Regulation of Telomerase by DNA and Protein Interactions

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Department of Medical Biophysics
University of Toronto

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2010

Abstract

In most eukaryotes, chromosomes ends are protected by telomeres which are formed by repetitive DNA, specialized binding proteins, and higher order structures. Telomeres become shorter following replication due to the positioning and degradation of terminal RNA primers, as well as resection by nucleases. Extensive telomere shortening over many cell cycles elicits a DNA damage checkpoint that culminates in senescence or, in the absence of tumor suppressor pathways, apoptosis. These effects block the expansion of cells with unstable genomes, but can also precipitate disease in tissues that rely on regeneration for function. In many unicellular eukaryotes and proliferative human cells including cancer cells, telomeres can be maintained by the telomerase reverse transcriptase (TERT) and its associated RNA (TR). The elongation of telomeric DNA by telomerase depends on the telomerase essential N-terminal (TEN) and C-terminal reverse transcriptase (RT) domains. We found that human TEN interacted with single-stranded telomeric DNA and restored function, in trans, to an hTERT mutant lacking hTEN. Telomerase required hTEN residues for activity, telomere maintenance, and extension of cellular replicative lifespan. Two inactive hTERT variants bearing mutations in TEN and RT domains, respectively, cooperated to regenerate telomerase activity in vitro. hTEN interacted with several regions of hTERT suggesting that dimerization may occur via TEN-TERT interactions. The in vivo defect of certain hTEN mutants may involve an inability to interact with factors that recruit the enzyme to the telomere and/or stimulate activity. Human homologs of the S. cerevisiae recruitment factor Est1 interacted with telomerase in a
species-specific manner. The TPR domain of hEST1A interacted with the N-terminus of hTERT. The TPR domain of ScEst1 was required for telomere length maintenance by telomerase, and, paradoxically, also negatively regulated telomere length. In preliminary experiments, hTERT interacted with hPOT1/hTPP1. This interaction may stimulate the elongation of telomeres by telomerase. The DNA and protein interactions described herein expand our knowledge of telomerase and present new targets for the manipulation of telomerase function in human disease.
Acknowledgments

The work described in this thesis would not have been possible without the support of my colleagues, family, and friends. Firstly, the encouragement that I received from my family – especially my parents, Robert and Faith Sealey, and brother, Sean Sealey – helped me to persevere through scientific and personal challenges. My supervisor, Dr. Lea Harrington, provided scientific mentorship, and a broad research program in which we explored many aspects of telomere biology. My deep thanks go to Kitty Chow for believing in me and our future, and for displaying my first research publications on the fridge.

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Several skilled and dedicated scientists contributed to the work described herein. On the TEN domain project, Dr. Mitsu Ikura and Le Zheng accepted the challenge of producing recombinant hTEN, stayed the course, and delivered. Michael Taboski performed cell culture and downstream work-up to characterize the functional deficits of hTERT mutants, and also welcomed my contribution to his study of the role of telomerase in tumorigenesis. Jennifer Cruickshank performed standard elongation assays of telomerase activity, and worked with me on several flier experiments in blind faith. Dr. Chris Marshall, Dr. Linda Holland, and Carol Liu provided technical assistance and advice. Dr. Rob Laister shared his experience with gel shift assays, and Dr. Susan McCracken provided advice about protein purification. Theo Goh and Dr. Thierry Le Bihan performed mass spectrometry analysis of recombinant hTEN, and went the extra mile to customize their workflow for our needs. On the telomerase dimerization project, I thank Michael Taboski for embarking on the project with me when we were late into our degrees. Bryan Snow and Natalie Erdmann expertly performed RT-PCR and q-FISH experiments, respectively (data not shown). I also thank Bryan for being open to my contributions of ChIP data and intellectual input to two PIF1 projects. On the EST1 project, Alex Kostic generated solid data and demonstrated exceptional dedication at the bench over two summers. Dr. Catherine LeBel designed the project with me, and took me on as a student of yeast biology. I also thank Catherine for her openness to my contributions to her study of telomerase- and RAD52-independent survival in yeast. I thank Dr. Fiona Pryde and Jennifer Dorrens for work on the EST1 and telomerase dimerization projects, respectively, beyond my hands-on presence in the lab.

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<th>Description</th>
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<tr>
<td><strong>STOP</strong> codon</td>
<td>STOP codon</td>
</tr>
<tr>
<td>A</td>
<td>adenosine; alanine</td>
</tr>
<tr>
<td>ARS</td>
<td>autonomously replicating sequence</td>
</tr>
<tr>
<td><em>A. thaliana</em>, At</td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BIO</td>
<td>biotin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cytosine; cysteine; (degrees) Celsius; CHAPS</td>
</tr>
<tr>
<td><em>C. albicans</em>, Ca</td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary (to mRNA) deoxyribonucleic acid</td>
</tr>
<tr>
<td>CEN</td>
<td>yeast centromere sequence</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>ChiP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Cl</td>
<td>chloride</td>
</tr>
<tr>
<td>C-terminus</td>
<td>carboxyl-terminus (of a protein)</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAT</td>
<td>dissociates the <em>in vitro</em> and <em>in vivo</em> activities of telomerase (domain in hTERT)</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td><em>E. aediculus</em>, Ea</td>
<td><em>Euplotes aediculus</em></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td><em>E. crassus</em>, Ec</td>
<td><em>Euplotes crassus</em></td>
</tr>
<tr>
<td>E1D</td>
<td>EST1-interaction domain (in hTERT)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td><em>e.g.</em></td>
<td><em>exempli gratia</em> [Latin]; for example</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>Est1</td>
<td>Ever shorter telomeres 1</td>
</tr>
<tr>
<td>Est2</td>
<td>Ever shorter telomeres 2</td>
</tr>
<tr>
<td><em>et al.</em></td>
<td><em>et alii</em> [Latin]; and others</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>fs</td>
<td>frameshift</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G</td>
<td>guanosine; glycine</td>
</tr>
<tr>
<td><em>GAPDH</em></td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>FLAG</td>
<td>peptide bearing the sequence DYKDDDDK</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin peptide bearing the sequence YPYDVPDYA</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HIS</td>
<td>histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td><em>H. sapiens</em>, Hs, h</td>
<td><em>Homo sapiens</em></td>
</tr>
<tr>
<td><em>HYG</em></td>
<td>hygromycin phosphotransferase (confers resistance to hygromycin)</td>
</tr>
<tr>
<td>IC</td>
<td>internal control (TRAP product)</td>
</tr>
<tr>
<td>IC50</td>
<td>inhibitory concentration(50)</td>
</tr>
<tr>
<td><em>i.e.</em></td>
<td><em>id est</em> [Latin]; that is</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SE</td>
<td>standard elongation (telomerase assay)</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride-sodium citrate</td>
</tr>
<tr>
<td>SFE</td>
<td>(telomere) signal-free end</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian immunodeficiency virus 40</td>
</tr>
<tr>
<td>T</td>
<td>thymidine; threonine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris Borate EDTA</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl) phosphine</td>
</tr>
<tr>
<td>Tel3</td>
<td>Telomeric DNA oligonucleotide primer, (TTAGGG)$_3$</td>
</tr>
<tr>
<td>TEN</td>
<td>Telomerase Essential N-terminal (domain in hTERT)</td>
</tr>
<tr>
<td>TERT</td>
<td>TElomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>t-loop</td>
<td>telomere loop</td>
</tr>
<tr>
<td>TPP1</td>
<td>TINTI/PTOP/PIP1</td>
</tr>
<tr>
<td>TPR</td>
<td>tetraetratricopeptide repeat (domain)</td>
</tr>
<tr>
<td>TR</td>
<td>Telomerase RNA</td>
</tr>
<tr>
<td>TRAP</td>
<td>Telomere Repeat Amplification Protocol</td>
</tr>
<tr>
<td>TRID</td>
<td>hTR-interaction domain (in hEST1A)</td>
</tr>
<tr>
<td>TRF</td>
<td>terminal telomere restriction fragment</td>
</tr>
<tr>
<td>TRF1</td>
<td>Telomere Repeat binding Factor 1</td>
</tr>
<tr>
<td>TRF2</td>
<td>Telomere Repeat binding Factor 2</td>
</tr>
<tr>
<td>Tris</td>
<td>2-Amino-2-(hydroxymethyl)propane-1,3-diol</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>T. castaneum</td>
<td>Tribolium castaneum</td>
</tr>
<tr>
<td>T. thermophila, Tt</td>
<td>Tetrahymena thermophila</td>
</tr>
<tr>
<td>URA3</td>
<td>orotidine-5'-phosphate decarboxylase/uridine 5'-monophosphate synthase</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WB</td>
<td>western blot</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>ZEO</td>
<td>Streptoalloteichus hindustanus ble (confers resistance to zeocin)</td>
</tr>
</tbody>
</table>
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INTRODUCTION

Telomere structure and function, replication, and maintenance by telomerase
TELOMERES

Chromosome end structure

Telomeres are specialized nucleoprotein structures that form the ends of linear chromosomes in most eukaryotes. Telomere DNA ranges in length from 100 to 300 bp in ciliates and yeasts, five to 15 kbp in humans, and up to (or over) 100 kbp in mice (Table I-1). Telomeres are comprised of simple, repetitive G-rich sequences (Moyzis et al., 1988). The G-rich tract is oriented in a 5′-to-3′ direction away from the centromere, and the C-rich tract is oriented 5′-to-3′ toward the centromere (reviewed in Henderson, 1995). Variant telomeric repeats (for example, TGAGGG and TCAGGG in humans) are located upstream of the telomere (Baird et al., 1995). Repetitive elements may occur further upstream that vary considerably in sequence and organization between species (Brown et al., 1990; Riethman et al., 2004). In S. cerevisiae, subtelomeres contain an X element and 0-4 distal Y' elements (Zakian, 1995; Zakian and Blanton, 1988).

The telomeric G-strand ends in a 3′ overhang that varies in length from <21 bases in ciliates to an average of ~150 bases in human cells (Huffman et al., 2000; Makarov et al., 1997; McElligott and Wellinger, 1997; Wright et al., 1997) (Table I-1), and the G- and C-strands terminate in a preferred sequence (Table I-1) (Jacob et al., 2003; Jacob et al., 2001; Lydall, 2003; Sfeir et al., 2005). The overhang length and terminal nucleotides are influenced by telomere end-binding proteins. For example, depletion of the human telomere end-binding protein POT1 reduces the length of the G-overhang, and alters the sequence at the 5′ end of the C-strand (Hockemeyer et al., 2005).

Chromosome end protection

As stable DNA ends in the nucleus, telomeres are distinguished from broken DNA by higher-order structures and specialized binding proteins. In mammals and trypanosomes, the 3′ overhang invades the telomere DNA duplex at an upstream site creating a large duplex telomere loop (t-loop) (de Lange, 2006; Griffith et al., 1999; Neidle and Parkinson, 2003; Nikitina and Woodcock, 2004). T-loop formation is facilitated by TRF2 (Stansel et al., 2001). TRF2 also guards against the excision of t-loops as
extrachromosomal telomeric circles (Wang et al., 2004). TRF1 and TRF2 homodimers bind to the telomere duplex and form a platform for other components of the shelterin complex: RAP1, TIN2, and POT1 (reviewed in Palm and de Lange, 2008). TRF1 and TRF2 are bridged by TIN2 (Liu et al., 2004a; Ye et al., 2004a). TIN2 interacts with TPP1, which in turn binds POT1 (Houghtaling et al., 2004; Liu et al., 2004b; Ye et al., 2004b). TPP1 stabilizes the binding of TIN2 to TRF1 and TRF2 (O'Connor et al., 2006), and POT1 also interacts with TRF2 (Barrientos et al., 2008; Yang et al., 2005). TPP1 does not bind DNA even though it contains an N-terminal oligonucleotide/oligosaccharide binding-fold (OB-fold) domain (Wang et al., 2007; Xin et al., 2007). In contrast, POT1 binds telomeric DNA in a sequence-specific manner through two N-terminal OB-fold domains (Kelleher et al., 2005; Lei et al., 2004; Loayza et al., 2004). Thus, POT1 is a component of both the duplex region of the telomere, by virtue of its interactions with TPP1 and TRF2, and the single-stranded region of the telomere via direct interaction with DNA (refer to Appendix A). POT1/TPP1 are substoichiometric with respect to other components of the shelterin complex (Takai et al., 2010). The CTC1/STN1/TEN1 complex binds the G-strand independently of POT1 (Miyake et al., 2009).

The shelterin complex, along with other associated proteins such as KU70/80, the MRE11/RAD50/NBS1 (MRN) complex, Apollo, RAP1 and WRN, protects telomeres and regulates the accessibility of enzymatic activities. TRF2 and POT1 protect the telomere by limiting the activation of ATM and ATR pathways, respectively, and downstream end-processing activities that normally accompany the DNA damage response (Buscemi et al., 2009; Denchi and de Lange, 2007; Guo et al., 2007; Karlseder et al., 2004). Removal of TRF2 from telomeres via over-expression of a dominant-negative allele (TRF2\textsuperscript{ABAM}) results in resection of the G-overhang by ERCC1/XPF, loss of RAP1 at telomeres, focal accumulation of phosphorylated of γ-H2AX, ATM, 53BP1 and MDC1 at telomeres, and activation of Chk1, Chk2, p53, p21, and p16. This signaling cascade can result in senescence, or telomere-end joining and chromosomal instability followed by apoptosis or tumorigenic conversion, depending on the genetic context (Artandi and DePinho, 2000; d'Adda di Fagagna et al., 2003; Dimitrova and de Lange, 2006; Jacobs and de Lange,
The suppression of telomere end-joining by TRF2 is mediated by RAP1 (Sarthy et al., 2009). Depletion of POT1 triggers a transient telomere damage response in G1 or telomere fusion and senescence or apoptosis, depending on the context (Veldman et al., 2004). Depletion of STN1 causes no overt DNA damage response or cell cycle arrest, but co-depletion of POT1 and STN1 results in an increased incidence of telomere damage, suggesting that POT1 and STN1 protect telomeres through partially redundant mechanisms (Miyake et al., 2009).

In *S. cerevisiae*, the duplex telomere is bound by Rap1 (a homolog of TRF1 and TRF2) (Berman et al., 1986; Buchman et al., 1988; Conrad et al., 1990). Through its C-terminus, Rap1 binds to Rif1 and Rif2 which repress telomere recombination (Hardy et al., 1992; Teng et al., 2000; Wotton and Shore, 1997). The single-stranded region of the telomere is bound by Cdc13 (Lin and Zakian, 1996; Nugent et al., 1996). Cdc13 binds Stn1 and Ten1, forming an end protection complex (Grandin et al., 2001; Grandin et al., 1997). Cdc13, Stn1, and Ten1 mutations can cause extensive resection of the C-rich strand and activation of a Rad9-dependent DNA damage checkpoint (Garvik et al., 1995; Grandin et al., 2001; Grandin et al., 1997; Puglisi et al., 2008; Xu et al., 2009). Deletion of *KU80* – which encodes a DNA end-binding protein – causes a similar phenotype (Gravel et al., 1998; Teo and Jackson, 2001). Therefore, the integrity and stability of chromosome ends depend on the suppression of cellular checkpoints and DNA repair activities by specialized binding proteins.

**TELOMERASE**

Telomerase is a reverse transcriptase formed by the telomerase reverse transcriptase (TERT) and the associated telomerase RNA (TR). TR bears the template for synthesis of the telomeric G-strand. TR was first identified in ciliates and then subsequently in yeast and mammals (Feng et al., 1995; Greider and Blackburn, 1989; Shippen-Lentz and Blackburn, 1990; Singer and Gottschling, 1994). Ciliate and human telomerases were purified using a combination of classical purification and crosslinking studies (Greider
and Blackburn, 1985; Hammond et al., 1997; Harrington et al., 1995; Lingner and Cech, 1996; Lingner et al., 1997b; Morin, 1989; Shippen-Lentz and Blackburn, 1989). *S. cerevisiae* telomerase was identified by genetic screening (Lendvay et al., 1996) and the human TERT gene was subsequently cloned based on sequence similarity with yeast and ciliate TERTs (Harrington et al., 1997; Kilian et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997; Nakayama et al., 1998). The encoded protein was proven to possess telomerase catalytic activity based on immunoprecipitation of endogenous TERT and mutagenesis of residues required for catalysis (Harrington et al., 1997). TERT and TR comprise the essential, minimal catalytic core of telomerase (Beattie et al., 1998; Weinrich et al., 1997). Much has been learned about evolutionarily conserved features and critical residues within TERT and the telomerase RNA (reviewed in Autexier and Lue, 2006).

There are several similarities and differences with respect to the structure and activity of telomerase between species. For example, the N-terminus of many TERTs contains a DNA binding domain that is required for telomerase activity and/or processivity (Chapter 1), but telomerase exhibits a non-universal ability to form and function as a dimer (Chapter 2). Several other proteins associate with telomerase (reviewed in Cong et al., 2002; Harrington, 2003). Of these proteins, only a few appear to be integral components of the enzyme, or to play an essential role in assembling telomerase or recruiting telomerase to the telomere. For example, ScEst1 (Chapter 3) and Est3 (Appendix A) are telomerase-associated proteins whose deletion leads to the same telomere-shortening phenotype and eventual cellular senescence as the deletion of either EST2 (TERT) or the telomerase RNA TLC1 (Hughes et al., 2000; Lendvay et al., 1996; Lingner et al., 1997a; Lingner et al., 1997b; Lundblad and Szostak, 1989; Singer and Gottschling, 1994). While the role of Est3 in the telomerase complex is unclear, Est3 interacts with the N-terminus of Est2, and senescence-inducing mutations in the N-terminus of Est2 can be suppressed by increased Est3 expression (Friedman et al., 2003; Hughes et al., 2000). Est3 can unwind DNA/RNA duplexes and dimerize in vitro (Sharanov et
al., 2006; Yang et al., 2006). Est1 recruits telomerase to the telomere (see below). The identification of Est1 homologs in S. pombe (Beernink et al., 2003), C. albicans (Singh and Lue, 2003; Singh et al., 2002), and humans (Reichenbach et al., 2003; Snow et al., 2003) has further supported an evolutionarily conserved role for Est1 in the telomerase complex (Chapter 3).

TELOMERE REPLICATION

Orientation of telomere DNA replication

The majority of telomere DNA is replicated by conventional DNA polymerases. In S. cerevisiae and ciliates, replication forks have been observed to originate at internal sites and progress toward chromosome ends (Newlon et al., 1993; Prescott, 1994; Tower, 2004; Wellinger et al., 1993a; Wellinger and Zakian, 1989). Although it was originally proposed that replication of gene-sized macronuclear chromosomes in ciliates could proceed from the ends (Zahler and Prescott, 1988; Zahler and Prescott, 1989), the replication of these small chromosome fragments also appears to proceed via internal origins (reviewed in Prescott, 1994; Tower, 2004). As stated above, the DNA strand bearing the G-rich telomere repeat sequence and single-stranded overhang is oriented 5′-to-3′ toward the end of the chromosome, and the complementary C-rich strand is oriented 5′-to-3′ toward the centromere. Thus, the G-strand is the template for the lagging strand replication process, in which a C-strand copy is produced; reciprocally, the daughter G-rich strand is synthesized as the leading strand (Figure I-1).

Telomere replication kinetics in S. cerevisiae

The activation of individual DNA replication origins throughout chromosomes is temporally regulated during the cell cycle. Significant differences exist between yeast and mammalian species with respect to the timing of telomere-proximal origin activation and telomere replication during S phase. The replication timing of chromosomal loci in S. cerevisiae has been surveyed by combining cell cycle synchronization with Meselson-Stahl dense isotope transfer or two-dimensional (2D) gel electrophoresis methods
(Ferguson et al., 1991; McCarroll and Fangman, 1988). Unique sequences adjacent to specific telomeres (on chromosomes III-L, V-R, VI, IX, X, XVI) are replicated in late S phase, while other segments of the genome, including centromeres, are replicated in early S phase (Makovets et al., 2004; McCarroll and Fangman, 1988; Yamashita et al., 1997). Most Y’-bearing telomeres are replicated near the end of S phase (McCarroll and Fangman, 1988), and upstream Y’ elements are replicated earlier than terminal Y’ elements (Makovets et al., 2004). Due to the repetitive sequence of the telomere, the methods described above cannot distinguish replication timing and fork progression at individual telomeres; however, genome-wide analysis of replication timing performed by hybridizing replicated DNA to oligonucleotide microarrays demonstrates that telomere-proximal sequences replicate late in S phase. Interestingly, the replication timing of the two ends of each chromosome is highly correlated (Raghuraman et al., 2001).

