amounts of contaminating nuclease (discussed below), and I have also noticed a telomerase-independent terminal transferase activity when Ku was complexed with duplex DNA substrates that lack 3’ overhangs. The transferase activity appears to originate from the RRLs and it is telomerase independent because the activity was not sensitive to RNase treatment (data not shown). Finally, increasing the 3’ overhang length alleviated the inhibition of telomerase extension in the presence of Ku (lanes 9,10 Figure C1A). Collectively these data suggest that the 3’ overhang length is the critical determinant of whether Mini T telomerase is able to extend a Ku-bound substrate.

In order to verify that duplex substrates were bound by Ku under telomerase assay conditions, dual telomerase and electrophoretic mobility shift assays were carried out (Figure C2). I carried out this experiment prior to the generation of TEV-cleavable Mini T telomerase, and so the telomerase assays were carried out with telomerase bound to IgG sepharose beads. Mini T telomerase was produced in RRLs as described for elutable telomerase in Appendix A, with a few minor changes. Beads were pre-equilibrated and washed in TMG (10 mM Tris (pH 8.0), 1 mM MgCl₂, 5% (v/v) glycerol, 0.1% Triton-X 100). Mini T telomerase was captured by nutating at 4ºC overnight. After washes, beads were resuspended in a dilute slurry and aliquoted evenly, and then snap frozen as a 1:1 TMG:bead slurry in liquid nitrogen and stored at -80ºC. Conditions for the telomerase assays carried out on beads were also completed as described in appendix A, with minor modifications. The 1X reaction buffer consisted of 80
mM Tris (pH 8.0), 100 mM NaCl, 5 mM MgCl₂, 1% glycerol, 1 mM spermidine, and 1 mM DTT. 7 μl of Mini T telomerase on IgG sepharose beads were used per reaction, TMG buffer was removed from beads prior to extension assay and the final reaction volumes were 30 μl.

**Figure C2. Duplex substrates are bound by Ku under telomerase assay conditions.** A. Native gel shift assays of primers depicted schematically below the gels. Ku protein concentration noted above the gel, Ku-bound and free DNA denoted by arrows. Ku-DNA reconstitutions for the gel
shift and primer extension assay in B. were carried out in parallel and under identical conditions. B. Matching Mini T telomerase extension assays carried out with decreasing concentrations of Ku, primer depicted below gel. Brackets on left indicate telomerase extension products for the gels. C. Same as described for figure C1B.

For the dual binding and telomerase assay the same primer mixes, Ku dilutions, and telomerase extension mixes were used. For the binding assays, a trace amount of 5’ end-labeled primer was added to the primer annealing mix before Ku binding. For both extension and binding assays, 200 nM primer was complexed with serial dilutions of Ku (diluted in Ku storage buffer) at room temperature for 2 hours. After reconstitution of Ku primer-complexes, telomerase extension mix (primers, dNTPs, buffer) was added to both binding and extension mixes. Telomerase was only added to the extension assays, but the binding assay was incubated alongside the telomerase extension at 30ºC for 20 min. Extension assays were processed as described in Appendix A. After 20 min, 5 µl of 50% glycerol was added to the binding reactions, which were loaded onto a native 40 to 20% TBE gel (Life technologies) and electrophoresed at 150 volts constant for 45 min in a gel box surrounded by ice. Binding gels were dried and visualized by phosphoimagery.