The mechanism by which telomere replication is restricted to late S phase is context-dependent. With respect to chromosome III-L (which contains X but not Y’ elements), the late replication of the region upstream of the X element is due to the delayed arrival of a replication fork from an early-activated centromere-proximal origin, despite the presence of an autonomously replication sequence (ARS) near the telomere (Button and Astell, 1986; Reynolds et al., 1989). In contrast, the telomere-proximal region of chromosome V-R is replicated in late S phase due, most likely, to the late activation of ARS501 which is located in the unique DNA upstream of the Y’ element. Alternatively, ARS501 may be activated in early S phase, but progression of the replication fork may be delayed until late S phase (Ferguson et al., 1991). The subtelomeric X element on chromosome V-R, as well as Y’ elements, has sequences that promote self-replication of plasmid DNA (Chan and Tye, 1983; Ferguson et al., 1991). However, X and Y’ ARSs are relatively inactive as origins in the chromosomal context and Y’ activation is probably not responsible for the late replication of the V-R telomere. Thus, depending on the chromosomal context, S. cerevisiae subtelomeric DNA and presumably telomeric repeats replicate late in S phase by the passage of replication forks emanating from distant, early-activated origins or from late-activated origins found near and/or within the subtelomeric region.
Telomere replication kinetics in *S. pombe* and *H. sapiens*

The late timing of telomere-proximal origin activation and telomere replication is observed in some but not all organisms. In *S. pombe*, a probe detecting telomere-adjacent sequences on chromosomes I and II hybridizes to replication intermediates fractionated by 2D gel electrophoresis from cells harvested in late S phase, indicating that telomeric regions are also replicated late in this organism. Also, at least one telomere-associated sequence contains an origin of replication, which is analogous to the origin-containing X and Y' elements of *S. cerevisiae* (Kim and Huberman, 2001). In contrast, studies in mammals, which generally have not employed the robust techniques described above, do not support a model in which telomere replication is limited to late S phase. The repetitive telomere sequence is detected in newly-replicated BrdU-labeled DNA extracted from human fibroblasts or lymphoblastoid cells throughout S phase (Ten Hagen et al., 1990; Wright et al., 1999). TRF1- and TRF2-containing chromatin is labeled with BrdU in S phase, and to a lesser extent in G2 (Verdun and Karlseder, 2006). Whole cell flow-fluorescence in situ hybridization (FISH) of normal and hematopoietic cell types, using propidium iodide as a marker for DNA replication, reveals increases in telomere signal intensity that rise markedly in early S phase or steadily alongside bulk DNA replication (Hultdin et al., 2001). These methods, which track the replication of all telomeres, do not elucidate subtle dynamics at individual telomeres. Using a sequence-specific FISH probe, the subtelomere and most telomeric 20 kbp of chromosome 16p were found to replicate in late S phase (Smith and Higgs, 1999). In contrast, microarray methods revealed that the subtelomeric region of chromosome 22 is replicated in early S phase (White et al., 2004; Woodfine et al., 2004). Thus, specific human telomeres replicate at distinct times in S phase such that replication of the repetitive telomeric sequence appears to occur throughout S phase. This pattern is also observed in Indian muntjac fibroblasts, in which each telomere undergoes replication at a distinct time in S phase. Further, in contrast to replication in *S. cerevisiae*, p- and q-arms of the same chromosome in these cells are not replicated at the same time (Zou et al., 2004).
Telomere proximity affects origin activation kinetics

Despite interspecies differences with respect to telomere replication timing, the proximity of origins to telomeric heterochromatin correlates with late replication in several systems. This effect can be demonstrated by moving ARS elements toward or away from telomeres and monitoring changes in replication timing. In *S. cerevisiae*, an early-replicating ARS becomes late-replicating when moved from its normal position near the centromere on chromosome IV to a position upstream of the chromosome V-R telomere. Reciprocally, the late-replicating ARS501 becomes early-replicating when moved, with 15 kbp of its surrounding sequence, from its normal position in the subtelomere of chromosome V-R to a centromeric circular plasmid containing no other ARS. Notably, if this circular plasmid is linearized by the addition of telomeres, the plasmid replicates in late S phase (Ferguson and Fangman, 1992). Likewise, when the origin from circular 2 micron DNA, a plasmid that normally replicates in early S phase, is moved to a linear plasmid containing telomeres, the origin is activated in late S phase (Wellinger *et al.*, 1993a). Analysis of genome-wide replication kinetics suggests that telomeres can impose late replication on sequences up to 35 kbp away (Raghuraman *et al.*, 2001). This finding is consistent with the observation that the early-replicating *RAD4* locus becomes late-replicating when moved from 66 kbp to 32 kbp away from a telomere (Ferguson and Fangman, 1992). Taken together, these observations indicate that telomeres exert a position effect on the activation of replication origins, causing them to fire in late S phase.

A similar position effect on replication timing has been observed in humans. In two variants of chromosome 16 associated with α thalassemia, in which the short arm has been truncated and capped with telomere DNA, α globin genes are juxtaposed toward telomeres and are replicated later than in the wild-type chromosome (Smith and Higgs, 1999). On a truncated chromosome 22q associated with mental deficiencies, whereby the terminal 130 kbp has been deleted and healed by telomere repeats, sequences upstream of the breakpoint shift from replicating in mid-S phase to late S phase (Ofir *et al.*, 1999). Therefore, a telomeric location can delay the activation of nearby origins or the progression of replication
forks from these origins, or both. The impact of telomeres on the replication kinetics of neighbouring DNA resembles the repressed transcription of telomere-proximal genes in yeast and humans (reviewed in Mondoux and Zakian, 2006).

**Chromatin status and telomere length affect telomere replication kinetics**

Heterochromatin is generally replicated late in S phase and genes located therein tend to be transcriptionally repressed (reviewed in Gilbert, 2002). Telomere-proximal and telomeric chromatin is formed in part, at least in *S. cerevisiae*, by the KU complex, SIR proteins and hypoacetylated core histones. These factors contribute to the transcriptional repression of telomere-proximal genes (Boulton and Jackson, 1998; Grunstein, 1998; Mishra and Shore, 1999). In addition to binding telomere DNA, KU is involved in non-homologous end joining of broken DNA (reviewed in Bertuch and Lundblad, 2003; Downs and Jackson, 2004), and Sir3 is part of a multi-protein complex that associates with, and is required for the establishment of transcriptionally silent chromatin (reviewed in Mondoux and Zakian, 2006; Taddei *et al.*, 2005). Remarkably, the late-replicating region upstream of the chromosome V-R X element, including ARS501, is replicated much earlier upon deletion of *KU70* or *SIR3*. In both cases, ARS501 becomes activated less often in favour of ARS activation within the Y′ element. Y′ elements replicate much earlier in the absence of *KU70* and somewhat earlier in the absence of *SIR3* (Cosgrove *et al.*, 2002; Stevenson and Gottschling, 1999). Interestingly, replication proceeds from ARSXII-32 (32 kbp away from the telomere on chromosome XII-L) when *KU70*, but not *SIR3*, is deleted. Further, deleting both genes does not have a greater effect on replication timing than deleting *KU70* alone (Cosgrove *et al.*, 2002). These observations suggest that Sir3 functions downstream of Ku70 in the control of replication timing, and that Ku70 may play a more global role in controlling telomere replication timing than does Sir3. The effect of telomeres on replication kinetics depends on telomere length. For example, Pol2, a component of DNA polymerase ε, associates with engineered VII-L and V-R telomeres earlier in S phase following Cre-mediated shortening (Bianchi and Shore, 2007a). This effect is consistent with the earlier replication of shorter telomeres in *KU* mutant cells (Fisher *et al.*, 2004; see above).
Although late-firing replication origins are not obligatorily tethered to the nuclear periphery, KU may also promote late replication through its role in tethering telomeres to the periphery (reviewed in Taddei et al., 2004). In addition, the histone deacetylase (HDAC) activity of the SIR complex may control replication via epigenetic changes. The effects of deleting the HDAC Rpd3 on the replication timing of telomere-proximal regions are context-dependent. ARS609, located 13.7 kbp from the chromosome VI-R telomere, replicates earlier in rpd3 cells (Vogelauer et al., 2002). Conversely, deletion of RPD3 or its corepressor SIN3, while allowing late-activated internal regions to replicate earlier, does not alter the late replication timing of ARS319 adjacent to the X element on chromosome III-R (Aparicio et al., 2004). Genome-wide analysis in mutant strains would address whether SIR proteins and HDACs control the replication timing of all or a subset of telomeres. How nucleosome modifiers such as HDAC inhibitors might affect telomere replication in mammalian cells is an important question given current investigations into the utility of HDAC inhibitors in cancer therapy (Marks et al., 2004).

**CHROMOSOME END REPLICATION PROBLEM AND REPLICATIVE SENESCEENCE**

Conventional DNA polymerases cannot fully replicate the very ends of linear chromosomes (Olovnikov, 1971; Olovnikov, 1973; Watson, 1972). Polymerization of the leading G-rich strand can theoretically continue until the very end of the C-rich template strand, generating a blunt-ended terminus (Figure I-1). However, the lagging strand is synthesized discontinuously in Okazaki fragments that are primed by 8-12 bases of RNA. As a result, the product of lagging strand replication is predicted to have a gap of 8-12 bases relative to the end of the template once the terminal RNA primer is removed (Newlon et al., 1974; Tseng et al., 1979). This terminal gap will be even longer if the primer is not initiated at the extreme 3′ end of the G-strand template. Consistent with these predictions, telomeres shorten by approximately 3-5 bp per cell division in telomerase-defective S. cerevisiae, which is about the rate expected if the terminal RNA primer is laid down at the very end of the DNA molecule (Lendvay et al., 1996; Lundblad and Szostak, 1989; Singer and Gottschling, 1994). In human somatic cells lacking telomerase activity, telomeres shorten by 50-100 bp per round of DNA replication (Allsopp et al., 1995; Allsopp et al., 1992;
Harley et al., 1990). This higher rate suggests that either the terminal RNA primer is not positioned at the end of the chromosome and/or that telomeres are degraded by other mechanisms (see below). In vitro experiments support the idea that Okazaki fragment synthesis at terminal sequences is inefficient, especially at G-rich telomeric repeats (Ohki et al., 2001).

Human cells without a means to maintain a minimal amount of telomeric DNA possess a finite replicative potential in vitro (Harley, 1997). In cells that undergo many cell divisions, extensive telomere shortening elicits a DNA damage response, including telomeric accumulation of γ-H2AX, 53BP1, ATM, MDC1, NBS1, MRE11, and SMC1-pS966. Downstream checkpoint activation is evidenced by the detection of CHK1-pS345, CHK2-pT68, RAD17-pS645, p53-pS15, p21, and decreased levels of phosphorylated pRb (d'Adda di Fagagna et al., 2003; Gire et al., 2004; Herbig et al., 2004; Sedelnikova et al., 2004). In the absence of ATM, senescence signals are transduced through ATR and CHK1 (Herbig et al., 2004). The senescence phenotype can be reversed or blunted by disruption of ATM, ATR, CHK1, CHK2, and/or expression of hTERT (see below) (d'Adda di Fagagna et al., 2003; Gire et al., 2004; Herbig et al., 2004). The senescence-associated DNA damage response is similar to that induced by the loss of telomere end protection caused by over-expression of dominant-negative TRF2 (discussed above; d'Adda di Fagagna et al., 2003; Takai et al., 2003), implying that short telomeres cannot protect chromosome ends.

Telomere erosion and the eventual activation of a DNA damage checkpoint act as barriers to tumorigenesis (Campisi, 2005). In cells capable of overcoming senescence (via the inactivation of p53 and/or pRb, for example), further telomere shortening can result in telomere instability, chromosome end-to-end fusion, and tumorigenic conversion (reviewed in Artandi and DePinho, 2000; Chin et al., 1999; Rudolph et al., 1999; Shay et al., 1991).
MECHANISMS OF TELOMERE MAINTENANCE

Telomere elongation by telomerase

In most single-cell eukaryotes and mice, for example, telomerase is nearly ubiquitously expressed. In humans, hTERT expression is restricted to early developmental stages and proliferative tissues (reviewed in Collins and Mitchell, 2002; Greider, 1998). In many somatic cell types, hTERT is transcriptionally repressed (Kim et al., 1994; Meyerson et al., 1997; Nakamura et al., 1997). Notably, most cancer cell types can divide indefinitely due to the maintenance of telomeres by the activation of hTERT transcription and telomerase activity (Kim et al., 1994; Meyerson et al., 1997; Shay and Bacchetti, 1997). As proof of concept, primary cells transduced with hTERT cDNA gain the ability to maintain telomeres and divide indefinitely (Bodnar et al., 1998; Vaziri and Benchimol, 1998). Thus, telomerase is an attractive target for the development of anti-cancer therapeutics. In some cases, telomeres can be maintained by the recombination-based alternative lengthening of telomeres (ALT; discussed below) (Cesare and Reddel, 2008).

The mutation of telomerase components (e.g., hTERT and hTR) and premature shortening of telomeres are linked to syndromes such as dyskeratosis congenita and idiopathic pulmonary fibrosis that may involve the exhaustion of stem cell compartments (reviewed in Armanios, 2009). In mice, deletion of either the telomerase RNA or Tert, while not immediately detrimental, leads to a loss of telomerase activity and progressive telomere shortening in all tissues. Eventually, detectable telomere DNA is lost from chromosome ends, leading to myriad consequences including genetic instability, infertility and increased apoptosis in germ cells, defects in the immune response due to reduced proliferation of activated T and B cells, premature hair loss, and impaired wound healing (reviewed in Hackett and Greider, 2002; Harrington, 2005). In general, short telomeres in a telomerase-null background hinder tumor formation. However, in the absence of Tp53, some malignancies are increased, especially in older animals (reviewed in Artandi, 2002; Artandi, 2003; Hackett and Greider, 2002).
**Telomere elongation by alternative lengthening of telomeres**

In a subset of human cells, telomeres are maintained in the absence of telomerase via homologous recombination between telomeric DNA tracts – the so-called ALT (alternative lengthening of telomeres) pathway (Cesare and Reddel, 2008; Reddel et al., 2001). Similarly, telomerase-deficient yeast can maintain telomeres by telomere-telomere recombination (Chen et al., 2001; refer to Chapter 3; Lundblad and Blackburn, 1993; Teng et al., 2000). In yeast and human cells that survive without telomerase activity, telomere lengths are characteristically long and heterogeneous. In ALT cells and other cell types, the presence of extrachromosomal telomeric circles suggests a possible mechanism to account for telomere length heterogeneity: intratelomeric invasion of the 3‘ G-overhang might lead to extensive telomere lengthening via rolling circle replication templated by the C-strand, or telomere deletion via the excision of t-loops (reviewed in Lustig, 2003; Tomaska et al., 2004; Wang et al., 2004).

**TEMPORAL PROGRAM OF TELOMERE END PROCESSING**

**Telomere replication and nucleolytic end-processing precede telomerase action**

Telomerase elongates single-stranded DNA but not blunt-ended duplex DNA (reviewed in Greider, 1995). An appealing model is that following telomere replication, nucleases generate the G-rich overhang which serves as a substrate for Cdc13 binding (see below), telomerase recruitment, and telomere elongation (Figure I-1). In support of this model, chromosome termini and telomere-containing linear plasmids bear G-rich overhangs of at least 30-40 bases following telomere replication during late S phase in *S. cerevisiae* (Wellinger et al., 1993a; Wellinger et al., 1993b). Since both ends of linear plasmids acquire G-rich overhangs, their occurrence is not limited to ends replicated by lagging strand synthesis. Moreover, replication appears to be obligatory for G-tail acquisition since overhangs are only detected on linear plasmids if they contain an ARS (Dionne and Wellinger, 1998). G-rich overhangs are detected even in ciliate, yeast and mouse strains lacking telomerase function, implicating a 5’-to-3’ exonuclease in the degradation of the C-strand (Dionne and Wellinger, 1996; Jacob et al., 2003; Wellinger et al., 1996; Yuan
et al., 1999). To further support that telomerase elongates the G-rich tract following DNA replication, artificially shortened S. cerevisiae telomeres are not elongated in G1-arrested cells or in the presence of hydroxyurea (Marcand et al., 1999; Marcand et al., 1997), and elongation of shortened telomeres on a linear plasmid lacking an ARS is partially blocked (Marcand et al., 2000). Also, telomere addition at an HO-induced double strand break occurs when cells are arrested in mitosis, but not in G1, and is abolished or greatly reduced in cdc17-2 (DNA Pol α), cdc2-2 (DNA Pol δ), or pri2-1 (DNA primase) strains (Diede and Gottschling, 1999). Therefore, the activities of DNA Pol α, δ, and DNA primase precede nucleolytic end processing and telomerase action.

This replication requirement suggests that the nuclease activity responsible for resection of the C-strand is associated with the DNA replication machinery or is dependent on the alteration of telomeric chromatin that occurs when the replication fork moves through the telomere. Perhaps as a demonstration of the latter possibility, loss of function of Cdc13 or deletion of KU80 leads to the appearance of long G-overhangs throughout the cell cycle, presumably because the C-rich strand is exposed to nucleases (Garvik et al., 1995; Gravel et al., 1998). Thus, both the end replication problem (see above) and nucleolytic resection cause telomere shortening and render the G-strand suitable for elongation by telomerase (Jacob et al., 2003; Olovnikov, 1973; Sfeir et al., 2005; Watson, 1972).

**Generation of the G-overhang by nucleolytic resection**

Candidate nucleases proposed to generate and/or recruit nucleases that generate the G-overhang in S phase include Exo1 and Rad24, since loss-of-function of these factors partially ameliorates the temperature sensitivity of ku and cdc13 mutations caused by extensive C-strand resection (Maringele and Lydall, 2002; Zubko et al., 2004). However, existing evidence does not support a critical role for any one nuclease in G-strand processing in a wild-type strain, and it seems likely that multiple nucleases may contribute to this process (reviewed in Bertuch and Lundblad, 2003; Lydall, 2003; Tran et al., 2004). Interestingly, in the absence of telomerase, deletion of EXO1 suppresses the genetic instability that arises
when telomeres become critically short and can promote recovery of a telomerase-deficient population (Bertuch and Lundblad, 2004; Hackett et al., 2001; Hackett and Greider, 2003; Maringele and Lydall, 2002; Maringele and Lydall, 2004a; Zubko et al., 2004).

Another candidate nuclease is the Mre11/Rad50/Xrs2 (MRX) complex. Strains lacking components of the MRX complex have shorter G-strand overhangs (Larrivee et al., 2004), shorter telomeres (Boulton and Jackson, 1998; Kironmai and Muniyappa, 1997; Ritchie and Petes, 2000), and delayed telomerase-mediated lengthening of an HO endonuclease-induced break in nocodazole-arrested cells (Diede and Gottschling, 2001), indicating that complex plays an important role at telomeres (or modeled telomeres) following replication.\(^1\) However, there is little evidence to suggest that the nuclease activity of MRX is essential for the generation of the overhang at telomeres, since a nuclease-inactive MRX complex does not alter G-strand overhangs or interfere with telomere length maintenance (Moreau et al., 1999; Takata et al., 2005; Tsukamoto et al., 2001). While the MRX complex is important for other processes such as intra-telomeric DNA excision and 5’ end-resection following a double-strand break, the nuclease activity of Mre11 is also dispensable for these processes (D’Amours and Jackson, 2002; reviewed in Haber, 1998; Williams et al., 2005). The nuclease-independent function of MRX at the telomere remains to be determined. One possibility is that MRX may recruit the nuclease that generates the G-strand overhang.

**Recruitment of telomerase to the telomere**

The passage of the replication fork through the telomere and formation of the G-overhang may generate a DNA damage signal adapted to recruit telomerase. (The telomerase recruitment pathway in *S. cerevisiae*, which culminates in the interaction of Cdc13, Est1 and Est2 with telomeres, has been most extensively characterized, and hence will be described first.) Cells lacking any one of Mre11, Rad50, Xrs2, Tel1 (homolog of vertebrate ATM), or Mec1 (homolog of vertebrate ATR) exhibit short telomeres (Boulton and Jackson, 1998; Kironmai and Muniyappa, 1997; Lustig and Petes, 1986; Ritchie et al., 1999).

\(^1\) In human cells, MRN co-purifies with TRF2, and NBS1 (the mammalian Xrs2 homolog) localizes to telomeres in S phase (Zhu et al., 2000).
suggesting that these proteins may function in the telomerase recruitment pathway. Cells lacking both Tel1 and Mec1 exhibit progressive telomere shortening and senescence, indicating that these kinases have non-redundant roles at telomeres (Ritchie et al., 1999). Cells lacking MRX and Mec1 exhibit a similar phenotype (Ritchie and Petes, 2000). [The essential function of Mre11 in this context does not depend on its nuclease activity (Tsukamoto et al., 2001).] Likewise, deletion of Tel1 and Rad3 (homolog of ATR) in *S. pombe* results in progressive telomere loss followed by chromosome circularization (Naito et al., 1998). In contrast, *S. cerevisiae* lacking MRX and Tel1 do not exhibit progressive telomere shortening or senescence, suggesting that these proteins function in the same pathway (Ritchie and Petes, 2000). The senescence phenotype of *mec1Δ mrxΔ* and *mec1Δ tel1Δ* strains can be rescued by the fusion of Cdc13 to Est1 or Est2 (see below); therefore, these pathways likely culminate in the recruitment of telomerase to the telomere (Tsukamoto et al., 2001). Indeed, one study reported that the telomeric levels of Est1 and Est2 in S phase are greatly reduced in the absence of MRE11 or TEL1, but not MEC1, while telomeric levels of Cdc13 in S phase are only modestly reduced in the absence of MRE11 or MEC1, but not TEL1 (Goudsouzian et al., 2006).

The MRX complex binds to telomeres in S phase, and may recruit Tel1 (Blackburn, 2001; Goudsouzian et al., 2006; Ritchie and Petes, 2000) in an analogous manner to the recruitment of Tel1 to DNA breaks (Lisby et al., 2004; Nakada et al., 2003; Shroff et al., 2004). Mec1 and Tel1 may phosphorylate key telomeric substrates; candidate targets include RPA and Cdc13 (Blackburn, 2001; Brush and Kelly, 2000; Brush et al., 1996; Takata et al., 2005). RPA is phosphorylated during the cell cycle and in response to DNA damage in a MEC1-dependent manner (and partially TEL1-dependent manner in the absence of MEC1) (Brush et al., 1996), and RPA interacts with telomeres in S phase (Schramke et al., 2004; Takata et al., 2004). Cells expressing an N-terminal deletion mutant of Rfa2 exhibit short telomeres and decreased telomeric levels of Est1 (but not Est2 or Cdc13) in S phase (Schramke et al., 2004). The telomere shortening in this context is not exacerbated by the absence of TEL1, and can be prevented by

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1 This result derives from analysis of *mec1Δ* and *ddc2Δ* strains.
fusion of Est1 to the DNA binding domain of Cdc13 (Schramke et al., 2004). These findings suggest that the recruitment of Est1 to the telomere is regulated by Tel1 and RPA. Also, Tel1 and Mec1 phosphorylate Cdc13 in vitro, and cells expressing mutants of Cdc13 that cannot be phosphorylated exhibit progressive telomere shortening and senescence (Tseng et al., 2006). This telomere shortening can be prevented by fusion of the Cdc13 mutants to Est1 (Tseng et al., 2006). These findings suggest that phosphorylation of Cdc13 by Mec1/Tel1 regulates the interaction of Est1 with the telomere. The phosphorylation of Cdc13 by Cdk1 also regulates the interaction of Est1 and Est2 with the telomere (Li et al., 2009; see below).

Cdc13 binds to single-stranded telomeric DNA (Lin and Zakian, 1996; Nugent et al., 1996), and associates with telomeres throughout the cell cycle, peaking in association in S phase (Fisher et al., 2004; Taggart et al., 2002). Est1 also binds to single-stranded telomeric DNA (Virta-Pearlman et al., 1996) and associates with telomeres in S phase (Fisher et al., 2004; Schramke et al., 2004; Taggart et al., 2002). Cdc13 and Est1 interact (Qi and Zakian, 2000). A cdc13-2 allele causes telomere shortening, senescence, and decreases the association of Est1 and Est2 with telomeres in S phase (Chan et al., 2008; Nugent et al., 1996; Taggart et al., 2002). The telomere shortening and senescence exhibited by this mutant can be rescued by expression of a Cdc13(DNA binding domain)-Est1 fusion, or an est1-60 allele (bearing a compensatory charge swap mutation) that, on its own, also causes telomere shortening and senescence (Evans and Lundblad, 1999; Pennock et al., 2001). Several findings suggest that Est1 recruits Est2 to the telomere in S phase by acting as an intermediary between Cdc13 and TLC1. The interaction of Est2 with the telomere in S phase is reduced upon deletion of EST1 (Chan et al., 2008). Also, the telomere shortening and senescence that occur in this context are rescued by expression of a Cdc13-Est2 fusion protein (Evans and Lundblad, 1999). Est1 binds to TLC1 (Lin and Zakian, 1995; Livengood et al., 2002; Seto et al., 2002; Steiner et al., 1996; Zhou et al., 2000), and the telomeric localization of Est2 in S phase is reduced when the Est1-interacting region of TLC1 is deleted (Chan et al., 2008). Reciprocally, the telomeric localization of Est1 in S phase is reduced when TLC1 is mutated or EST2 is deleted (Chan et al., 2008).
Cdc13 is phosphorylated at T308 by Cdk1 in late S phase/G2 (Li et al., 2009). In support of the model whereby Est2 is brought to the telomere in S phase via Cdc13-Est1-TLC1 interactions, cells expressing a Cdc13-T308A mutant (which cannot be phosphorylated at residue 308) exhibit the following defects: reduced interaction between Cdc13 and Est1/TLC1; reduced binding of Est1 and Est2 to telomeres in S phase; and shorter telomeres (Li et al., 2009).

In addition to interacting with telomeres in S phase, Est2 also associates with telomeres in G1 (Smith et al., 2003; Taggart et al., 2002). When KU70 or the Ku80-interacting region of TLC1 is deleted, the association of Est2 with the telomere in G1 is lost, and the associations of Est1 and Est2 with the telomere in S phase are reduced by half (Fisher et al., 2004). Both mutations result in shorter telomeres, demonstrating the functional importance of these recruitment pathways (Peterson et al., 2001; Stellwagen et al., 2003). Although telomere-bound Est2 does not lengthen telomeres in G1, in conjunction with Ku, it may promote telomere capping and inhibit NHEJ (Chan and Blackburn, 2003). In ku70Δ cells, the association of Cdc13 with the telomere is increased and occurs earlier in S phase, and the association of Est1 and Est2 with the telomere in S phase occurs earlier (Fisher et al., 2004). These effects are consistent with the accelerated timing of telomere replication and increased G-strand overhang length that occur in ku mutant cells (see above).

With respect to humans, the composition of telomeric chromatin changes through the cell cycle. Telomeric levels of TRF1 (a negative regulator of telomerase; see below) drop in S phase (Smith et al., 2003; Verdun et al., 2005). Activated ATM (pS1981) and ATR bind telomeres transiently in S phase, apparently in advance of the arrival of NBS1-pS343 and MRE11 in early G2 (Verdun et al., 2005; Verdun and Karlseder, 2006). After a slight decrease in association, activated ATM and ATR bind telomeres again in G2, concomitant with a decrease in POT1 association (see below) and susceptibility of telomeres to enzymatic labeling (Verdun et al., 2005; Verdun and Karlseder, 2006). While ATM and ATR (and other proteins) are involved in signaling telomere dysfunction upon extensive telomere shortening and/or loss of end protection (see above), these factors may also instigate the recruitment of
telomerase, resolution of telomere replication blocks, and/or assembly of a protected chromosome end structure \( (i.e. \text{t-loop}) \) (Verdun and Karlseder, 2006).

The interaction of endogenous human TERT and telomerase-associated proteins with the telomere through the cell cycle has not been described, although the recruitment pathway may share some similarities with the pathway in \( S. \text{cerevisiae} \). For example, KU70 and KU80 interact with telomerase, telomere DNA, and telomere binding proteins (Bianchi and de Lange, 1999; Chai \textit{et al.}, 2002; Hsu \textit{et al.}, 2000; Song \textit{et al.}, 2000; Ting \textit{et al.}, 2005). Human homologs of ScEst1 (refer to Chapter 3) interact with telomerase, telomere DNA, and/or telomeric chromatin (Azzalin \textit{et al.}, 2007; Reichenbach \textit{et al.}, 2003; Snow \textit{et al.}, 2003). Also, peripheral blood lymphocytes derived from patients with ataxia telangiectasia (\( \text{ATM}^/- \)) or Nijmegen breakage syndrome (\( \text{NBS}^/- \)) exhibit shorter telomeres relative to normal controls (Metcalfe \textit{et al.}, 1996; Ranganathan \textit{et al.}, 2001), which is consistent with a role for human ATM and NBS1 in telomerase recruitment.

\textit{Cis-acting length-dependent controls on telomere elongation}

Whether telomerase is engaged to act at a given telomere is governed by factors that bind the duplex telomere and the single-stranded G-overhang. The frequency of telomere elongation is inversely proportional to telomere length. As described above, \( S. \text{cerevisiae} \) Rap1 binds to the double-stranded region of the telomere and interacts with Rif1 and Rif2 through its C-terminus. Telomeres in strains lacking Rif1, Rif2, or the C-terminus of Rap1 become elongated by telomerase in a Tel1-dependent manner (Craven and Petes, 1999; Hardy \textit{et al.}, 1992; Kyrion \textit{et al.}, 1992; Levy and Blackburn, 2004; Teng \textit{et al.}, 2000; Wotton and Shore, 1997) and the frequency of telomere elongation in a given cell cycle is increased (Teixeira \textit{et al.}, 2004). Also, short telomeres, which by definition contain less Rap1, are more frequently extended by telomerase than long telomeres (Marcand \textit{et al.}, 1999; Teixeira \textit{et al.}, 2004). In contrast, artificially increasing the complement of Rap1, Rif1, or Rif2 at a given telomere results in telomere shortening (Levy and Blackburn, 2004; Marcand \textit{et al.}, 1997). However, cells lacking Tel1
cannot prevent the lengthening of Rap1-tethered telomeres by telomerase (Ray and Runge, 1999) and fail to elongate the shortest telomeres in an otherwise normal cell (Arneric and Lingner, 2007). These findings are consistent with a role for Tel1 in promoting the selective elongation of short telomeres. Interestingly, telomerase repeat addition processivity is increased at short telomeres in a Tel1-dependent manner (Chang et al., 2007). In contrast to these length-dependent controls, Stn1 limits telomere elongation independently of telomere length, and may function to block telomerase at any telomere (Puglisi et al., 2008a).

The selective elongation of short telomeres involves the loss of an inhibitory pathway (i.e., Rap1/Rif1/Rif2) and the increased activity of the Tel1 recruitment pathway (described above). Artificial shortening of an engineered telomere results in decreased binding by Rif2, and preferential binding by Tel1, Est1 and Est2 (but not Cdc13) in late S phase (Bianchi and Shore, 2007b; Sabourin et al., 2007). This selective recruitment to short telomeres depends on the C-terminus of Xrs2, again suggesting that Tel1 acts downstream of Xrs2 (Sabourin et al., 2007). Similarly, when telomeres become shorter in the absence of telomerase, Tel1 associates with the shortest telomeres (Hector et al., 2007). Also, over-expression of Tel1 results in the accumulation of Tel1 at telomeres, binding of Est2 to Tel1-bound telomeres, and Mre11- and Rad50-dependent telomere elongation (Hector et al., 2007; Viscardi et al., 2007). Therefore, the MRX-Tel1 pathway exerts dominant control over telomere length. In the absence of Tel1, telomeres are elongated less frequently (Arneric and Lingner, 2007). Finally, recent studies suggest that Rif1/Rif2 compete with Tel1 for binding to DNA ends and Xrs2, and inhibit Tel1-mediated telomere elongation (Hirano et al., 2009; Viscardi et al., 2007). Thus, telomere length homeostasis is achieved by a balance of telomere shortening, and telomere lengthening via selective recruitment of telomerase to short telomeres.

In human cells, the elongation of telomeres by telomerase is regulated by the shelterin complex (described above). TRF1 and TRF2, which are related to ScRap1 through their C-terminal Myb-like DNA-binding domains, are the major duplex telomere-binding proteins (Broccoli et al., 1997). Over-expression of
TRF1 or artificially tethering it to telomeres results in telomere shortening, whereas over-expression of a dominant-negative TRF1 causes telomere lengthening (Ancelin et al., 2002; van Steensel and de Lange, 1997). Similarly, over-expression of TRF2 causes telomere shortening (Smogorzewska et al., 2000), whereas depletion of TRF2 by RNA interference causes telomere lengthening (Takai et al., 2010). Depletion of TIN2 by RNA interference or over-expression of tankyrase1 causes ADP-ribosylation of TRF1, removal of TRF1 from telomeres, and telomere elongation (Smith and de Lange, 2000; Ye and de Lange, 2004). TRF1 and ATM act antagonistically in the control of telomere length in a similar manner to the competition between Rif1/Rif2 and Tel1 in S. cerevisiae. Small molecule-mediated inhibition of ATM causes an increase in telomeric TRF1 levels in an NBS1-dependent manner, as well as telomere shortening, whereas over-expression of NBS1 reduces telomeric TRF1 levels (Wu et al., 2007b). Thus, ATM and MRN function in the same pathway, akin to the Tel1/MRX pathway in S. cerevisiae. ATM-dependent phosphorylation of TRF1 reduces the binding activity of TRF1, which may explain how ATM regulates telomere length (Wu et al., 2007b).

The shelterin complex ultimately controls telomere length through TPP1 and POT1 which can accumulate on long telomeres via protein-protein interactions and also bind directly (as for POT1) or indirectly (through POT1, as for TPP1) to the G-rich overhang, thereby limiting the ability of telomerase to access its substrate (Takai et al., 2010). These interactions and the functions of POT1 and TPP1 in telomere length control are discussed in Appendix A. Human RAP1, unlike its yeast counterpart, does not bind directly to DNA but rather binds to TRF2 (Li et al., 2000). Over-expression of RAP1 causes telomere elongation, perhaps by titrating telomere length control complexes away from the telomere (Li and de Lange, 2003; Li et al., 2000). Unlike in budding yeast, the human ortholog of Rif1 does not appear to function in telomere length regulation, but does have a role in the telomere and DNA damage responses (Silverman et al., 2004; Xu and Blackburn, 2004).

In mice as in yeast, short telomeres appear to be preferentially elongated by telomerase. mTert+/− embryonic stem cells are haploinsufficient for the maintenance of average telomere length, but retain a
minimal amount of telomeric DNA at all chromosome ends (Erdmann et al., 2004; Liu et al., 2002). Also, re-introduction of mTert or mTerc (the telomerase RNA gene) into mTert<sup>−/−</sup> or mTerc<sup>−/−</sup> mice, respectively, while unable to prevent shortening of the bulk telomere population, restores telomere DNA to chromosome ends lacking detectable telomeres (Erdmann et al., 2004; Hemann et al., 2001). These findings suggest that limiting levels of telomerase are sufficient to prevent genetic instability by preferentially elongating the shortest telomeres. Atm is not required for the maintenance of minimal telomere DNA in this context, in apparent contrast to the involvement of Tel1 in recruiting telomerase to short telomeres in S. cerevisiae (Feldser et al., 2006).

In human primary lung fibroblasts transduced with hTERT, short telomeres are rapidly and selectively elongated over several population doublings until they have become sufficiently long, at which point telomeres are maintained at an equilibrium length (Britt-Compton et al., 2009). Interestingly, most telomeres in cancer cells are elongated during each round of telomere replication suggesting that, in this context, telomere length-sensing mechanisms are uncoupled from regulatory pathways (Zhao et al., 2009). However, telomeres in cancer cells are often maintained at a relatively short length (de Lange et al., 1990; reviewed in Shay and Wright, 2005), and hence may be prime substrates for telomerase. The minimum telomere length that no longer elicits telomerase recruitment and telomere elongation in normal or cancer cells has not been determined.

**G-strand product of telomerase becomes the template for lagging strand synthesis**

Once engaged to act at a telomere, telomerase extends the G-overhang in the 5′-to-3′ direction by reverse transcribing the template sequence contained in the telomerase RNA (TLC1 or hTR in S. cerevisiae or humans, respectively). In human cells that contain telomerase activity, enough DNA is added to the G-strand to counterbalance telomere shortening, or at least to prevent the loss of telomere end protection and/or senescence (Ouellette et al., 2000; Zhao et al., 2009; Zhu et al., 1999). In S. cerevisiae, fewer than 10% of telomeres are lengthened by telomerase in a given cell cycle, and those telomeres that are
lengthened gain a few to 100 nucleotides of telomeric DNA (Teixeira et al., 2004). The extension of G-overhangs is followed by polymerization of the complementary lagging C-rich strand (Dionne and Wellinger, 1996; Wellinger et al., 1993a; Wellinger et al., 1993b). A C-strand fill-in reaction limits the presence of recombination-prone, checkpoint-inducing single-stranded regions (Carson and Hartwell, 1985; Garvik et al., 1995), and contributes to telomere elongation by maintaining the duplex architecture.

**Recruitment of lagging strand synthesis to the nascent G-strand**

C-strand synthesis is likely initiated by DNA primase and DNA Pol α, since DNA Pol α is the only polymerase known to initiate DNA replication (Waga and Stillman, 1998), and appears to be coupled to telomerase and the nascent G-strand. During macronuclear development in the ciliate *E. crassus*, when thousands (to millions) of new telomeres are synthesized at the ends of gene-sized chromosome fragments, DNA primase (p48) and Pena, the Pol δ processivity factor, are associated with telomerase (Greene and Shippen, 1998; Ray et al., 2002). In *S. pombe*, Pol α and Trt1 (TERT) can be reciprocally co-immunoprecipitated (Dahlen et al., 2003). Therefore, complexes containing the ability to both elongate telomeres and replicate the lagging strand exist, and DNA replication machinery may accompany telomerase during telomere elongation and commence lagging strand synthesis at a given point.

The stimulus to initiate lagging strand synthesis at the telomere may be a sufficient template length, or a protein complex assembled on the nascent G-strand (Fan and Price, 1997; Ray et al., 2002). In addition to its roles in protecting telomeres from degradation (Garvik et al., 1995) and recruiting telomerase in concert with Est1 (Pennock et al., 2001; Qi and Zakian, 2000), Cdc13 may also recruit the Pol α/primase complex to the telomere. Cdc13 interacts with Pol1, the catalytic subunit of DNA Pol α (Hsu et al., 2004; Qi and Zakian, 2000). The N-terminus of Stn1 interacts with Ten1 to protect telomere ends, while the C-terminus of Stn1 interacts with Cdc13 and Pol12 – another subunit of the Pol α complex (Grandin et al., 1997; Grossi et al., 2004; Pennock et al., 2001; Puglisi et al., 2008). Thus, Cdc13 may act as a molecular switch to promote telomere elongation by telomerase or semi-conservative replication of
telomeres depending on its binding partner (Chandra et al., 2001; Grandin et al., 2000; Puglisi et al., 2008; Qi and Zakian, 2000; Ray et al., 2002).

Less is known about the C-strand fill-in reaction in humans. CTC1 and STN1 are components of the alpha accessory factor that stimulates Pol α/primase (Casteel et al., 2009; Miyake et al., 2009; see below; Surovtseva et al., 2009). Interestingly, in cancer cells, C-strand fill-in is delayed until late S phase and proceeds in a discontinuous manner, suggesting the involvement of a process other than conventional lagging strand synthesis (Zhao et al., 2009).

**C-strand fill-in negatively regulates telomerase**

The synthesis of the C-strand by lagging strand replication affects the ability of telomerase to elongate telomeres. *E. crassus* has been a particularly useful organism in which to study this phenomenon. The generation of telomeres during macronuclear development occurs after general DNA synthesis (Prescott, 1994). Remarkably, when DNA polymerases are inhibited with aphidicolin just prior to telomere formation, the G-strand becomes much longer while the C-rich strand becomes heterogeneous in length, suggesting that telomere C-strand synthesis by DNA polymerases normally inhibits extensive G-strand elongation by telomerase (Fan and Price, 1997). Mutations in some *S. cerevisiae* DNA replication factors also affect telomere length. Numerous alleles of the two subunits of DNA Pol α, Pol1 and Pol12, including many alleles with intact catalytic activity, cause telomere lengthening. In some of these mutants, lengthening has been confirmed to depend on telomerase (Adams and Holm, 1996; Adams Martin et al., 2000; Carson and Hartwell, 1985; Chandra et al., 2001; Grossi et al., 2004; Ohya et al., 2002; Pennock et al., 2001; Qi and Zakian, 2000). Consistent with a defect in lagging strand synthesis and failure to generate duplex telomere DNA, some of these mutants exhibit long G-strand overhangs, increased rates of telomere-proximal recombination, and/or a decreased silencing of telomere-proximal genes. Telomerase-dependent telomere lengthening also occurs upon mutation of replication factor C (Cdc44) (Adams and Holm, 1996), suggesting that the switch from DNA Pol α to Pol δ may be important
for complete lagging strand synthesis and inhibition of telomerase. On the other hand, mutation of the catalytic subunit of DNA Pol δ (Cdc2) does not result in abnormal telomere lengthening, nor does mutation of thymidylate kinase (Cdc8), DNA ligase (Cdc9) or thymidylate synthetase (Cdc21) (Adams and Holm, 1996).