The electrophoretic mobility shift assays indicate that Ku binds the substrate duplexes under telomerase assay conditions. As described, substrates with 14 nt single-stranded overhangs were not extended by Mini T telomerase when bound by Ku (left gels, Figure C2). However if the 3’ overhang was extended to 28 nt Mini T extension was restored even in the presence of saturating amounts of Ku. Note at the highest concentrations of protein, the Ku-DNA complex super-shifts in the native gel. The reason for the super-shift is not known but may represent multiple Ku heterodimers binding each DNA or the formation of Ku-DNA multimers. It should be noted that the telomerase extension or inhibition did not appear to be sensitive to the phenomenon causing the super-shift in the binding assay. One
other complicating factor in this experiment is the presence of low molecular weight products that are visible at the bottom of the gel in lanes with high concentrations of Ku. After carrying out the experiments shown in Figure C2, I found that my Ku preps had a nuclease activity, discussed more at length in appendix D. Thus, it is possible that telomerase did actually extend primers with a 14 nt overhang, but the products were subsequently degraded by nucleases. The dual binding and extension experiments need be repeated with nuclease-free Ku and the TEV-cleaved Mini T telomerase in an attempt to eliminate the lower molecular weight products.

Based on these data, I suggest an initial model in which telomerase is able to extend Ku-bound substrates with sufficiently long overhangs (Figure C3). Ku binds at the single-stranded to duplex DNA transition. When the overhang is short (≤ 14 nt), Ku effectively shortens the 3’ overhang that is accessible to telomerase and thereby inhibits telomerase action. Conversely when the 3’ overhang is sufficiently long (28 nt), telomerase is able to act on a Ku-bound substrate. Currently, there is no formal evidence demonstrating that Ku physically encircles telomeric DNA in live budding yeast. However a number of studies support a model in which Ku directly binds telomeric DNA, and mutants that disrupt this function also disrupt telomere maintenance (Ribes-Zamora et al. 2007; Lopez et al. 2011; Pfingsten et al. 2012; Balestrini et al. 2013). Even if Ku does encircle telomeric DNA, it is possible that Ku does not participate in capping at the time of telomerase action. Ku must be removed from telomeric DNA at some point to allow passage of the replication fork. Despite these caveats, Ku is required for telomeric capping and preventing nucleolytic resection of the C-strand during G1 of the cell-cycle (Vodenicharov et al. 2010). Prevention of nucleolytic resection also appears to be dependent on direct DNA binding by Ku (Balestrini et al. 2013). During this stage of the cell cycle, 3’ overhang lengths are thought to be 12-15 nt in vivo (Wellinger and Zakian 2012). Here we show that Mini T telomerase action is inhibited when Ku is bound to telomeric substrates with a 14 nt overhang, raising the intriguing possibility that Ku serves as a
redundant negative regulator of telomerase action during G1. In addition, during S phase in budding yeast the 3’ telomeric overhangs lengthen to 30 to 100 nt (Wellinger and Zakian 2012); our results demonstrate that a Ku-bound telomeric substrate with an overhang of 28 nt can be extended, suggesting that Ku would not be a negative regulator of telomerase action at S-phase.

**Figure C3. Model for extension of Ku-bound DNA substrates.** Summary of Mini T telomerase direct extension assays with Ku-bound substrates. The effective 3’ overhang length dictates extension of the substrates. In top two panels the overhang length is not sufficiently long to allow telomerase access. In the bottom panel the longer 3’ overhang allows for productive extension of Ku-bound substrates, denoted by red arrows on 3’ end of the substrate. Brackets above primers signify the effective overhang length. Primer coloring same as described figure C1.
Appendix D: The Ku heterodimer binds single-stranded DNA with sequence selectivity

As noted in Appendix C, the appearance of low molecular weight products in telomerase assays containing Ku suggested that a contaminating nuclease was carried through from the Ku prep or from the RRLs. Incubation of Ku with single-stranded DNA resulted in degradation of DNA as monitored by native and denaturing electrophoresis. The nuclease activity was specific for single-stranded DNA, required a 5’ phosphate, and appeared to degrade DNA in a 5’ to 3’ direction (data not shown). I attempted to identify the nuclease by mass spec, but I did not obtain statistically significant matches to peptides from known nucleases. I also attempted to heat inactivate the nuclease in Ku preps; however, Ku binding and nuclease activity were simultaneously reduced between 46-48ºC. Nuclease contamination was significantly reduced by modification of the anion exchange step in the Ku purification (detailed in materials and methods of chapter 2). In the original purification scheme, Ku was passed over the anion exchange column in 500 mM NaCl. Ku eluted from the anion column in the void volume and resulted in the removal of nucleic acids associated with Ku. To remove nuclease contamination, Ku was bound to the anion exchange column in low salt, and then subjected to a gradient elution of increasing ionic strength; this modification also removed nucleic acids bound to Ku. This modified anion exchange significantly reduced the single-stranded DNA degradation observed with previous Ku preps.