Providing further evidence that lagging strand synthesis activities are recruited to a newly generated G-strand, mutations in Pol1 or Cdc13 that disrupt their mutual interaction result in telomerase-dependent telomere lengthening (Qi and Zakian, 2000). Furthermore, the lengthening observed in some Pol1 mutants can be suppressed by the fusion of the Cdc13 DNA-binding domain to Pol1, or over-expression of Stn1 (Chandra et al., 2001; Pennock et al., 2001). Interestingly, the pol12-109 mutation, which results in telomere lengthening (see below), and the stn1-13 mutation, which results in longer G-strand overhangs, are synthetically lethal (Grossi et al., 2004). It would be interesting to determine whether this lethality is rescued by removing telomerase.¹ In S. pombe, telomeres become longer when DNA primase, DNA Pol α or Pol δ is mutated. The elongation of telomeres in DNA primase mutants is telomerase-dependent, while the progeny of a cross between pol α and trt1 mutants exhibit a synthetic lethal phenotype (Dahlen et al., 2003). In human cells, the G-strand overhang becomes longer upon depletion of CTC1 or STN1 (telomere binding components of the alpha accessory factor; see above) in telomerase-positive cells (Casteel et al., 2009; Miyake et al., 2009; Surovtseva et al., 2009), suggesting that the inhibitory relationship between lagging strand synthesis and telomere elongation by telomerase is conserved. Taken together, these data suggest that recruitment of lagging strand replication proteins to telomeres displaces or inhibits telomerase. According to this model, if activities that initiate lagging strand synthesis are impaired in their localization to telomeres, then telomerase can continue to act and telomeres become hyper-extended. One appealing mechanism to explain this effect is that C-strand fill-in of telomerase-generated single stranded DNA may generate a substrate for double-stranded telomere-binding proteins and their partners that inhibit, in cis, further lengthening of telomeres. When C-strand

¹ Over-elongation of the G-overhang by telomerase in this context may have deleterious consequences.
fill-in does not occur, a lack of negative feedback from double-stranded telomeric chromatin may allow telomerase to continue extending the G-strand (Adams and Holm, 1996; Fan and Price, 1997). However, this model conflicts with the observation that the frequency, not the extent, of telomerase-mediated telomere elongation is affected by Rif proteins (Teixeira et al., 2004). Observing changes in the complement of proteins bound to telomeres in the lagging strand replication mutants described above may illuminate the inhibitory mechanism.

**REGULATION OF TELOMERASE BY DNA AND PROTEIN INTERACTIONS**

Despite our knowledge of telomere structure and function, telomere replication, and the role of telomerase in telomere maintenance, several critical aspects of telomere biology are yet to be discovered. This thesis describes the elucidation of key DNA and protein interactions that regulate human and *S. cerevisiae* telomerases. In Chapter 1, we identify roles for the essential N-terminus of hTERT in binding telomeric DNA and enabling telomerase function in cells. In Chapter 2, we describe an ability of human telomerase to operate as a dimer *in vitro*, and identify TERT-TERT interactions that may mediate this dimerization. In Chapter 3, we explore the functional conservation of human homologs of *S. cerevisiae* Est1. We map the interaction between hEST1A and hTERT, and identify roles for a conserved domain in ScEst1 in regulating telomerase. Finally, in Appendix A, we identify an interaction between hTERT and hPOT1 that may affect the recruitment of telomerase to the telomere and/or the processivity of telomerase at the telomere.
Table I-1. Telomere sequences and structure in different organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>G-rich sequence</th>
<th>Duplex (bp)</th>
<th>G-overhang (nt)</th>
<th>3' terminus</th>
<th>5' terminus</th>
<th>T-loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>*<em>Ciliates</em></td>
<td></td>
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<tr>
<td>E. crassus</td>
<td>GGGTTTTT</td>
<td>80-100</td>
<td>13-15</td>
<td>TGGGGTTT-3'</td>
<td>AAAACCC-5'</td>
<td>N.D.</td>
</tr>
<tr>
<td>T. thermophila</td>
<td>TTGGGG</td>
<td>250-300</td>
<td>14-21</td>
<td>TGGGGT-3'</td>
<td>AACCC-5'</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Flagellates</strong></td>
<td></td>
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<tr>
<td>Trypanosoma</td>
<td>TTAGGG</td>
<td>10-20k</td>
<td>75-225</td>
<td>N.D.</td>
<td>N.D.</td>
<td>~1 kbp</td>
</tr>
<tr>
<td><strong>Yeasts</strong></td>
<td></td>
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<td></td>
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<tr>
<td>S. cerevisiae</td>
<td>TG$<em>{2-3}$(TG)$</em>{1-6}$</td>
<td>~300</td>
<td>12-14 outside of S &gt;30 (S phase)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>S. pombe</td>
<td>TTAC(A)G$_{2-5}$</td>
<td>~300</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Plants</strong></td>
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</tr>
<tr>
<td>A. thaliana</td>
<td>TTTAGGG</td>
<td>2-4.5k</td>
<td>20-30</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Mammals</strong></td>
<td></td>
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<tr>
<td>H. sapiens</td>
<td>TTAGGG</td>
<td>5-15k</td>
<td>35-600</td>
<td>GGTTAGG-3'</td>
<td>CCAATC-5'</td>
<td>&lt;25 kbp</td>
</tr>
<tr>
<td>M. musculus</td>
<td>TTAGGG</td>
<td>10-100k</td>
<td>35-600</td>
<td>N.D.</td>
<td>CCAATC-5'</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. not determined. * Macronuclear DNA ends are listed. # Not yet determined to exist in vivo. Underlined nucleotides indicate the most frequent positions at which the chromosome ends, e.g. AACCC-5' indicates that the majority of 5' ends terminate in AACCC-5' or AACC-5'.
Figure I-1. Telomere replication and telomerase-mediated extension. (I) Schematic representation of a linear chromosome end. The G-rich telomere strand protrudes beyond the duplex yielding a 3’ single-stranded overhang. (II) DNA replication proceeds bi-directionally from an origin located upstream of the telomere. The terminal Okazaki fragment is initiated at or upstream of the 3’ end of the G-rich template (A). Leading strand synthesis may completely replicate the C-rich template (B). (III) Removal of the terminal lagging strand RNA primer (A) and C-strand resection by a nuclease (A and/or B) result in telomere shortening, and create a single-stranded G-rich substrate for telomerase. (IV) Telomerase reverse transcribes G-rich telomere repeats onto the end of the G-rich strand according to the template sequence specified by the associated telomerase RNA. (V) The G-rich telomere extension product becomes the template for lagging strand replication. (VI) Removal of the RNA primers initiating lagging strand synthesis and subsequent nucleolytic trimming of C- and/or G-strands generate a mature telomere end structure. (Legend) Ori, origin of replication; solid line, parental DNA; broken line, nascent DNA; thin light grey, subtelomeric and genomic DNA; black, G-rich telomere DNA; dark grey, C-rich telomere DNA; thick black arrow, RNA primer; rounded broken black, nascent G-rich telomere DNA; ?, uncertain primer alignment or extent of DNA end processing.
CHAPTER 1

The N-terminus of hTERT contains a DNA binding domain and is required for telomerase activity and cellular immortalization.

The experiments described in this Chapter were designed, executed and interpreted by me in the laboratory of Lea Harrington, with the following exceptions, for which credit is shared as indicated. **Figure 1-2.** Theo Goh, David Sealey, Le Zheng. **Figure 1-3.** (A-B) Le Zheng, David Sealey. (C) David Sealey, Le Zheng. **Figure 1-4.** (A) Le Zheng, David Sealey. (B-C) David Sealey, Le Zheng. **Figures 1-5 to 1-9.** David Sealey, Le Zheng. **Figure 1-10.** (B) David Sealey, Jennifer Cruickshank, Le Zheng. **Figure 1-11.** (A-C) Le Zheng, David Sealey. (D) David Sealey, Le Zheng. **Figure 1-12.** Michael Taboski, David Sealey. **Figure 1-13.** (A-C) Michael Taboski, David Sealey. (D) David Sealey, Michael Taboski. **Figure 1-14.** Michael Taboski, David Sealey. For additional details, refer to Statement of Contributions.

Portions of this Chapter appear in the following publication: (Sealey et al., 2009).
INTRODUCTION

TERT contains several evolutionarily conserved domains. Domains in the C-terminal half of hTERT (amino acids 601-936) share homology with other reverse transcriptases and form the active site of the enzyme (Harrington et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997). Domains in the central region of the protein (CP, QFP, T; amino acids 397-594) bind the telomerase RNA (reviewed in Kelleher et al., 2002). A conserved N-terminal domain (GQ), identified by multiple sequence alignment of mammalian, yeast, ciliate and plant TERT sequences (Xia et al., 2000), is separated from the CP domain by a non-conserved region that varies in length between organisms (Kelleher et al., 2002).

Telomerase exhibits nucleotide addition processivity by adding one nucleotide at a time onto the 3′ end of a DNA primer up to the 5′ end of the RNA template. While S. cerevisiae telomerase, for example, has the ability to add only a limited number of telomeric repeats onto a DNA primer in vitro (Bosoy and Lue, 2004), human and T. thermophila telomerases exhibit an ability to add multiple telomeric repeats without dissociating from the primer (Greider, 1991; Prowse et al., 1993). Observations that the 5′ end of the primer can influence substrate utilization led to the notion that a so-called “anchor site” outside of the reverse transcriptase domain of TERT contacts the DNA primer upstream of the active site and may facilitate iterative copying and repositioning of the RNA template (Collins and Greider, 1993; Hammond et al., 1997; Harrington and Greider, 1991; Lee and Blackburn, 1993; Morin, 1989; Morin, 1991).

Mounting evidence suggests that the N-terminus of TERT contains this anchor site for DNA (reviewed in Lue, 2004) that is important for both telomerase activity and repeat addition processivity.

Structural determination of the N-terminus of T. thermophila TERT revealed a groove on one side of the domain that may accommodate single-stranded DNA (Jacobs et al., 2006). The domain can crosslink to single-stranded DNA primers, and removal of the domain or mutation of exposed residues along the groove reduces primer crosslinking and impairs telomere DNA elongation in vitro (Finger and Bryan, 2008; Jacobs et al., 2006; Romi et al., 2007). The N-termini of S. cerevisiae and human TERTs also
interact with DNA (Lue, 2005; Wyatt et al., 2007; Wyatt et al., 2009; Xia et al., 2000), and mutations in the region have been identified that impair telomerase activity/processivity and, in some cases, the ability of telomerase to immortalize cells (Armbruster et al., 2001; Beattie et al., 2000; Beattie et al., 2001; Friedeman and Cech, 1999; Lee et al., 2003; Lue, 2005; Lue and Li, 2007; Moriarty et al., 2004; Moriarty et al., 2005; Wyatt et al., 2007; Wyatt et al., 2009; Xia et al., 2000). Thus, this region has been dubbed the telomerase essential N-terminal (TEN) domain (Jacobs et al., 2006).

Although the N-terminus of TERT contains a region of homology (GQ), the sequence conservation between human and T. thermophila TERTs in this region is especially low. Previous studies have shown that TtTERT and hTERT generated in reticulocyte lysates in the absence of hTR interact with telomeric DNA oligonucleotides in crosslinking and biotinylated oligonucleotide capture assays, respectively (Sperger and Cech, 2001; Wyatt et al., 2007; Wyatt et al., 2009; Lea Harrington, unpublished); however, conclusive evidence that hTERT contacts DNA directly has not been described.

We hypothesized that hTERT contains one or more domains outside the active site that contact DNA independently of hTR and contribute to the ability of telomerase to elongate DNA primers and extend the replicative lifespan of cells. This Chapter describes a structure-function study of hTERT that resulted in the identification of a DNA binding domain and characterization of its function in vitro and in cells.

**MATERIALS AND METHODS**

**Biotinylated oligonucleotide capture assay**

Neutravidin resin (Pierce) was equilibrated in CHAPS buffer (0.5% w/v CHAPS, 10 mM Tris-Cl pH 7.5, 10% v/v glycerol, 100 mM NaCl, 1 mM MgCl₂). 20 µL aliquots of a 50% w/v slurry were incubated with 1 µg of 5'-BIO-(TTAGGG)₄TTAG-3' (PAGE-purified telomeric DNA oligonucleotide biotinylated at the 5' end, Operon) in 300 µL of CHAPS buffer for 30 min at 4°C. Unbound oligonucleotides were removed by washing three times in CHAPS buffer. Coupled in vitro transcription-translation reactions (rabbit reticulocyte lysates, RRL; Promega Corp.) were prepared with 0.01 µg/µL pCR3-FLAG-hTERT-FLAG
cDNA, and incubated for 90 min at 30°C. Mock reactions did not contain a cDNA template. 20 µL of the indicated RRL reaction was added to DNA-coupled neutravidin resin in 500 µL CHAPS buffer containing 14.3 mM β-mercaptoethanol and EDTA-free Complete protease inhibitor cocktail tablet (Roche). Mixtures were rocked at 4°C for 1 hr. Unbound protein was washed away by washing the resin three times in 1 mL cold CHAPS buffer (with β-mercaptoethanol and protease inhibitor cocktail) at 4°C. The final pellet was boiled in SDS-PAGE loading dye and proteins were resolved through 4-20% w/v Tris-Glycine Novex gels, transferred to PVDF membranes (Invitrogen), and subjected to immunoblotting with anti-FLAG M2 (Sigma) and sheep anti-mouse IgG-HRP (GE Healthcare) antibodies. Reactive bands were detected with ECL reagents (Amersham) and chemiluminescent signals were captured on film (Kodak).

**hTERT cDNA sequence optimized for expression in *E. coli***

To express hTERT in *E. coli*, the DNA sequence encoding hTERT(amino acids 1-200) was optimized by correcting for *E. coli* codon bias and minimizing mRNA secondary structure (GenScript Corp.). The custom DNA sequence, as follows, was synthesized (Blue Heron Biotechnology):

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5′-ATGCCGCAGCAGCCAGC GTTGGCAGCAGCGAGGTG CCGAGCTGCTGCTGT AGCCATTATACGAA
GTGCTGCCCTGCTGC ACCTTTGCTGCCGCG CTGGGGCTGCCAGGTG TGGCGTCTGCTGCAG
CGTGGCGATCCCGCC GCCTTTGCTGCCGCT GTGGCGCAGTCTCCTG GTGGCCTGGCGCTGG
GATGGGCGTCCCGCC CGGGCGCGCGCGGAGC TTTGGCTAGGTCAGC TGCCTGAAAGA CTG
GTGGCGGCTGCTGTC CAGCGCTGCTGCGAA CGCGCGCGCGAAAT TGGCGTCGCTTCTG
TTTGGGCTGCTGCGT GGCGCCGCAGGGCTG CCGCCAGAAGCTGT ACCACCCAGCTGCC
AGCTATCTGCCGAAT ACCGCTGCCAGATCG CTGGCGGTAGCGGC CGTGCTGGGCCTGCTG
CTGGCGTGGCTGCGC GATGATGCTGCTGCT CATCTGCTGGCGCGC TGGCGGCTGCTGCTT
CTGGGCTCAGCCGAGC TGGCCGTATCGAAGG TGGCCGCCGGCCG TGATCGCTGCTGCTGT
GCGACCAGGCAGCGT CGCGCCGCAGATCG AGCGGTGCCGTGCTG CTGGCTGGCGCGA
3′
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**Expression and purification of recombinant hTERT**

The optimized hTERT coding sequence shown above was subcloned into BamHI and XhoI sites of a modified pET32a vector to generate a Thioredoxin(Trx)-HIS6-hTERT(1-200) coding sequence. The
fusion protein of approximately 36 kDa was expressed in BL21(DE3) codon plus *E.coli* (Stratagene). Cells were grown at 37°C to an optical density (at 600 nm) of 1.0 and expression was induced with 0.2 mM IPTG at 15°C overnight. Cells were harvested by centrifugation and frozen at -80°C. Cell pellets were resuspended in lysis buffer (50 mM Tris-Cl pH 7.5, 25% v/v glycerol, 500 mM NaCl, 0.2% v/v NP40, 10 mM imidazole, 1 mM DTT, 0.2 mM TCEP, Roche Complete protease inhibitor cocktail) and incubated with 0.1 mg/mL lysozyme and 0.05 mg/mL DNaseI at 4°C. Cell debris was removed by centrifugation at 26,000 x g for 30 min. Soluble lysate was incubated with Ni-NTA resin (Qiagen) at 4°C. After washing with 20 column volumes of wash buffer (50 mM Tris-Cl pH 7.5, 25% v/v glycerol, 1.5 M NaCl, 20 mM imidazole, 1 mM DTT, 0.2 mM TCEP), bound proteins were eluted in 50 mM Tris-Cl pH 7.5, 25% v/v glycerol, 500 mM NaCl, 300 mM imidazole, 1 mM DTT, and 0.2 mM TCEP.

Trx-HIS6-hTERT(1-200) was further purified by HiTrap SP cation exchange (GE Healthcare) in a buffer of 50 mM Tris-Cl pH 7.5, 25% v/v glycerol, 5 mM DTT, 0.2 mM TCEP with a 0.08 to 1.0 M NaCl gradient. The protein could not be dialyzed to a low-salt buffer or concentrated without undergoing precipitation. The control Trx-HIS6 protein was expressed from pET32a and purified over Ni-NTA resin.

To analyze protein composition, elution fractions were boiled in SDS-PAGE loading dye and resolved by denaturing electrophoresis alongside the RPN5800 (GE Healthcare) or Benchmark (Invitrogen) molecular weight markers through 4-20% w/v Tris-Glycine Novex gels (Invitrogen). Gels were stained with Deep Purple Total Protein Stain (GE Healthcare) or Coomassie Brilliant Blue (Fisher Scientific), or transferred to PVDF membranes (Invitrogen) and subjected to immunoblotting with anti-Trx primary (Novagen) and sheep anti-mouse IgG-HRP secondary (GE Healthcare) antibodies and ECL Plus detection reagents (GE Healthcare). Deep Purple and ECL Plus fluorescent signals were captured below saturation using a Typhoon Trio variable mode imager (GE Healthcare). Coomassie Brilliant Blue signals were captured using a LI-COR infrared imaging system.
DNA oligonucleotide electrophoretic mobility shift assay

Single-stranded DNA oligonucleotide bearing the telomeric sequence (TTAGGG)$_3$ was labeled at the 5’ end with $^{32}$P. Residual $^\gamma-^{32}$P-ATP was removed by centrifugation though a G25 spin column (GE Healthcare). DNA was boiled for 5 min and cooled on ice to minimize secondary structures. Each 20 μL binding reaction contained 1 nM $^{32}$P-(TTAGGG)$_3$ (or 1.25 nM in Figures 1-4B, 1-5A, 1-6), 50 mM Tris-Cl pH 7.5, glycerol (15% v/v, Figure 1-3C; 11.9%, Figure 1-4B; 12.5%, Figure 1-5A; 10%, Figure 1-5B; 17.5%, Figure 1-11D), 450 mM NaCl, 5 mM DTT (270 mM NaCl and 2.5 mM DTT in Figure 1-5A; 300 mM NaCl and 2 mM DTT in Figure 5-B), and recombinant Trx-HIS6-hTERT(1-200) protein. For the heat denaturation control, protein was denatured on a 95-100°C heating block for 5 min then cooled on ice prior to mixture with DNA. Protein-DNA mixtures were incubated for 30 min at room temperature, then resolved at 15 V/cm at 4°C in cold 0.5X TBE through a pre-cooled gel containing 5% w/v 19:1 acrylamide:bisacrylamide, 0.5X TBE and 5% v/v glycerol. Gels were dried at 80°C for 1 hr and exposed to a phosphorimager screen which was then scanned on a Typhoon Trio variable mode imager. Intensity of the DNA shift signal was quantified using ImageQuant software (GE Healthcare). For competition assays, unlabeled DNA of different sequence/length was mixed with $^{32}$P-(TTAGGG)$_3$ prior to the addition of protein. DNA oligonucleotides were obtained from Integrated DNA Technologies after polyacrylamide gel electrophoresis (PAGE) purification.

Mass spectrometry analysis of recombinant Trx-HIS6-hTERT(1-200)

Proteins expressed in *E. coli* and purified as described above were boiled in SDS-PAGE loading dye, resolved through a 4-20% w/v Tris-Glycine Novex gel and stained using the SilverQuest Silver Staining Kit according to the manufacturer’s instructions (Invitrogen). The gel was scanned using an Epson Perfection 3490 Photo flatbed optical scanner using Epson Scan software (version 2.75A). Gel bands were excised manually by scalpel and destained by incubation in 50% v/v 50 mM NH$_4$HCO$_3$ / 50% v/v methanol for 10 min on ice. Gel pieces were dehydrated in 10% 50 mM NH$_4$HCO$_3$ / 90% methanol for
10 min on ice with occasional mixing. Disulphide bonds were reduced by the addition of 10 mM DTT in 50 mM NH₄HCO₃ solution for 45 min at 56°C. Gel bands were again dehydrated in 10% v/v 50 mM NH₄HCO₃ / 90% v/v methanol for 10 min on ice with occasional mixing. Cysteines were alkylated by incubating gel bands in 50 mM NH₄HCO₃ solution containing 55 mM iodoacetamide in darkness for 30 min at 25°C. Gel pieces were washed with 50 mM NH₄HCO₃ for 10 min on ice. Gel pieces were dehydrated in 10% v/v 50 mM NH₄HCO₃ / 90% v/v methanol for 10 min on ice with occasional mixing. Proteins were digested by adding 15 µL 2.5 ng/µL trypsin in 50 mM NH₄HCO₃ solution at 37°C overnight. 10 µL of 50 mM NH₄HCO₃ solution was added to prevent gel bands from drying out. After transferring the supernatant to fresh tubes on ice, peptides were extracted by the addition of 20 µL 5% v/v formic acid at room temperate for 30 min. Extracts were transferred to fresh tubes on ice, and dehydrated in 100% v/v methanol for 30 min at room temperature with occasional mixing. Extracts were transferred to fresh tubes on ice, and subjected to speed-vacuum under low heat until dry. Peptides were dissolved in buffer A (2.5% v/v acetonitrile, 0.1% v/v formic acid). Peptides were subjected to reversed-phase gradient separation through C18 micro-columns (inner diameter = 75 µm, length = 10 cm) into buffer B (90% v/v acetonitrile, 0.1% v/v formic acid, 0.02% v/v trifluoroacetic acid) for at least 1 hr on an Agilent 1100 HPLC instrument, and analyzed in-line using a Thermo-Fisher LTQ-Orbitrap mass spectrometer. Bands in Figure 1-2 were analyzed in the following order: 8 to 1, 18 to 9.¹

Reconstitution of telomerase in reticulocyte lysates

Telomerase activity was reconstituted in rabbit reticulocyte lysates (RRL) as described (Beattie et al., 1998; Weinrich et al., 1997), with modifications. Human telomerase RNA (hTR) was transcribed in vitro using the RiboMax kit (Promega) and gel-purified. Coupled in vitro transcription-translation reactions (Promega Corp.) were prepared with 0.01 µg/µL pCR3-FLAG-hTERT-FLAG cDNA and 0.01 µg/µL hTR RNA, and incubated for 90 min at 30°C. To generate hTERT fragments, the following cDNAs were

¹ LC-MS runs for Bands 2 and 9 crashed, resulting in loss of most of the samples (Figure 1-2). Few additional peptides were identified during a second run.
expressed in separate RRL reactions for 60 min: pCR3-FLAG-hTERT(201-1132)-FLAG; pBiEx3-hTERT(1-200)-S-HIS8; pET32a-Trx-HIS6-hTERT(1-200). To combine fragments, 15-20 µL of each reaction was mixed and incubated for an additional 60 min. Mock reactions did not contain a cDNA template. RRL mixtures were then diluted to 500 µL with cold CHAPS buffer (0.5% w/v CHAPS, 10 mM Tris-Cl pH 7.5, 10% v/v glycerol, 100 mM NaCl, 1 mM MgCl₂ 1 mM DTT, Roche EDTA-free Complete protease inhibitor cocktail) and rocked for 1 hr at 4°C. FLAG-tagged complexes were immunoprecipitated with anti-FLAG M2 affinity resin (Sigma) and washed three times with 1 mL cold CHAPS buffer. For PCR-based analysis of telomerase activity, 1 µL of the immunoprecipitate was assayed by the TRAP assay (described below). The remainder of the pellet was boiled in SDS-PAGE loading dye and proteins were resolved through 4-20% w/v Tris-Glycine Novex gels, transferred to PVDF membranes (Invitrogen) and subjected to immunoblotting. Primary antibodies employed were as follows: S protein-HRP (Novagen); rabbit anti-Trx antibody (Sigma); and mouse anti-FLAG M2 antibody (Sigma). Secondary antibodies employed were donkey anti-rabbit IgG-HRP and sheep anti-mouse IgG-HRP (GE Healthcare). Reactive bands were detected with ECL Plus reagents (GE Healthcare) and fluorescent signals were scanned on a Typhoon Trio variable mode imager. For direct analysis of primer elongation activity, the entire immunoprecipitate was analyzed by the standard elongation assay (described below).

**Telomerase activity assays**

Telomerase activity was assessed using the Telomere Repeat Amplification Protocol (TRAP) or Standard Elongation (SE) assay. TRAP assays were performed using the TRAPEze kit (Millipore) with modifications to the manufacturer’s protocol. Cell lysate or in vitro-reconstituted telomerase was incubated with the TS primer at 30°C for 30 min prior to heat inactivation at 95°C for 2 min. Reactions were held at 95°C while Taq DNA polymerase (New England Biolabs) was added. Primer extension products were amplified through 25 cycles of 30 s at 94°C, 30 s at 50°C and 90 s at 72°C. 20 µL of each 50 µL reaction was combined with DNA loading dye and resolved through a 10% w/v 19:1
acrylamide:bisacrylamide gel in 0.6X TBE at 15-20 V/cm. DNA was stained with SYBR Green I (Sigma) and fluorescent signals were scanned on a Typhoon Trio variable mode imager.

For SE assays, *in vitro*-reconstituted telomerase was incubated with 200 pmol PAGE-purified (TTAGGG)$_3$ (IDT), 40 µCi of [$\alpha$-$^{32}$P]-dGTP (Perkin Elmer, 6000 Ci/mmol, 20 mCi/mL) and 1X SE buffer (2 mM dATP, 2 mM dTTP, 1 mM MgCl$_2$, 1 mM spermidine, 5 mM $\beta$-mercaptoethanol, 50 mM potassium acetate, 50 mM Tris acetate pH 8.5) in a 40 µL volume for 2 hr at 30°C. Samples were treated with RNase A at 37°C for 15 min, followed by Proteinase K at 37°C for 15 min. Samples were phenol:chloroform extracted. 150 cpm $^{32}$P-end-labeled 96-mer DNA was added to each reaction as a loading control. DNA was precipitated overnight at -20°C by the addition of 1/10 volume sodium acetate pH 5.2, 2 volumes cold ethanol and 5 µg GenElute linear polyacrylamide (Sigma). DNA was pelleted by centrifugation and resuspended in 3 µL gel loading buffer (100% v/v formamide, 0.6X TBE). Samples were boiled for 3 min and resolved by electrophoresis in 0.6X TBE through a 10% w/v denaturing polyacrylamide gel (29:1, acrylamide:bisacrylamide, 7 M urea, 0.6X TBE). The gel was dried at 80°C for 1 hr, and then exposed to a phosphorimager screen which was then scanned on a Typhoon Trio variable mode imager.

**Mutagenesis of hTERT(1-200)**

The structure-based sequence alignment of the N-terminus of TERT (Jacobs *et al.*, 2006) was used as a guide to select residues in hTERT(1-200) that may be exposed to the surface of the protein. The design of PCR primers for site-directed mutagenesis was aided by The Primer Generator (Turchin and Lawler, 1999). Primers were obtained from Operon and IDT. The incorporation of intended mutations into the cDNA (and the absence of unwanted mutations) was confirmed by DNA sequencing.

**Generation and passaging of stable cell lines**

pcDNA3.1(HYG)-hTERT vectors were linearized with SspI, treated with calf intestinal phosphatase (New England Biolabs), and gel-purified. Human embryonic kidney HA5 cells were transfected with either
wild-type or mutant hTERT DNA using FuGENE6 reagent (Roche). Cells were grown in alpha-Minimal Essential Medium (α-MEM) containing 10% v/v fetal bovine serum and 2 mM L-glutamine, and then selected with 200 µg/mL (Figure 1-12) or 100 µg/mL hygromycin (Figure 1-13A). Cells undergoing mock transfection (with no DNA) did not survive selection. After 4-5 weeks of selection, colonies were treated with TrypLE Express (Invitrogen) and pooled. Polyclonal populations were passaged in media containing hygromycin by plating 2 x 10^5 cells in a 10 cm plate at regular intervals. The number of cumulative population doublings at each passage was determined by the formula as described (Hayflick, 1973). The experiment was performed with late-passage cells approaching crisis (Figure 1-12), and also with cells at an earlier passage (Figure 1-13). Apoptosis was assessed using the TiterTACS assay (R&D Systems) and by visual inspection of cellular morphology under microscopy.

**Protein extraction**

Cells were washed with ice cold PBS and dislodged from tissue culture plates using a cell scraper. Cells were resuspended and lysed in 4-5 volumes of ice-cold cold CHAPS buffer (0.5% w/v CHAPS, 10 mM Tris-Cl pH 7.5, 10% v/v glycerol, 1 mM MgCl₂, 5 mM β-mercaptoethanol, Roche EDTA-free Complete protease inhibitor and 400 U Roche RNase Inhibitor) for 30 min on ice. Insoluble material was pelleted by centrifugation at 13,000 rpm for 30 min. The protein concentration of the cleared whole cell extract was determined by the Bradford Assay (BioRad).

**Analysis of mRNA by RT-PCR**

Total RNA was isolated from cells using TRIzol reagent (Invitrogen), and then treated with DNase I (Roche). cDNA was generated from RNA using Superscript II reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen). Residual RNA was removed from cDNA products using RNase H (Invitrogen). cDNAs were amplified by PCR using Taq DNA polymerase (New England Biolabs) under the following conditions: 94°C for 5 min, followed by 32 cycles of 94°C for 45 s, 57°C for 45 s and 72°C for 1 min, followed by a final extension of 72°C for 4 min. The following gene-specific primers were
used: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and 5′-TGCTAAGCAGTTGGTGTGCAAGA-3′; hTERT, 5′-AAGTTCCCTGCACTGGCTGATGAG-3′ and 5′-TCGTagTTGAGCACCCTAAACAG-3′; hygromycin-resistance gene (HYG, hygromycin phosphotransferase), 5′-CGCAAGGAATCGGTCAATAC-3′ and 5′-ACATTGTGGAGCGCGAAAC-3′. DNA was resolved through a 0.8% w/v agarose gel, stained with ethidium bromide, and imaged on a Typhoon Trio variable mode imager.

Telomere length analysis

Genomic DNA was isolated from cells using the DNeasy kit (Qiagen) and digested with RsaI and HinfI. Restriction fragments were resolved through a 0.5% w/v agarose gel at 45 V (2 V/cm) for 24 hr. DNA was denatured in buffer containing 0.5 M NaOH, 1.5 mM NaCl for 30 min, and neutralized in buffer containing 1.5 M NaCl, 0.5 mM Tris-Cl pH 7.5 for 30 min. DNA was transferred to Hybond-N+ membrane in 20X SSC. Following transfer, DNA was UV-crosslinked to the membrane, which was then rinsed in 2X SSC. Telomeric DNA was hybridized to a 32P 5′-end-labeled (CCCTAA)3 probe in Church buffer (0.5 M NaPO4 pH 7.2, 1% w/v BSA, 7% w/v SDS, 1 mM EDTA), then washed with 1X SSC, 0.1% w/v SDS. The membrane was exposed to a phosphorimager screen which was then scanned using a Typhoon Trio variable mode imager. The weighted mean telomere length in each lane was calculated according the following formula, as previously described (Chai et al., 2005): \( \frac{\sum \text{OD}_i}{\sum (\text{OD}_i/L_i)} \), where \( \text{OD}_i \) is the signal intensity and \( L_i \) is the length in nucleotides of DNA at position \( i \) as determined by comparison to the molecular mass standards.

RESULTS

Preliminary identification of DNA binding domains in hTERT

To investigate the interaction of hTERT with DNA, I generated fragments of hTERT in reticulocyte lysates and assayed for precipitation onto a biotinylated 28-mer telomeric DNA oligonucleotide. The N-terminus of hTERT (amino acids 1-350), was captured onto the telomeric oligonucleotide (Figure
These results are consistent with findings that unpurified hTERT(1-1132), (1-300), and (1-350) produced in reticulocyte lysates interact with telomeric DNA oligonucleotides (Wyatt et al., 2007; Wyatt et al., 2009). I also observed that hTERT(601-1132) was captured onto the telomeric oligonucleotide (Figure 1-1A-B). The apparent interaction of the C-terminus of hTERT with the telomeric oligonucleotide was recapitulated with hTERT(928-1132), indicating that hTERT may contain at least two interaction sites for DNA that do not require hTR. hTERT(601-928) precipitated onto the resin non-specifically (Figure 1-1B, lanes 2-3), suggesting that this fragment (containing the reverse transcriptase domain) may not be soluble in reticulocyte lysates.

Production of recombinant hTERT(1-200) in a bacterial expression system

To clarify these apparent hTERT:DNA interactions, we developed and tested many strategies to produce recombinant hTERT. Although efforts to express the C-terminus of hTERT in E. coli were not successful, we expressed Trx-HIS6-hTERT(1-200) in E. coli and, despite a low expression level and limited solubility (less than 5% of expressed protein was recovered in the soluble fraction of the cell lysate), the protein was purified over two chromatographic steps to a modest degree of homogeneity (see

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1 We attempted multiple strategies to express and purify recombinant hTERT alone or in combination with other protein and RNA binding partners. Most of these strategies (described below) were ineffective, due to low expression and/or solubility of the target protein(s), protein fragmentation, and/or co-purification with host proteins. I designed and tested four vectors for the expression of hTERT(1-200) (hTERT-N) or smaller fragments and one vector for the expression of hTERT(936-1132) in E. coli. I attempted to express one of the hTERT-N constructs in RRL, and three of the hTERT-N constructs in SF9 cells. Le Zheng validated my unfavourable results using one of the hTERT-N constructs in E. coli, and also attempted to express the construct in a rapid translation system (Roche). In consultation with me and Chris Marshall, Le Zheng designed and tested three constructs for the expression of hTERT-N in E. coli. With advice from Susan McCracken, Le Zheng and I evaluated four protocols for the purification of hTERT-N expressed from one of these constructs; one of these protocols (refer to Materials and Methods) yielded a sufficient quantity of hTERT-N of adequate purity for use in experiments. Also, in consultation with me, Chris Marshall designed and tested more than one construct for the expression of the C-terminus of hTERT (hTERT-C) in E. coli. The optical densities of bacteria expressing hTERT-C, as well as the yields of hTERT-C, were very low, suggesting a toxic effect. Given that hTERT-N and hTERT-C interact (refer to Chapter 2), Le Zheng and Chris Marshall, in consultation with me, attempted to co-express hTERT-N and hTERT-C using aforementioned constructs that they had developed. Following additional consultation with Mitsu Ikura, Le Zheng and Chris Marshall designed and tested three vectors encoding hTERT-N and hTERT-C fused through a flexible polypeptide sequence. Given our interest in reconstituting the telomerase complex in a mammalian expression system, and our finding that hTERT and hEST1A interact (refer to Chapter 3), Le Zheng, in consultation with me, attempted to express the following using a T7-based vaccinia virus/HeLa cell expression system: hTERT, hTERT in combination with hEST1A and/or hTR, hTERT-N, and hTERT-N in combination with the hTERT-interacting region of hEST1A.
Materials and Methods). The fusion protein was detected at the expected size of 36 kDa (Figures 1-2A, 1-3A). The presence of Trx fused at the N-terminus was confirmed by mass spectrometry (MS) analysis and western blotting (Figures 1-2B, 1-3B), and the identity of TERT was confirmed by MS (Figure 1-2B). Minor bands of greater molecular weight were identified as the bacterial chaperones DNAK and DNAJ and the translation elongation factor Tu (Figure 1-2C). The majority of peptides derived from the lower molecular weight bands, some of which were reactive to anti-Trx antibody, corresponded to fragments of Trx-HIS6-hTERT(1-200) (Figures 1-2B, 1-3B). The purity of full-length Trx-HIS6-hTERT(1-200) in a typical purification was determined to be 75% based on Coomassie staining (Figure 1-11C, lane 4). Based on Deep Purple staining (which is more sensitive than Coomassie staining), purity was determined to range from 20% (Figure 1-3A, lane 5) to 40% (Figure 1-11A, lane 4).

**Trx-HIS6-hTERT(1-200) interacts with telomeric DNA in vitro**

To determine whether hTERT(1-200) interacts with DNA, I performed electrophoretic mobility shift assays. Recombinant Trx-HIS6-hTERT(1-200) was mixed with radiolabeled single-stranded DNA oligonucleotides containing three full telomeric repeats and subjected to native polyacrylamide gel electrophoresis. A single, lower mobility complex contained labeled DNA, and this complex increased in intensity upon addition of increasing amounts of protein (Figure 1-3C). The DNA shift reached a maximum when the binding mixture contained 450 mM NaCl, and persisted in 950 mM NaCl (Figure 1-4). Heat denaturation of the protein inactivated the apparent DNA-binding activity (Figure 1-3C, lane 6). The gel shift observed with radiolabeled DNA was, as expected, competed by an excess molar ratio of unlabeled, specific oligonucleotide (Figure 1-3C, lanes 9-11). An extract prepared from cells expressing Trx-HIS6 and purified over Ni-NTA resin (in the same manner as Trx-HIS6-hTERT(1-200)) did not alter the mobility of the telomeric DNA (Figures 1-3A-B, lane 6; 1-3C, lane 7). These data argue that the DNA:protein interaction was not conferred by the tag alone or co-purifying bacterial proteins, and suggest a specific interaction between the N-terminus of hTERT and telomeric DNA.
Addressing the protein specificity of the apparent hTERT:DNA interaction

To determine whether the gel-shifted complex containing the telomeric DNA oligonucleotide contained a Trx-containing protein (i.e., Trx-HIS6-hTERT(1-200)), I attempted antibody-supershift EMSA experiments. In theory, the migration of a ternary complex of DNA, Trx-HIS6-hTERT(1-200) and anti-Trx antibody would be slower in native PAGE than a complex of DNA and Trx-HIS6-hTERT(1-200). Incubation of a polyclonal anti-Trx antibody with Trx-HIS6-hTERT(1-200) before or after mixture of hTERT with the telomeric DNA oligonucleotide slowed the migration of the oligonucleotide to a second, lower mobility position (Figure 1-5A, lanes 3-4, ii). Even though a negative control antibody did not similarly alter the migration of the telomeric DNA probe (Figure 1-5A, lanes 6-7), the significance of the result was unclear. Firstly, the anti-Trx antibody slowed the migration of the telomeric probe to a similar position in the absence of Trx-HIS6-hTERT(1-200) (Figure 1-5A, lane 5), although the intensity of the signal at this position was weaker than in the presence of Trx-HIS6-hTERT(1-200). Secondly, despite formation of the “supershifted” complex (Figure 1-5A, lanes 3-4, ii), the apparent complex of Trx-HIS6-hTERT(1-200) and the telomeric probe remained (position iii). This result suggests that the supershifted complex (position ii) is not specific to Trx-HIS6-hTERT(1-200). Alternatively, the anti-Trx antibody may have activated or stabilized an additional pool of Trx-HIS6-hTERT(1-200); the supershifted complex may have contained this newly activated pool of Trx-HIS6-TERT(1-200) in complex with anti-Trx and the telomeric probe. Further, the supershifted complex was most intense when the anti-Trx antibody was mixed Trx-HIS6-hTERT(1-200) prior to addition of the telomeric probe (Figure 1-5A, lane 3), which provides some indication of protein-specificity. In an attempt to clarify these results, I performed a similar experiment using a monoclonal anti-Trx antibody (Figure 1-5B). In contrast to the first experiment, no additional complexes were observed; however, the result must be interpreted with caution. The pool of Trx-HIS6-hTERT(1-200) containing the epitope recognized by the monoclonal anti-Trx antibody may have been limited, as suggested by the numerous co-purifying fragments of the full-length fusion protein (Figure 1-2), and the lower reactivity of denatured Trx-HIS6-hTERT(1-200) to the monoclonal antibody.
compared to the polyclonal antibody (compare Figure 1-3B to Figures 1-6, 1-8). Therefore, the interpretation of the antibody supershift experiments is unclear.

I also attempted to address the protein specificity of the gel shift by monitoring changes in the mobility of the DNA-containing complex after cleaving Trx-HIS6 from hTERT(1-200) using thrombin (which targets an intervening cleavage site) (Figure 1-6). Incubation of Trx-HIS6-hTERT(1-200) with thrombin resulted in removal of Trx-HIS, but a pool of full-length Trx-HIS6-hTERT(1-200) remained, even in the presence of excess thrombin and under various reaction conditions (Figure 1-6, top; data not shown). Incubation of Trx-HIS6-hTERT(1-200) with thrombin before or after the addition of the telomeric DNA oligonucleotide did not alter the migration of the apparent protein-DNA complex in the native gel (Figure 1-6, bottom). Thus, the pool of Trx-HIS6-hTERT(1-200) that was resistant to cleavage may have been responsible for the electrophoretic mobility shift of the DNA. A faint, lower mobility complex of unknown identity (Figure 1-6, bottom, lane 3, position #) was detected when Trx-HIS6-hTERT(1-200) was incubated with thrombin prior to addition of the telomeric oligonucleotide. Collectively, these results do not address the protein specificity of the apparent hTERT:DNA interaction (refer to Discussion).

hTERT(1-200) interacts preferentially with telomeric DNA of a particular register and sufficient length

To determine the DNA binding specificity of hTEN, I tested the ability of unlabeled competitor DNA oligonucleotides of different length and/or telomeric register to compete with labeled (TTAGGG), for binding to Trx-HIS6-hTERT(1-200), and expressed the relative apparent binding affinities as the amount of oligonucleotide required to reduce the signal intensity of the mobility shift by 50% (Figure 1-7A-C). Single-stranded oligonucleotides 18 nucleotides (nt) in length did not require a specific 5’ or 3’ end in the telomeric register to facilitate binding (Figure 1-7B, compare oligonucleotides I, II). Oligonucleotides ending in GGG required at least 13 nt of telomeric sequence to bind comparably to the 18 nt (TTAGGG), probe (I, III, IV, V). For oligonucleotides 13 nt in length, a specific telomeric register was preferred: in
decreasing order of apparent affinity, oligonucleotides terminating in GGG, GG, and G (IV, VI, IX).
Reducing the length to 12 nt in any register reduced the apparent binding affinity (compare IV and V; VI and VII; IX and X). Oligonucleotides 12 nt in length were preferentially bound when they contained, in decreasing order of apparent affinity, terminal GG, GGG, G and no G (VII, V, X, XIII). Reducing oligonucleotide length to 11 nt reduced the apparent affinity for DNA ending in GG (VII and VIII); when ending in G, a further decrease in apparent affinity was observed only upon decreasing the length to 10 nt (X, XI, XII). Surprisingly, for oligonucleotides ending in TTA, 11 nt (but not 10 nt) bound with a greater apparent affinity than did 12 nt (XIII, XIV, XV). According to this dataset, single-stranded telomeric DNA 13 nt in length and terminating in GGG or GG was a preferred substrate for hTEN binding (IV, VI), whereas oligonucleotides of this length with different telomeric registers, or shorter oligonucleotides, did not bind with a comparable apparent affinity.