After implementation of the new ion exchange protocol to remove the nuclease activity, I noticed that single-stranded DNA unexpectedly underwent a gel-shift in the presence of Ku. Electrophoretic mobility shift assays were carried out by incubating trace amounts of 5´-32P-labeled single-stranded DNA oligos with increasing concentrations of Ku. Final binding reactions contained 15
mM Tris (pH 8.0), 150 mM NaCl, 5 mM MgCl₂, 17% glycerol, 0.5 mM EDTA, 1 mM DTT, 0.25 μg yeast tRNA (sigma), and 0.5 μg BSA. Ku was complexed with single-stranded DNA at room temperature for 2.5 hours and then loaded onto a native 40 to 20% TBE gel (Life technologies) and electrophoresed at 150 volts constant for 45 min at room temperature. Although Ku is predominantly known as a double-stranded DNA end-binding protein, I observed that Ku-bound single-stranded DNA with telomeric sequence with moderately high affinity ~ 5 nM (Top panel Figure D1A,B). Single-stranded DNA binding was both length dependent and sequence dependent. A single-stranded DNA primer of 14 nt in length was not bound by Ku except at very high concentrations (Bottom panel Figure D1A). Ku-bound 35 nt oligos that were either poly dT, or 21 nt poly dT plus 14 nt of telomeric sequence. Interestingly, Ku did not bind to 35 nt poly dA or poly U oligos except at very high Ku concentrations (Figure D1B,C), suggesting sequence specificity. In addition, Ku binding of oligos containing poly T did not conform to ideal binding behavior for a single receptor ligand interaction (Middle panels Figure D1A,B). Sequence specific binding to single-stranded DNA has been observed for human Ku (Torrance et al. 1998; Bianchi and de Lange 1999; Uliel et al. 2000).
Figure D5. Ku binds single-stranded DNA with sequence specificity. A. Top gel: Ku binds oligos with random sequence and one telomeric repeat (5’ GTGTGGTGTTGGG 3’) with high affinity. Middle gels: Ku binds a fully poly dT oligo and oligo with poly dT appended to one telomeric repeat. Bottom gel: Ku does not bind a single telomeric repeat of shorter length. Very high concentrations of Ku are necessary to bind. B. Binding curves for top three gels in panel A. The fraction bound was calculated treating all bound species equivalently. Fraction bound for random sequence with one telomeric repeat was fit to the Langmuir isotherm (black), the fraction bound for the poly dT oligo (orange) and poly dT oligo appended to one telomeric repeat (blue) were fit to the equation for multiple non-equivalent binding sites where Fraction bound = (n_1/1+(K_{d1}/[Ku])) + (n_2/1+(K_{d2}/[Ku])), n represents the number of each type of binding site. C. poly dA oligos and D. poly U oligos. Ku concentrations noted above gels.
Appendix E: hTERT C-DAT mutant telomerase is stimulated by TPP1-POT1 but has low enzymatic activity in vitro

Previous reports suggested that mutations in the CTE of hTERT dissociate the activities of telomerase (C-DAT). C-DAT mutants were claimed to retain high enzymatic activity while being defective at maintaining telomeres in vivo (Banik et al. 2002). The CTE was subsequently proposed to interact with the TEL-patch of TPP1 to mediate telomerase recruitment in vitro (Zhong et al. 2012). As TPP1 stimulation and in vivo recruitment are thought to be manifestations of the same interaction between TPP1 and telomerase, we decided to test whether TPP1 stimulates RAP of a C-DAT mutant telomerase. This project was carried out in collaboration with Dr. Ctirad Hofr. Ctirad generated the C-DAT mutant by quick-change mutagenesis, I prepped the telomerase, and Ctirad carried out the telomerase assays. I analyzed the data and produced the figure presented here. The experimental protocols are described and data analysis were carried out as described in chapter four, except that the concentration of telomerase was increased five-fold to boost the signal (10 μl of lysate was used opposed to the usual 2 μl).