To determine the sequence specificity of the DNA interaction, I performed another set of competition experiments with oligonucleotides containing non-telomeric substitutions (Figure 1-7C). Telomeric DNA 18 nt in length in which the middle G at position 11 was replaced with C had a seven-fold lower apparent affinity for hTEN (compare I and I.C11). For telomeric DNA 13 nt long ending in a GG register (VI), replacing G with C significantly reduced apparent affinity at some positions (VI.C1, C6, C7, C12, C13) but not others (VI.C2, C8). Interestingly, replacing the central GGG with TTA did not reduce apparent affinity (VI.TA6), whereas replacing the GG with TA at the 5′ and 3′ ends did reduce apparent affinity (VI.TA1.12). Combining these central, 5′ and 3′ G-to-T/A replacements reduced apparent affinity (VI.TA1.6.12), suggesting that GG residues form critical contacts with hTEN and that the central GGG, while not required, does stabilize the interaction in the absence of other G contacts. Inverting the GGG and TA positions across the 13 nt also reduced relative binding affinity (VI.INVERT), again suggesting that GG ‘bookends’ facilitate the hTEN:DNA interaction, and that the relative positions of internal G residues are also important. Taken together, these data indicate that the interaction of hTEN with 13 nt oligonucleotides depends on G-rich character at certain positions – a demonstration of telomere sequence
specificity (Figure 1-7C, lower), and in keeping with previous observations on the preferred substrate composition for elongation by telomerase (refer to Discussion).

**hTERT(1-200) can restore activity to the inactive hTERT(201-1132) fragment in trans**

N-terminal fragments of hTERT can restore enzymatic function, *in trans*, to inactive fragments of the remainder of hTERT in combination with hTR (Beattie et al., 2001; Moriarty et al., 2004). Since hTERT(201-1132) exhibits an activity defect (Beattie et al., 2000), we reasoned that providing the hTEN DNA binding domain *in trans* may restore its activity. I reconstituted telomerase activity in rabbit reticulocyte lysates by expressing FLAG-hTERT cDNA in the presence of hTR, and assayed anti-FLAG immunoprecipitates for telomerase activity (see Materials and Methods). Whereas wild-type hTERT displayed a characteristic ability to generate long extension products (lane 1 in Figures 1-8, 1-9, 1-10A-B), removal of the first 200 amino acids nearly abolished this activity (lane 2 in Figures 1-8, 1-9, 1-10A-B), as demonstrated previously (Beattie et al., 2000; Beattie et al., 2001). However, when hTERT(201-1132) was mixed with hTERT(1-200) (containing Trx or an S-tag at the N- or C-terminus, respectively), the two fragments interacted with one another and catalytic activity was restored (Figure 1-8, lanes 3, 7; Figure 1-9, lane 3; Figure 1-10A, lane 3 and 1-10B, lanes 3, 9). Interestingly, hTR was not required for the interaction of hTERT N- and C-termini (Figure 1-8, lanes 5, 13, 14; refer to Chapter 2). Therefore, hTERT(1-200) is essential for telomerase activity *in vitro*, and can function as a separable domain to restore activity to an inactive fragment of hTERT. While recombinant Trx-HIS6-hTERT(1-200) purified from *E. coli* interacted with hTERT(201-1132), the protein was unable to regenerate activity in combination with hTERT(201-1132) (Figure 1-8, lane 8; Figure 1-9, lane 4; Figure 1-10B, lane 10). Incubation of purified Trx-HIS6-hTERT(1-200) with mock RRL did not confer an ability to regenerate in activity in combination with hTERT(201-1132) (Figure 1-9, lane 5; refer to Discussion).

**Identification of amino acids in hTERT(1-200) that are critical for telomerase activity *in vitro***

Although the first 200 amino acids of human and *T. thermophila* TERTs share less than 15% identity and
25% similarity, the N-terminus of hTERT may still adopt a similar three-dimensional fold. Guided by the sequence alignment of the N-terminus of hTERT to the *T. thermophila* TERT TEN domain structure (Jacobs *et al.*, 2006), I generated point mutations in acidic, basic and polar residues in hTEN that are potentially exposed to the surface of the domain, and assayed mutants in the context of hTEN for the ability to restore function to hTERT(201-1132). Many of the mutations, including T116A/T117A/S118A, exhibited no discernible effect on telomerase activity or the ability to interact with hTERT(201-1132) + hTR (Figure 1-10A-B, lanes 5 and 7; summarized in Table 1-1). In contrast, mutation of Q169A in hTERT(1-200)-S-HIS8 or Trx-HIS6-hTERT(1-200) impaired telomerase function in the TRAP assay (Figure 1-10A, lane 8 and data not shown; Table 1-1). Also, Q169A rendered telomerase unable to catalyze nucleotide addition in the SE assay – a demonstration that Q169 is required for catalytic activity (Figure 1-10B, lane 8; Table 1-1) similar to the requirement of Q168 in *T. thermophila* TERT (Jacobs *et al.*, 2006). The H17A/Y18A/R19A mutant displayed a similar inability to elongate telomeric DNA, although the defect in the TRAP assay was not as pronounced (Figure 1-10A-B, lane 4; Table 1-1). The defect of the triple mutant was attributed to a single amino acid change at Y18, but not H17 or R19 (Figure 1-10A-B, lanes 5-7). None of the mutations described above disrupted the interaction with hTERT(201-1132) + hTR (Figure 1-10A).

**Y18 and Q169 are dispensable for the interaction of hTERT(1-200) with DNA**

To further study the effect of hTEN mutation on the biochemical activities of telomerase, we produced hTEN mutants in bacteria alongside wild-type hTEN in the same manner as described above. Purification of Trx-HIS6-hTERT(1-200) wild-type, Y18A, T116A/T117A/S118A, and Q169A yielded a prominent band at the expected size that was recognized by anti-Trx antibody (Figure 1-11A-C). (For discussion of G100V, refer to Appendix A.) Lower molecular weight fragments of the fusion protein co-purified with the intact protein, as in the purification of wild-type hTEN (see also Figure 1-3B). Q169A contained a prominent band at 31 kDa that was recognized by anti-Trx antibody and was present in equal abundance to the full-length 36 kDa fusion protein (Figure 1-11A-C).
To determine whether the residues in hTEN required for activity are required for DNA binding, I performed electrophoretic mobility shift assays with hTEN mutants. As before, Trx-HIS6-hTERT(1-200) shifted the single-stranded telomeric DNA oligonucleotide in a native gel in a concentration-dependent manner (Figure 1-11D, lanes 2-4). The T116A/T117A/S118A mutant also bound telomeric DNA (Figure 1-11D, lanes 8-10), which is consistent with the predicted position of these residues on the surface of the domain opposite the putative DNA binding groove, and an apparent lack of involvement in the catalytic reaction cycle (Table 1-1). Interestingly, mutation of Y18 or Q169, each of which impaired telomerase activity, did not affect the ability of hTEN to bind telomeric DNA (Figure 1-11D, lanes 5-7, 11-13). One could infer that these residues do not contact DNA, or that these residues may contact DNA (based on their predicted positions in/near the putative DNA binding groove) but that neither Y18 nor Q169 is the sole determinant of a DNA interaction – at least not to an extent that can be resolved by the EMSA assay. The ability of hTEN(Q169A) to bind telomeric DNA in an EMSA differs from the reduced ability of T. thermophila TERT(Q168A) to crosslink to primer (Jacobs et al., 2006) (refer to Discussion). (For analysis of the G100V mutant, refer to Appendix A.)

**Residues in hTERT(1-200) are required for telomerase-mediated extension of cellular lifespan**

To determine whether Y18 and Q169, as determinants of telomerase activity in vitro, are also required for telomerase function in cells, we performed a cellular immortalization experiment. We created polyclonal HA5 cell lines\(^1\) stably expressing wild-type or mutant hTERT, and monitored population doubling levels at regular intervals. Cells that received wild-type hTERT cDNA continued to divide through the duration of the experiment, whereas cells receiving empty vector (pcDNA3.1) or hTERT(D868A/D869A) (mutations that inactivate reverse transcriptase activity) succumbed to apoptosis within approximately 10 population doublings (Figure 1-12), which is consistent with previous observations (Harrington et al.,

\(^1\) HA5 is a mortal, SV40-transformed human embryonic kidney cell line that bypasses senescence and encounters crisis – characterized by telomere instability and apoptosis – and does not undergo spontaneous immortalization (Counter et al., 1992).
Cells receiving hTERT(H17A/Y18A/R19A) (n=2), Q169A (n=2), or T116A/T117A/S118A mutants were also unable to divide beyond 10 population doublings.

To confirm these results, we derived a second set of stable lines from HA5 cells at an earlier passage. As before, cells receiving wild-type hTERT survived through the duration of the experiment. In contrast, cells receiving any of the mutant hTERT cDNAs failed to survive beyond 15-20 population doublings (Figure 1-13A). This delay in reaching crisis relative to HA5 cells transduced at a later passage (Figure 1-13A versus Figure 1-12) is in keeping with the different initial telomere lengths of these two populations (Harley et al., 1990). We assayed telomerase activity in lysates prepared from cells at day 35 in the experiment (Figure 1-13B). Lysates from cells containing H17A/Y18A/R19A, Y18A, Q169A, or D868A/D869A mutants did not display telomerase activity (Figure 1-13B), which is consistent with the reduced activity of these mutants in reticulocyte lysates (Figure 1-10A-B, Table 1-1; Beattie et al., 1998; Harrington et al., 1997). Although T116A/T117A/S118A reconstituted telomerase activity in vitro, cells containing this mutant did not display telomerase activity (Figure 1-13B, Table 1-1).

Analysis of mRNA transcript levels confirmed expression of the vector-encoded hygromycin-resistance gene at early and late time points in the experiment (Figure 1-13C). Expression of hTERT(WT), H17A/Y18A/R19A, T116A/T117A/S118A, and D868A/D869A alleles was maintained at later population doublings. Although Y18A phenocopied H17A/Y18A/R19A in vitro and H17A/Y18A/R19A-transduced cells did not survive (Table 1-1), expression of Y18A in cells was not maintained (Figure 1-13C, lanes 7-8); thus, we were unable to conclude that this mutant cannot immortalize cells. Q169A was expressed at a lower level than wild-type hTERT in the second experiment, but at a comparable level in the first experiment (Figure 1-13C, lanes 11-12 versus 3-4; Figure 1-14, lanes 3-4 and 5-6 versus 2). Regardless of the level of expression, Q169A did not reconstitute activity in cell lysates (Figure 1-14, lanes 3-5). Therefore, we conclude that hTERT-Q169A cannot immortalize cells (Table 1-1).

To determine whether mutations in hTEN interfere with the ability of telomerase to maintain telomeres, I
analyzed telomere length changes in HA5 stable cell lines by Southern blotting of the terminal restriction fragments (TRF). Average telomere lengths in cells expressing wild-type hTERT were maintained up to population doubling 16 (Figure 1-13D, lane 4), whereas telomeres in cells expressing H17A/Y18A/R19A, T116A/T117A/S118A, Q169A or D868A/D869A mutants became shorter as the populations reached crises (Figure 1-13A, D). Therefore, the mutations in hTEN that we have described render telomerase unable to maintain telomeres or extend cellular lifespan. The average telomere length of cells expressing wild-type hTERT became shorter beyond population doubling 16 (Figure 1-13D, lanes 5-6), but the population did not encounter crisis (Figure 1-13A). This result is consistent with previous findings that the maintenance of minimal telomere DNA by hTERT is sufficient to extend cellular replicative potential (Ouellette et al., 2000; Zhu et al., 1999). Analysis of telomere length changes in cells from the first immortalization experiment (Figure 1-12) revealed similar results (data not shown).

**DISCUSSION**

In this study, we described the ability of the N-terminus of hTERT (hTEN, amino acids 1 to 200) to interact with telomeric DNA (Figures 1-1, 1-3, 1-7), and complement the activity defect of hTERT(201-1132) + hTR as a separable domain (Figure 1-8). Mutational analysis identified that Y18 and Q169 residues were required for primer extension activity *in vitro* (Figure 1-10). These residues, as well as T116/T117/S118, were required for telomere length maintenance and telomerase-mediated extension of cellular lifespan (Figures 1-12, 1-13), but not for the interactions of hTEN with DNA or hTERT(201-1132) + hTR (Figures 1-10, 1-11).

Many previous studies have used telomerase activity as a read-out for investigating telomerase-primer interactions, in part due to the difficulty of producing sufficient quantities of purified hTERT to measure DNA binding directly. By EMSA (Figures 1-3C, 1-11D), we demonstrated that the interaction between purified Trx-HIS6-hTERT(1-200) and telomeric DNA was independent of hTR, other domains of hTERT, and telomerase activity. The interaction was reproducible across independent purifications of
protein and many experimental replicates. The recombinant protein also interacted with hTERT(201-1132) (Figures 1-8, 1-9). Despite these interactions, Trx-HIS6-hTERT(1-200) produced in bacteria did not regenerate telomerase activity in combination with RRL-hTERT(201-1132) + hTR (Figures 1-8, 1-9), suggesting that E. coli may not provide the folding pathways, modification activities, or binding partners required for telomerase activity that a eukaryotic system can provide (Bachand and Autexier, 1999; Beattie et al., 1998; Holt et al., 1999; Masutomi et al., 2000). This inability to support catalysis was not caused by interference of the N-terminal Trx-HIS6 tag, since Trx-HIS6-hTERT(1-200) produced in RRL reconstituted activity in complex with hTERT(201-1132) as well as RRL-hTERT(1-200)-S-HIS8 did (Figures 1-8, 1-9). My attempt to activate bacterial Trx-HIS6-hTERT(1-200) with RRL components was unsuccessful (Figure 1-9).

Our method of purification of hTEN represents the most successful of several strategies we attempted. Codon optimization for expression in E. coli improved yield significantly, but we were unable to obtain enough concentrated Trx-HIS6-hTERT(1-200) to conduct crystallization trials or determine an affinity constant for DNA. In EMSA experiments, we were limited by the total amount and concentration of purified protein, and thus did not observe a quantitative shift of the probe that would be necessary to estimate binding affinities. Nevertheless, I determined the relative affinity of hTEN for DNA oligonucleotides of different sequence and length (Figure 1-7). DNA 13 nt long ending in either GGG or GG containing G residues at specific positions was a preferred substrate for Trx-HIS6-hTERT(1-200) binding, which establishes that hTEN exhibits length and sequence preference for binding telomeric DNA (Figure 1-7). This sequence specificity is in accord with telomeres being the substrates of telomerase (Bodnar et al., 1998; Cristofari and Lingner, 2006; Jady et al., 2006; Tomlinson et al., 2006; Vaziri and Benchimol, 1998). Also, the preference of hTEN for telomeric DNA parallels that of endogenous telomerase – a substrate preference that has been, in some instances, attributed to the putative anchor site. For example, oligonucleotides longer than 10-12 nt are necessary for high affinity binding and processive elongation, and substitution of 5′ residues (particularly guanines) for non-telomeric residues impair

The recombinant protein preparation, in which the most abundant protein was Trx-HIS6-hTERT(1-200), shifted telomeric DNA to a single band in the native gel (Figures 1-3C, 1-4B, 1-5, 1-6, 1-7, 1-11D). Fragments of Trx-HIS6-hTERT(1-200) (which may be degradation and/or premature termination products), bacterial chaperones, and a small number of other proteins were retained through the purification (Figures 1-2, 1-3A-B). Bacterial DNA binding proteins were not detected in the material by mass spectrometry (Figure 1-2C). I also attempted several experiments to address the hTERT-specificity of the DNA interaction. Using monoclonal or polyclonal anti-Trx antibodies to supershift the apparent Trx-HIS6-hTERT(1-200)-DNA complex in the native gel yielded inconclusive results; the antibodies either did not supershift the apparent TERT:DNA complex, or caused an electrophoretic mobility shift of telomeric DNA even in the absence of Trx-HIS6-hTERT(1-200) (Figure 1-5).¹ We also attempted to analyze the protein components of the shifted DNA band by subjecting the native gel to western blotting, mass spectrometry, or elution-activity complementation of hTERT(201-1132) + hTR; however, we obtained no results, likely because the amount of protein present in the band (in the attomole range) was below the limit of detection by these methods. To exclude the possibility that DNA interacted with the Trx-HIS6 portion of the fusion protein, we attempted to separate Trx-HIS6 from hTERT(1-200) at an internal cleavage site using thrombin. When thrombin was added to the fusion protein while it was adhered to NiNTA resin, hTERT(1-200) would not elute (Le Zheng), suggesting that hTERT(1-200) relied on the N-terminal tag for solubility. When Trx-HIS6-hTERT(1-200) was treated with thrombin in solution following purification, a pool of Trx-HIS6-hTERT(1-200) resisted cleavage (Figure 1-6). In addition, treatment of Trx-HIS6-hTERT(1-200) with thrombin before or after incubation with DNA did not alter the migration of the apparent protein-DNA complex in the native gel (Figure 1-6). These results

¹ In subsequent experiments conducted by Lea Harrington, the electrophoretic mobility of telomeric DNA upon mixing with hTERT(1-200)-S-HIS8 or Trx-HIS6-hTERT(1-200) was specifically altered by a concentrated anti-HIS antibody from Roche (Sealey et al., 2009).
suggest that thrombin did not recognize or cleave well-folded Trx-HIS6-hTERT(1-200) — possibly the active entity in the mixture. Because Trx-HIS6 purified as a separate protein did not shift the telomeric probe (Figure 1-3A-C), we concluded that the apparent interaction of Trx-HIS6-hTERT(1-200) with DNA occurred through hTERT(1-200).

Despite the limited sequence similarity among the N-termini of ciliate, yeast and human TERTs (Xia et al., 2000), the DNA binding function of the domain appears to be conserved. The recombinant N-termini of Est2 and TtTERT have been shown to interact with DNA in filter binding and crosslinking assays, respectively (Finger and Bryan, 2008; Jacobs et al., 2006; Xia et al., 2000). Telomerase purified from yeast cells forms crosslinks to DNA via the N-terminus of Est2 (measured in an RNA-dependent assay) (Lue, 2005). Consistently, crosslinking of TtTERT (purified from RRL) to DNA is reduced upon removal of the N-terminus (Finger and Bryan, 2008; Jacobs et al., 2006). Also, the extent of crosslinking of TtTERT to DNA is reduced upon mutation of residues in the N-terminus of TtTERT, including Q168, F178, and W187 (Jacobs et al., 2006), and W187 has been identified as a bona fide protein contact with telomeric DNA (Romi et al., 2007). With respect to human telomerase, hTERT(1-300) and (1-350) expressed in reticulocyte lysates can be captured onto biotinylated telomeric DNA (Figure 1-1; Wyatt et al., 2007; Wyatt et al., 2009). The capture of full-length hTERT in this assay is reduced upon removal or mutation of the N-terminus (Wyatt et al., 2007). Previous EMSA studies demonstrated an ability of purified ciliate telomerase to bind specifically to telomeric DNA; in this context, telomere DNA binding was telomerase RNA-dependent (Harrington et al., 1995). To our knowledge, our study represents the first EMSA developed for a specific fragment of recombinant telomerase.

Mutations in the N-termini of TERTs have been identified that impair the activity of human (Armbruster et al., 2001; Beattie et al., 2000; Beattie et al., 2001; Lee et al., 2003; Moriarty et al., 2004; Moriarty et al., 2005; Wyatt et al., 2007; Wyatt et al., 2009), T. thermophila (Jacobs et al., 2006; Jacobs et al., 2005; Miller et al., 2000; Romi et al., 2007; Zaug et al., 2008) and S. cerevisiae telomerases (Friedman and Cech, 1999; Lue, 2005; Lue and Li, 2007; Xia et al., 2000). We identified two mutations in hTERT that
attenuated catalytic activity. I targeted hTERT-Q169 for mutation based on the conservation of this residue with TrTERT-Q168 and the position of the latter on the floor of a putative DNA binding groove on the surface of the N-terminal domain (Jacobs et al., 2006). Like TrTERT-Q168A, and in agreement with a recent report, hTERT-Q169A impaired telomerase activity (Jacobs et al., 2006; Wyatt et al., 2009; Figure 1-10). However, unlike TrTERT-Q168A, hTERT-Q169A did not impair the interaction with an 18-mer telomeric DNA oligonucleotide in the EMSA (Jacobs et al., 2006; Romi et al., 2007; Figure 1-11D) or a biotinylated DNA protein capture assay (Wyatt et al., 2009). This apparent discrepancy may be due to the different assays employed. Jacobs et al. observed that mutation of Q168 reduced the crosslinking of immuno-purified RRL-TrTERT to a 20 nt-long iodouracil-substituted telomeric primer following exposure to UV light and denaturing PAGE (Jacobs et al., 2006). Romi et al. observed that mutation of Q168 reduced primer affinity in an assay that measured catalytic activity of RRL-TrTERT following UV-crosslinking to a 6 nt-long iodouracil-substituted telomeric primer (Romi et al., 2007). The formation of TERT:DNA crosslinks in these experiments depends on the proximity of iodouracil to reactive amino acid side chains. The interaction between hTEN and an 18 nt-long telomeric primer detected by native gel EMSA (Figures 1-3 to 1-7) was not subject to these parameters. Therefore, whereas previous studies have documented the involvement of TrTERT-Q168 in binding DNA, I did not find similar evidence for hTERT-Q169. Either there are species-specific differences in the function of this conserved residue, or a role for Q169 in binding DNA could not be ascertained by analyzing the behaviour of the single amino acid mutant (in the context of the TEN domain) in the native EMSA.¹

Notably, mutation of hTERT-Q169A reduces, but does not eliminate, the capacity of hTERT(1-300) to be captured onto a 6-mer biotinylated telomeric DNA oligonucleotide (or non-telomeric oligonucleotides) (Wyatt et al., 2009), suggesting that the role of Q169 in binding DNA may be DNA length-dependent (see below).

¹ The phenotype of the hTERT-Q169A mutation is similar to that of TtTERT-D94A and L174A mutations that impair catalytic activity but not DNA binding/crosslinking (Jacobs et al., 2006).
We also identified hTERT-Y18 as a residue (at a non-conserved position but that may lie near the end of the DNA binding groove) that is required for normal catalytic activity but not necessarily DNA binding (Figures 1-10, 1-11). Following from the discussion above, it is possible that Y18 and Q169 are not essential mediators of the DNA interaction. It is also possible that both Y18 and Q169 can compensate along with other DNA binding residues, for the loss of any one DNA contact in a DNA binding assay. The loss of any one DNA contact may yet render the enzyme incapable of action on a primer. The contributions of Y18 and Q169 to the catalytic reaction cycle may be distinct, given that mutation of each residue impaired activity to a different degree, but the exact functions of these residues remain to be determined.

Mutation of hTEN at critical sites interfered with the ability of telomerase to immortalize human embryonic kidney cells. Residues H17/Y18/R19 and Q169 were required for the generation of telomerase activity in cell lysates, as they were in vitro, and also for the maintenance of telomere length and the continued replication of cells in culture (Figures 1-12, 1-13A). Q169 is similarly required for the generation of telomerase activity, maintenance of telomeres and continued replication in culture of foreskin (BJ) fibroblasts (Wyatt et al., 2009). The defect of hTERT-Q169A in cells may stem from a reduced ability to bind a short segment of the G-strand overhang and/or an inability to act on telomeres. The importance of the contribution of Q169 to the binding of short substrates (Wyatt et al., 2009) for telomerase action in vivo is not known, given a lack of knowledge about how much of the long G-strand is accessible to hTERT during S phase. We were not able to formally identify a requirement for Y18 in extending the replicative lifespan of cells because transgene expression was lost during propagation of the culture (Figure 1-13C, lanes 7-8).

Interestingly, the T116A/T117A/S118A triple mutant, which did not exhibit deficits in DNA binding or activity in vitro (Figure 1-10, Table 1-1), did not regenerate telomerase activity in cell lysates, maintain telomeres, or immortalize cells (Figures 1-12, 1-13). An hTERT mutant lacking residues 110-119 displays normal activity in vitro on telomeric primers 18 nt in length (although repeat addition
processivity is reduced on shorter primers), but reduced activity in cell lysates and an inability to immortalize HA5 cells (Moriarty et al., 2004; Moriarty et al., 2005). Collectively, these results suggest that residues 116-118 may interact with cellular factors that influence recruitment to the telomere or telomerase activity in vivo (refer to Appendix A). This possibility is consistent with our prediction that residues 116-118 may be exposed to the non-DNA binding surface of hTEN. By the criteria of Armbruster et al., hTERT(T116A/T117A/S118A) fulfills the characteristics of a so-called “DAT” mutant that dissociates the in vitro and in vivo activities of telomerase (Armbruster et al., 2001). The human POT1/TPP1 complex, which interacts with, and can stimulate telomerase, is a candidate modulator of telomerase in vivo (Wang et al., 2007; Xin et al., 2007). Notably, A. thaliana POT1A interacts with telomerase activity in cells and the N-terminus of AtTERT in vitro and in two-hybrid assays (Rossignol et al., 2007; Surovtseva et al., 2007). In preliminary experiments, we identified a possible interaction between the N-terminus of hTERT and hPOT1/hTPP1 in vitro (refer to Appendix A).

In addition to binding DNA, hTR, and presumably other cellular factors, the N-terminus of hTERT interacts with the C-terminal portion of hTERT in complex with hTR (Beattie et al., 2001; Figures 1-8, 1-9, 1-10). Although N- and C-terminal portions of hTERT each contact hTR, hTR or other nucleic acids are not required to bridge this interaction (Arai et al., 2002; Armbruster et al., 2001; Moriarty et al., 2004; Figure 1-8, Chapter 2). Co-expression of the N-terminus of hTERT with the hTERT C-terminus-hTR complex activates telomerase (Beattie et al., 2001; Figures 1-8, 1-9, 1-10). There is also evidence to suggest that C-terminal regions of TERT interact with DNA (Figure 1-1; Finger and Bryan, 2008; Hossain et al., 2002; Jacobs et al., 2006; Romi et al., 2007; Wyatt et al., 2007), and that C-terminal regions are required for normal telomerase function in vitro and in vivo (Banik et al., 2002; Beattie et al., 2000; Hossain et al., 2002; Huard et al., 2003; Lee et al., 2003). Thus, it is possible that N- and C-terminal regions of TERT combine to form one DNA binding site. Alternatively, N- and C-terminal regions of TERT may bind DNA separately, perhaps in a cooperative manner. In the latter scenario, the N-terminus of TERT, which has been described as a low affinity DNA binding domain (Finger and
Bryan, 2008), may not recruit telomerase to primers, but once DNA is bound to another site in hTERT, the local concentration of DNA available to the N-terminus may be sufficiently high for a stable interaction to occur. In turn, the N-terminus may contribute specificity (Figure 1-7), along with the RNA template in hTR, to the primer sequences that can be bound and elongated efficiently by telomerase. This hypothesis is consistent with the crosslinking of *E. aediculatus* telomerase to primers at both 5′ and 3′ ends (Hammond *et al*., 1997), and the observation that telomerase cannot extend short primers or primers with non-G-rich 5′ ends efficiently (Collins and Greider, 1993; Greider and Blackburn, 1987; Harrington and Greider, 1991; Lee and Blackburn, 1993; Morin, 1989; Morin, 1991). Finally, whether the functional multimerization of N- and C-terminal regions of hTERT reflects an intra- or inter-molecular interaction in the context of native telomerase remains to be determined (refer to Chapter 2).

The exact telomerase reaction mechanism has yet to be fully elucidated. Structure-function analysis is beginning to shed light on how various domains of TERT, TR, other proteins, and telomeric DNA associate and work together. For example, TtTERT-W187 can crosslink to the same nucleotide in telomeric primers with different registers, suggesting that N-terminus of TERT may form a static interaction with DNA as the RNA template is iteratively copied (Romi *et al*., 2007). The sequence-specificity of the DNA interaction with the N-terminus of hTERT at different nucleotide positions may reflect this property (Figure 1-7). Also, mutation of TtTERT-L141 impairs telomere repeat addition processivity but not DNA binding, which led Zaug *et al*. to propose models of how the N-terminus may behave during primer translocation (Zaug *et al*., 2008). These findings, along with the knowledge that TtTERT-Q168, the N-terminus of Est2, and now human TERT-Q169 and Y18 are required for productive elongation of DNA (Jacobs *et al*., 2006; Lue and Li, 2007; Romi *et al*., 2007; Wyatt *et al*., 2009; this work), are beginning to inform an understanding of the molecular events of the telomerase reaction cycle. Deciphering exactly how the N-terminus of hTERT enables telomerase function may provide an

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1 hTERT-L13A and hTERT-L14A mutants exhibit reduced activity but intact telomere repeat addition processivity, whereas the hTERT-L13A/L14A mutant exhibits reduced telomere repeat addition processivity. The role of TtTERT-L14 in processivity is apparently shared between L13 and L14 residues in hTERT (Zaug *et al*., 2008).
additional target for inhibiting telomerase function in cancer.

**FUTURE DIRECTIONS**

**In vitro studies of hTEN**

We demonstrated the basic requirements for the interaction of hTEN with DNA (Figure 1-3). We purified hTEN under high concentrations of NaCl (up to 1 M) and found that binding to telomeric DNA oligonucleotides was maximal in the presence of 450 mM NaCl (Figure 1-4). To further study this interaction, the effect of different salts/ions relevant to normal physiology could be tested. For example, active human telomerase was extracted from cells in 300 mM KCl (Cohen et al., 2007). Although telomerase processivity is impaired at higher potassium concentrations (Cohen et al., 2007; Sun et al., 1999), evaluating the interaction of hTEN with DNA in the presence of this ion may better reflect the behaviour of telomerase in the intracellular milieu. Although hTEN did not require the addition of magnesium – a co-factor of telomerase catalysis – to bind DNA (Figure 1-3), the effect of this and other metal ions on DNA binding activity could be investigated. Manganese, which confers yeast and human telomerase with an ability to add nucleotides onto primers in the absence of hTR (Lue et al., 2005), is another interesting candidate. Because manganese-dependent, template-independent nucleotide addition by Est2 is most efficient on telomeric primers (Lue et al., 2005), manganese may not alter the DNA sequence preference of TERT-DNA interactions. Nevertheless, an investigation of the effect of manganese on the DNA sequence specificity of hTEN is warranted.

Deciphering the three-dimensional structure of hTEN may reveal the DNA binding surface. Other strategies to express and purify hTEN, beyond the many that we tried, may yield recombinant hTEN of sufficient yield and purity for crystallization and structure determination. For example, the folding and expression of hTERT is improved by targeting the protein to the endoplasmic reticulum in insect cells (Wu et al., 2007a). Such a system may also host post-translational modification pathways required for the formation of activity-competent hTEN (Figure 1-9 and Discussion). Also, in a manner similar to that
employed previously in the study of TtTERT (Jacobs et al., 2005), a bacterial fluorescence screen of GFP-fused hTERT fragments could be used to identify soluble domains that are suitable for expression.

My mutational analysis of the N-terminus of hTERT revealed amino acids that are required for telomerase activity in vitro (Figure 1-10, Table 1-1), but I was unable to identify amino acids that are required for the DNA interaction (Figure 1-11). To this end, combining mutations that did not impair the DNA interaction, such as Y18A and Q169A, may yet define a role for these residues in binding DNA. Further, the hTEN-DNA interaction was relatively insensitive to high salt concentrations (Figure 1-4), suggesting that mutational analysis of non-polar, surface-exposed residues may lead to the identification of DNA-coordinating residues.

hTEN exhibited a preference for binding single-stranded DNA oligonucleotides of a specific length with telomeric residues at specific positions. Whether mutation of hTEN alters these binding specificities is not known. For example, a mutant such as Q169A may not be able to bind shorter oligonucleotides as well as wild-type hTEN does, and may exhibit reduced tolerance for substitution of non-telomeric nucleotides. Preliminary findings along these lines were reported for non-purified hTERT(1-300) generated in reticulocyte lysates (Wyatt et al., 2009). Also, if a larger quantity of purified hTEN were obtained, a determination of absolute binding affinity could be performed. Future studies could also directly correlate the affinity of hTEN for different substrates with the activity of telomerase on those substrates. Also, the ability of hTEN to bind double-stranded, overhang, and G-quadruplex, and RNA substrates could be examined. A study of RNA binding is particularly relevant, given that hTEN contains a minor site (RID1) for the binding of hTR (Moriarty et al., 2002; Moriarty et al., 2004). Whether hTEN mutation impairs interaction with hTR could also be determined.

**In vivo studies of hTEN**

We identified several mutations that impair telomerase activity in vitro and/or the ability of hTERT to extend cellular replicative potential (Table 1-1). Although none of these mutations affected interaction
with an 18-mer telomeric DNA oligonucleotide in vitro, the effect of these mutations on the interaction of hTERT with the telomere remains unknown. With the exception of one report (Sharma et al., 2003), the detection of hTERT at the telomere by ChIP or immunofluorescence has been limited to over-expression systems (Cristofari and Lingner, 2006; Tomlinson et al., 2006). In any case, these systems could be used to study potential intracellular trafficking or telomere interaction defects. An inability of mutants to interact with the telomere would suggest a biogenesis defect or recruitment defect, possibly involving an inability to bind telomere DNA and/or other telomere binding proteins. Interaction of non-functional mutants with the telomere would suggest a post-recruitment defect, possibly involving loss of catalytic function or inability to associate with proteins that stimulate telomerase processivity.

Several hTERT mutants that exhibited no telomerase activity defect in vitro were not tested for the ability to immortalize cells (Table 1-1). A determination of whether these mutants have a DAT phenotype might further define the residues that are required for the in vivo functions of telomerase (Armbruster et al., 2001). Whether DAT mutants, including T116A/T117A/S118A, exhibit an inability to interact with cellular cofactors such as hEST1A (refer to Chapter 3), hEST1B, or POT1/TPP1 (refer to Appendix A) could be investigated.

**Telomerase catalytic reaction cycle**

How the N-terminus of hTERT enables catalysis and telomere repeat addition processivity remains unclear. For example, both hTERT-Y18 and Q169 are required for telomerase activity, but neither residue is required for the interaction of hTEN with DNA or the C-terminal portion of the protein (Figures 1-10, 1-11; Wyatt et al., 2009). Recently, hTERT-L13/L14 were found to be required for telomere repeat addition processivity (Zaug et al., 2008), which led Zaug et al. to propose models of how telomerase achieves this processivity. One of these models refers to an “intramolecular switch” mediated by an interaction between the TEN domain and another domain in telomerase (Zaug et al., 2008; refer also to Chapter 2). Investigating the effect of mutating hTERT-L13/L14 on the interaction and functional trans-
complementation between hTEN and the C-terminus of hTERT may lend support for this model. Similarly, identifying any mutation in hTEN that interferes with the interaction with the hTERT C-terminus would be interesting.

**Protein engineering**

Many proteins are modular in nature and contain several domains that mediate distinct functions or interactions. That the N-terminus of TERT may function as an accessory domain in telomerase is supported by the observation that it interacts with, and complements a mutant of telomerase lacking the domain (Figures 1-8, 1-9, 1-10). Interestingly, fusion of telomere binding domains of other proteins (e.g., Cdc13, TRF2, POT1) to yeast and human telomerases can compensate for the functional deficits of telomerase mutants (Armbruster et al., 2003; Armbruster et al., 2004; Evans and Lundblad, 1999). We tried to fuse different DNA binding domains to hTERT(201-1132) in place of hTEN, but these heterologous fusion proteins did not consistently exhibit telomerase activity (David Sealey and Jennifer Cruickshank, unpublished). It may still be possible to identify DNA binding domains that can function in place of the hTEN domain, perhaps altering the DNA sequence specificity and reaction kinetics of telomerase, or targeting telomerase to non-telomeric sites in the cell. This approach may reveal unique features of the hTEN domain, and address the functional importance of the interaction between the N- and C-termini of hTERT (which would be lost upon replacement of the hTEN domain by a domain from a different protein; refer to Discussion). In a reciprocal manner, the hTEN domain could be fused to other proteins or nucleic acid modifying enzymes to confer telomeric specificity. Our one such attempt was unsuccessful (Emanuel Rosonina, Lea Harrington, and David Sealey, unpublished), but the general strategy seems reasonable. Future attempts at protein engineering may benefit from the use of flexible linker sequences to accommodate proper domain architecture.

**Targeting hTEN in disease**

The interactions of hTEN with DNA and the C-terminus of hTERT are likely required for the ability of
telomerase to maintain cellular longevity. Therefore, these interactions represent potential therapeutic
targets for cancer therapy. The assays described in this Chapter could be exploited in a search for small
molecule or biological inhibitors of the DNA binding and trans-complementation activities of hTEN.
Also, previous studies have demonstrated an ability of mutant telomerase to exert dominant interference
with endogenous telomerase, resulting in loss of telomerase activity, telomere shortening, apoptosis, and
loss of tumorigenicity (Hahn et al., 1999b; Zhang et al., 1999), although resistance to the interference has
been reported (Delhommeau et al., 2002). We investigated the potential use of hTERT transgenes
harboring mutations in the TEN domain as dominant-negative inhibitors, but levels of transgene
expression were likely too low in our experiments to have an effect (David Sealey and Michael Taboski,
unpublished). Boosting expression levels via retroviral infection may improve future attempts.

Mutations in the first 200 amino acids of hTERT (P33S, L55Q, frame shift P112, V144M) have been
linked to idiopathic pulmonary fibrosis (Armanios et al., 2007; Tsakiri et al., 2007). P33S and V144M do
not impair telomerase activity in vitro (Tsakiri et al., 2007), while L55Q decreases activity by 50%
(Armanios et al., 2007). The effects of these mutations on the ability of hTEN to bind DNA and the
C-terminus of hTERT could be examined. Also, given that hTEN can trans-complement hTERT(201-
1132) as a separable domain, I recommend testing whether co-expression of wild-type hTEN can alleviate
the in vitro and in vivo defects of these disease-associated hTERT mutants.
Table 1-1. Summary of hTERT mutant phenotypes.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Telomerase activity</th>
<th>DNA binding</th>
<th>Cell lifespan extension</th>
<th>Predicted surface</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TRAP</td>
<td>SE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal activity, binds DNA, extends cellular lifespan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>+</td>
<td>(17, 4)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Normal activity, binds DNA, does not extend cellular lifespan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T116A/T117A/S118A</td>
<td>+</td>
<td>(3)</td>
<td>+</td>
<td>- (2) A / B</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defective activity, binds DNA, does not extend cellular lifespan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H17A/Y18A/R19A</td>
<td>-</td>
<td>(8)</td>
<td>-</td>
<td>- (3) A / B . G</td>
</tr>
<tr>
<td>Y18A</td>
<td>-</td>
<td>(3)</td>
<td>+</td>
<td>? (1) A / B . G</td>
</tr>
<tr>
<td>Q169A</td>
<td>-</td>
<td>(10, 2)</td>
<td>-</td>
<td>- (3) A . G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Normal activity (not tested: DNA binding, cellular lifespan extension)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R15A/S16A</td>
<td>+</td>
<td>(3)</td>
<td>+</td>
<td>A / B . G</td>
</tr>
<tr>
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<td>+</td>
<td>(5)</td>
<td>+</td>
<td>A / B</td>
</tr>
<tr>
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<td>+</td>
<td>A / B . G</td>
</tr>
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<td>B</td>
</tr>
<tr>
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<td>(5)</td>
<td>+</td>
<td>B</td>
</tr>
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<td>+</td>
<td>(3)</td>
<td>+</td>
<td>A / B</td>
</tr>
<tr>
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<td>(3)</td>
<td>+</td>
<td>A / B</td>
</tr>
<tr>
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<td>(3)</td>
<td>+</td>
<td>B</td>
</tr>
<tr>
<td>D105A</td>
<td>+</td>
<td>(3)</td>
<td>+</td>
<td>B</td>
</tr>
<tr>
<td>E113A</td>
<td>+</td>
<td>(3)</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>S121A/Y122A</td>
<td>+</td>
<td>(3)</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>D129A</td>
<td>+</td>
<td>(3)</td>
<td>+</td>
<td>A . G</td>
</tr>
<tr>
<td>S134A</td>
<td>+</td>
<td>(3)</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>R155A</td>
<td>+</td>
<td>(3)</td>
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<td>B</td>
</tr>
<tr>
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<td>+</td>
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</tr>
<tr>
<td>H189A/S191A</td>
<td>+</td>
<td>(3)</td>
<td>+</td>
<td>A / B . G</td>
</tr>
</tbody>
</table>

*+* no defect or *-* defect. Phenotype representative of (n) experimental replicates.

a Telomerase was reconstituted by mixing RRL-expressed hTERT(1-200)-S-HIS8\textsuperscript{a} or RRL Trx-HIS6-hTERT(1-200)\textsuperscript{b} with FLAG-hTERT(201-1132)-FLAG in the presence of hTR. Anti-FLAG immunoprecipitates were assayed for telomerase activity by TRAP or SE protocols.

b Trx-HIS6-hTERT(1-200), purified from bacteria, was mixed with (TTAGGG)\textsubscript{3} and complexes were analyzed by electrophoretic mobility shift assay (EMSA).

c Predicted surface position based on alignment of the hTERT sequence to the structure of the *T. thermophila* TERT TEN domain (Jacobs et al., 2006). Side ‘A’ includes the putative DNA binding groove. Side ‘B’ faces opposite Side ‘A.’ A/B represents an edge between both sides. G indicates a location in/near the putative DNA binding groove.