To test whether the previously described C-DAT mutant FKT1127NAA is a separation-of-function mutant which has high enzymatic activity but is not stimulated by POT1-TPP1, direct telomerase extension assays were carried out. Activity and RAP stimulation by wild-type TPP1-POT1 were tested for the C-DAT mutant, wild-type telomerase, and R132E mutant telomerase. R132E was previously shown to be a separation-of-function mutant that retains moderately high enzymatic activity but is not stimulated by TPP1-POT1 (Schmidt et al. 2014). The extension assays revealed that the enzymatic activity of the C-DAT mutant is severely compromised in vitro, 27±10% of wild-type telomerase (Figure E1A, C). In contrast, R132E retained 44±2% of wild-type activity. The activity for R132E is lower than
previously reported and may reflect batch to batch variability in the telomerase preparations (R132E was prepared separately from WT and C-DAT mutants). The C-DAT mutant and R132E mutant telomerases both had minor reductions in processivity $81 \pm 2\%$ and $82 \pm 1\%$, respectively (Figure E1D). In contrast to the reduction in activity, the addition of TPP1-POT1 to the extension assay resulted in RAP stimulation of the C-DAT mutant, but not the R132E mutant. TPP1-POT1 RAP stimulation of C-DAT was $74 \pm 4\%$ of the stimulation of wild-type telomerase, and $19 \pm 1\%$ for the R132E telomerase (Figure E1E). Based on these data we conclude that C-DAT has low enzymatic activity but retains the ability to for RAP stimulation by TPP1-POT1 in vitro. This is the opposite result of the separation of function that is observed for the R132E mutant which retains moderate enzymatic activity, but has strong RAP stimulation defects, this study, (Schmidt et al. 2014). In addition, R132E mutant telomerase has in vivo recruitment defects (Schmidt et al. 2014).

Our data are in agreement with a previous report which demonstrated that the C-DAT mutant has low enzymatic activity in vitro (Jurczyluk et al. 2011). We also show that TPP1-POT1 stimulates the RAP of C-DAT which suggests that the mutant telomerase is competent to interact with the TPP1-POT1 complex. In addition, a previous report demonstrated that a disease-associated hTERT mutant F1127L also shows moderately high RAP stimulation in the presence of TPP1-POT1 (Zaug et al. 2013), further supporting the notion that this region of the CTE is not necessary for the interaction with TPP1. Based on these data we hypothesize that recruitment of the C-DAT mutant telomerase is not compromised due to a failure to interact with TPP1 in vivo. In support of this hypothesis, the C-DAT mutant telomerase was previously shown to co-localize with TPP1 tethered to a non-telomeric locus $88\%$ of the time in vivo (Zhong et al. 2012). Interestingly in the same study, the C-DAT mutant also had telomere localization defects suggesting that this mutant telomerase has another defect such as misfolding or proper telomerase assembly that interfere with proper telomere maintenance.
Figure E1. The C-DAT region of hTERT is important for activity but not interaction with TPP1-POT1. A. Direct telomerase activity assays in the absence and presence of the TPP1-POT1 heterodimer for wild-type (WT) and mutant telomerases. LC, loading control. Numbers on left, telomeric repeats added. B. Western blot of the relative quantities of wild-type, C-DAT, and R132E mutant hTERTs after immuno-purification of the telomerases. C-D. Bar graphs representing the quantification of activity, RAP, and RAP stimulation (decay method) by wild-type POT1-TPP1. Values are normalized to WT telomerase, and to WT telomerase with WT TPP1-POT1 for RAP stimulation (n = 2, Mean ± SD).