Refer to Materials and Methods for additional details.
Figure 1-1. N- and C-termini of hTERT interact with a telomeric DNA oligonucleotide. (A-B) A biotinylated telomeric DNA oligonucleotide (+, 5′-BIO-(TTAGGG)$_4$TTAG-3′) was affinity-captured onto neutravidin resin. As a control, resin was prepared without oligonucleotide (-). FLAG-tagged hTERT truncations were generated in reticulocyte lysates and incubated with the resin in CHAPS buffer. Unbound proteins were washed away, and bound proteins were analyzed by western blotting using anti-FLAG M2 and anti-mouse IgG-HRP antibodies. 20% of input protein (In) was analyzed, as indicated. Mock reactions (mk) containing no cDNA were assayed as negative controls. Molecular mass is indicated at the left (kDa). (A) and (B) represent separate experiments. In (B), lanes 1-7 and 8-10 were analyzed on separate gels. Irrelevant lanes between 3 and 4 were omitted. (C) Schematic representation of hTERT fragments that precipitated onto neutravidin specifically in the presence of 5′-BIO-(TTAGGG)$_4$TTAG-3′ (solid line), or non-specifically (dashed line). Conserved regions are indicated by white and black boxes.
Figure 1-2. Mass spectrometry analysis of recombinant Trx-HIS6-hTERT(1-200). (A) Trx-HIS6-hTERT(1-200) was expressed in E. coli and purified (refer to Materials and Methods). Proteins were boiled in SDS-PAGE loading dye, resolved through a 4-20% w/v Tris-Glycine Novex gel and stained with silver. The mass (ng) of Trx-HIS6-hTERT(1-200) (*) present in lanes 2-3 was approximated by comparing the load volumes to those of Figure 1-3A, where mass was determined by comparison of the band intensity (*) to the average intensity of bands in the RPN5800 molecular size marker (M, 30 ng/band). Molecular mass is indicated at the left (kDa). (B-C) Tryptic peptides identified by mass spectrometry analysis of proteins extracted from bands shown in (A). (B) Schematic representation of peptides corresponding to Trx-HIS6-hTERT(1-200). Lowercase letters identify peptides as follows: a, SDKIIHLTDDSFDTDVLK or IIHLTDDSFDTDVLK; b, MIAPILDEIADEYQGK; c, LNIDQNPGTAPK; d, GIPTLLLFK; e, EVLPLATFVR; f, LGPQGWR; g, NVLAFGALLDGAR; h, GGPPEAFTTSVR; i, SYLPTNTDALR; j, GSGAWGILLR; k, VGDVVLHLLAR. (C) Peptides corresponding to proteins other than Trx-HIS6-hTERT(1-200). All of these proteins are of bacterial origin.
Figure 1-3. Recombinant hTERT(1-200) interacts with telomeric DNA. (A) Analysis of Trx-HIS6-hTERT(1-200) expressed in E. coli and purified as described in Materials and Methods. Proteins were boiled in SDS-PAGE loading dye, resolved through a 4-20% w/v Tris-Glycine Novex gel and stained with Deep Purple. The mass (ng) of Trx-HIS6-hTERT(1-200) present in lanes 2-5 was determined by comparing the band intensity of the full-length fusion protein (*) to the average intensity of bands in the RPN5800 molecular size marker (M, lane 1, 30 ng/band). Molecular mass is indicated at the left (kDa). Lane 6, Trx-HIS6 (**) purified as described in Materials and Methods. (B) Proteins prepared as in (A) were transferred to PVDF and immunoblotted with anti-Trx (Novagen) and anti-mouse IgG antibodies. Lane 1 (not shown) contains Benchmark molecular size marker. [Figures 1-6, 1-8, 1-9, 1-10 were probed with a different anti-Trx antibody (Sigma).] (C) Electrophoretic mobility shift assay of telomeric DNA. $^{32}$P-(TTAGGG)$_3$ was mixed with the following components as described in Materials and Methods: lane 1, DNA alone; lanes 2-5, recombinant Trx-HIS6-hTERT(1-200) (same amounts as in A-B expressed as molar ratio to radiolabeled probe); lane 6, pre-boiled (b) Trx-HIS6-hTERT(1-200); lane 7, Trx-HIS6 (same amount as in A-B expressed as molar ratio to radiolabeled probe); lanes 9-11, Trx-HIS6-hTERT(1-200) in the presence of unlabeled specific competitor oligonucleotide (at molar ratio to radiolabeled probe). Lanes 5 and 8 are duplicates. Complexes were resolved by native polyacrylamide gel electrophoresis.
Figure 1-4. Effect of salt concentration on the interaction of recombinant hTERT(1-200) with telomeric DNA.

(A) Analysis of Trx-HIS6-hTERT(1-200) expressed in E. coli and purified as described in Materials and Methods. This material was produced in a separate experiment. Proteins were boiled in SDS-PAGE loading dye, resolved through a 4-20% w/v Tris-Glycine Novex gel and stained with Deep Purple. The mass (ng) of Trx-HIS6-hTERT(1-200) present in lane 2 was determined by comparing the band intensity of the full-length fusion protein (*) to the average intensity of bands in the RPN5800 molecular size marker (M, lane 1, 30 ng/band). Molecular mass is indicated at the left (kDa). Irrelevant lanes between 1 and 2 were removed. (B) Electrophoretic mobility shift assay of telomeric DNA as described in Materials and Methods, with the following modifications: all lanes contained 1.25 nM \(^{32}\text{P}-(\text{TTAGGG})_{3}\), 28:1 molar ratio of Trx-HIS6-hTERT(1-200) to probe, and varying concentrations of NaCl as indicated. Complexes were resolved by native polyacrylamide gel electrophoresis. Results are representative of two experiments. (C) Plot of the intensity of the shifted band in (B) (less background; determined using ImageQuant software) versus salt concentration. Circles, duplicate measurements; line, average values.
Figure 1-5. Attempts to address the protein specificity of the apparent Trx-HIS6-hTERT:DNA complex using anti-Trx antibodies. (A) Polyclonal anti-Trx antibody shifts the telomeric DNA oligonucleotide to a lower mobility position in presence or absence of Trx-HIS6-hTERT(1-200). (B) Monoclonal anti-Trx antibody does not alter the migration of the telomeric DNA oligonucleotide in the presence or absence of Trx-HIS6-hTERT(1-200). (A-B) The following components were added to a 20 μL final volume at 0, 30 or 60 min: ThT, Trx-HIS6-hTERT(1-200); D, $^{32}$P-(TTAGGG)$_3$; antibodies (A: Trx, rabbit polyclonal anti-Trx, Sigma; E, rabbit polyclonal anti-hEST1A peptide, Snow et al., 2003. B: Trx, mouse monoclonal anti-Trx isotype IgG2b, Novagen; HA, mouse monoclonal anti-HA isotype IgG2b, clone 12CA5, Roche.). Refer to Materials and Methods for reaction conditions. At 90 min, reactions were analyzed by EMSA as described in Materials and Methods. The radiolabeled telomeric DNA oligonucleotide probe migrated to the following positions: i, gel well; ii, in complex with anti-Trx antibody in presence or absence of Trx-HIS6-hTERT(1-200) (A only); iii, in complex with Trx-HIS-hTERT(1-200); iv, free oligonucleotide. Trx-HIS6-hTERT was present at an approximate 29:1 (A) or 73:1 (B) molar ratio to probe. Trx-HIS6-hTERT protein preparations used in (A) and (B) are depicted in Figures 1-4 and 1-3, respectively.
Figure 1-6. Thrombin does not completely digest Trx-HIS6-hTERT(1-200) and does not alter migration of the complex containing telomeric DNA. (Top) Trx-HIS6^hTERT(1-200) (*, thrombin cleavage site) was incubated with thrombin for 1 hr at room temperature (A) or 16 hr at 4°C (B), and subjected to SDS-PAGE followed by Deep Purple staining or western blotting with anti-Trx (Sigma) and anti-rabbit IgG-HRP antibodies. *, position of Trx-HIS6^hTERT. **, position of Trx-HIS6. M, RPN5800 molecular size marker (30 ng/band). Molecular mass is indicated beside each panel (kDa).

(Bottom) Trx-HIS6^hTERT(1-200) was incubated with the telomeric DNA oligonucleotide before or after treatment with thrombin for the indicated time. Trx, Trx-HIS6 control protein. Complexes were subjected to analysis by EMSA (refer to Materials and Methods). The radiolabeled telomeric DNA oligonucleotide migrated to the following positions: i, gel well; ii, in complex with Trx-HIS-hTERT(1-200); iii, free oligonucleotide; #, unknown entity. Trx-HIS6-hTERT(1-200) was present at an approximate 29:1 (A) or 73:1 (B) molar ratio to probe. Trx-HIS6-hTERT protein preparations used in (A) and (B) are depicted in Figures 1-4 and 1-3, respectively.
Figure 1-7. DNA sequence and length preferences for binding of hTERT(1-200). (A) Representative example of an EMSA testing the effect of competitor DNA on the interaction of hTERT(1-200) with telomeric DNA. $^{32}$P-(TTAGGG)$_3$ (1 nM) was mixed with unlabeled competitor oligonucleotide at the indicated molar ratio to radiolabeled probe prior to incubation with Trx-HIS6-hTERT(1-200) (at a 110:1 molar ratio to radiolabeled probe, as depicted in Figure 1-3). Data for different oligonucleotide competitors are summarized in (B-C).
Figure 1-7. DNA sequence and length preferences for binding of hTERT(1-200). (B-C) X-axis indicates the molar ratio of unlabeled competitor oligonucleotide relative to 32P-(TTAGGG) required to reduce the Trx-HIS6-hTERT(1-200)-dependent gel shift signal by 50% (Relative IC50). Y-axis indicates the sequence, length (L), number of replicates (n), and name of oligonucleotides tested. To confirm the reproducibility of results, (B) and (C) were performed with different elution fractions from the same purification. (B) Relative apparent binding affinities of DNA oligonucleotides with different lengths and telomeric registers. A one-tailed t-test for samples with equal variance was used to conclude significance (P* <0.05; compare VI and VII). (C) Relative apparent binding affinities of telomeric DNA oligonucleotides with non-telomeric substitutions. A two-tailed t-test for samples with unequal variance was used to identify significant decreases in affinity relative to the unmodified telomeric oligonucleotide of identical length (P* <0.05, ** <0.025, *** <0.006). The bottom schematic indicates positions at which nucleotide substitutions decrease (-) or do not decrease (+) the interaction with hTERT(1-200).
Figure 1-8. hTERT(1-200) expressed in RRL or bacteria interacts with hTERT(201-1132) with or without hTR; hTERT(1-200) expressed in RRL but not bacteria regenerates telomerase activity in combination with hTERT(201-1132) + hTR. Telomerase activity was reconstituted in reticulocyte lysates (RRL) with hTR and wild-type hTERT or combined fragments of hTERT as indicated (see Materials and Methods). RRL FLAG-tagged hTERT(1-1132)+hTR (lane 1), (201-1132)+hTR (lanes 2, 3, 7-9), (201-1132)-hTR (lanes 5, 13-15) or mock RRL (lane 4, 10-12) was mixed with RRL hTERT(1-200)-S-HIS8 (lanes 3-5), RRL (lanes 7, 10, 13) or bacterial (lanes 8, 11, 14) Trx-HIS6-hTERT(1-200), bacterial Trx-HIS6 (lanes 9, 12), or mock RRL (lanes 1, 2, 4). Anti-FLAG immunoprecipitates (lanes 1-5, 7-15) were analyzed by western blotting using S protein-HRP (top left) or anti-Trx (Sigma) and anti-rabbit IgG-HRP antibodies (top right). Input protein was analyzed (lanes 6, 16, 15%; lane 17, 60%; lane 18, 100%). Blots were re-probed using anti-FLAG M2 and anti-mouse IgG antibodies (middle). Bands of interest are indicated with an arrowhead. Telomerase activity was assessed by TRAP assay (bottom). Lane ‘C’, buffer alone (CHAPS) was assayed as a negative control. Top and middle: lanes 1-6 and 7-18 were contained on separate blots. Bottom: lanes C-3 and 7-9 were analyzed on the same gel; blank spacer lanes were omitted to align lanes to top/middle panels. IC, internal control product.
Figure 1-9. Treatment of bacterial Trx-HIS6-hTERT(1-200) with mock RRL does not confer an ability to regenerate telomerase activity in combination with hTERT(201-1132) + hTR. Telomerase activity was reconstituted in reticulocyte lysates (RRL) with hTR and wild-type hTERT or combined fragments of hTERT as indicated (see Materials and Methods). RRL FLAG-tagged hTERT(1-1132)+hTR (lane 1) or (201-1132)+hTR (lanes 2-6) was mixed with mock RRL (lanes 1, 2), RRL Trx-HIS6-hTERT(1-200) (lane 3), bacterial Trx-HIS6-hTERT(1-200) (lane 4), bacterial Trx-HIS6-hTERT(1-200) + mock RRL (lane 5), or bacterial Trx-HIS6 + mock RRL (lane 6). Anti-FLAG immunoprecipitates (lanes 1-6) were analyzed by western blotting using anti-Trx (Sigma). Input protein was analyzed (lanes 8-9, 10%; lane 10, 20%; lane 11, 100%). Blots were re-probed using anti-FLAG M2 and anti-mouse IgG antibodies (middle). Bands of interest are indicated with arrowheads. Telomerase activity was assessed by TRAP assay (bottom). Lane 7, buffer alone (CHAPS) was assayed as a negative control. IC, internal control product.
**Figure 1-10. hTERT(1-200) rescues the activity defect of hTERT(201-1132) in a Y18- and Q169-dependent manner.** FLAG-tagged hTERT(201-1132) was mixed with mock RRL (lane 2), wild-type (lane 3) or mutant hTERT(1-200)-S-HIS8 containing substitutions at the indicated positions (lanes 4-8) and immunoprecipitated onto anti-FLAG resin (IP). FLAG-hTERT(1-1132) was mixed with mock RRL as a positive control (lane 1). (A) Telomerase activity in the IPs was assessed by TRAP assay. Irrelevant lanes between 8 and 9 were removed. Buffer alone (CHAPS) was assayed as a negative control (lane 9). Immunoprecipitates (IP) were analyzed by western blotting using S protein-HRP (WB1), followed by anti-FLAG M2 and anti-mouse IgG-HRP antibodies (WB2). 15% of input protein (In) was analyzed on a separate blot with S protein-HRP. (B) Telomerase activity in the IPs was analyzed using a radioactive, linear standard elongation (SE) telomerase assay. Lane 11, position of the \((TTAGGG)_3\) DNA oligonucleotide upon 5' end-labeling with \(^{32}\)P (bottom arrowhead). Lane 12, 96 nt loading control (top arrowhead) that was added to each reaction prior to the recovery of extension products. Bottom panel: 15% of input protein was analyzed by western blotting using S protein-HRP (lanes 3-8). 15% and 60% of input protein were analyzed in lanes 9 and 10, respectively, using anti-Trx (Sigma) and anti-rabbit IgG-HRP antibodies. Lanes 9-10 are copied from lanes 16-17 of Figure 1-10, as the experiments were performed at the same time.
Figure 1-11. Selected mutations in hTERT(1-200) do not abrogate DNA binding activity. (A-C) Analysis of Trx-HIS6-hTERT(1-200) (wild-type or mutant, as indicated) expressed in E. coli and purified as described in Materials and Methods. Proteins were boiled in SDS-PAGE loading dye, resolved through 4-20% w/v Tris-Glycine Novex gels, and stained with Deep Purple (A), transferred to PVDF and immunoblotted with anti-Trx (Novagen) and anti-mouse IgG-HRP antibodies (B), or stained with Coomassie Brilliant Blue (C). The mass (ng) of Trx-HIS6-hTERT(1-200) present in lanes 2-13 was determined by comparing the band intensity of the full-length fusion protein (*) to the average intensity of bands in the RPN5800 molecular size marker (M) (Panel A, lane 1, 30 ng/band). Molecular mass is indicated at the left (kDa).
Figure 1-11. Selected mutations in hTERT(1-200) do not abrogate DNA binding activity. (D) Electrophoretic mobility shift assay of telomeric DNA. $^{32}$P-(TTAGGG)$_3$ was mixed with the following components as described in Materials and Methods: lane 1, DNA alone; lanes 2-13, increasing amounts of wild-type or mutant Trx-HIS6-hTERT(1-200) (same amounts as in A-B; expressed as molar ratio to radiolabeled probe). Complexes were resolved by native polyacrylamide gel electrophoresis.
Figure 1-12. Selected mutations in the N-terminus of hTERT interfere with the ability of telomerase to extend the replicative lifespan of late-passage primary human (HA5) cells. Polyclonal cell lines containing wild-type or mutant hTERT cDNA (or pcDNA3.1 negative control) were derived from HA5 cells (at later passage than cells used in Figure 1-13). Cells were passaged under selection with hygromycin. Y-axis contains a break spanning populations doublings (PD) 35-55 in order to display the uninterrupted growth of cells expressing wild-type hTERT.
Figure 1-13. Selected mutations in the N-terminus of hTERT interfere with the ability of telomerase to extend the replicative lifespan of early-passage primary human (HA5) cells. (A) Polyclonal cell lines containing wild-type or mutant hTERT cDNA (or pcDNA3.1 negative control) were derived from HA5 cells (at earlier passage than cells used in Figure 1-12). Cells were passaged under selection with hygromycin. Y-axis contains a break spanning populations doublings (PD) 35-55 in order to display the uninterrupted growth of cells expressing wild-type hTERT. (B) Lysates of polyclonal cell lines containing wild-type or mutant hTERT cDNA were prepared in CHAPS buffer at day 35 and assayed for telomerase activity by TRAP assay. CHAPS buffer was assayed as a negative control (lane 8). HeLa cell lysate was assayed as a positive control (lane 9). IC indicates TRAP assay internal control product. Lanes containing irrelevant samples were omitted. (C) Analysis of mRNA expression level at early and late passage by RT-PCR. Hygromycin-resistance gene (HYG, upper), hTERT (middle), and GAPDH cDNAs were amplified using gene-specific primers (refer to Materials and Methods). Lanes containing irrelevant samples were omitted.
Figure 1-13. Selected mutations in the N-terminus of hTERT interfere with the ability of telomerase to maintain telomeres in primary human (HA5) cells. (D) Analysis of the terminal telomere restriction fragments (TRF) at the indicated population doubling (PD) of polyclonal HA5 cell lines receiving wild-type or mutant hTERT. Genomic DNA was isolated, digested with Rsal and Hinfl, and subjected to Southern blotting using a (CCCTAA)$_3$ probe (refer to Materials and Methods). Molecular size (kbp) is shown at left according to the migration of 1 kb+ DNA ladder (not shown). Left, middle, and right panels were analyzed separately and aligned according to duplicate samples contained on individual blots (not shown). The weighted mean telomere length (kbp) is indicated at the bottom of each lane. The presence of an interstitial, cross-hybridizing band at 3 kbp can be used to estimate the relative DNA loading of each lane.
Figure 1-14. hTERT(Q169A) expressed at low or comparable level to wild-type hTERT does not reconstitute telomerase activity in cell lysates. (Top) Analysis of hTERT mRNA levels in HA5 polyclonal stable cell lines generated in the first (Figure 1-12, n=2) and second (Figure 1-13) transfection experiments. Cells were subjected to analysis at the indicated population doubling (PD). Hygromycin-resistance gene (HYG, upper), hTERT (middle), and GAPDH cDNAs were amplified using gene-specific primers (refer to Materials and Methods). Untransfected HA5 cells (lane 8) and an independent stable HA5 cell line expressing hTERT and the hygromycin-resistance gene (lane 7, Michael Taboski and Lea Harrington, unpublished) were analyzed as controls. All reactions were analyzed on one gel, and irrelevant regions of the gel between upper, middle, and lower panels were removed. (Bottom) Cells were lysed in CHAPS buffer and assayed for telomerase activity by TRAP assay. Lanes 1-5, labels continue from above. Lane 6, CHAPS buffer was assayed as a negative control. Lane 7, HeLa cell lysate was assayed as a positive control. Irrelevant lanes were omitted.
STATEMENT OF CONTRIBUTIONS

Figures 1-2A, 1-3A-B. Le Zheng, Dr. Chris Marshall (members of Dr. Mitsu Ikura’s laboratory) and I designed and prepared the pET32a-Trx-HIS6-hTERT(1-200) vector. Le Zheng, with input from me, developed the purification protocol (refer to Materials and Methods) and purified the protein. Carol Liu assisted with the purification. I analyzed the protein by SDS-PAGE followed by silver staining (Figure 1-2A), Deep Purple staining (Figure 1-3A), and western blotting (Figure 1-3B) and prepared the Figures. (This protein material was also used in Figures 1-5B, 1-6B, and 1-7.) I calculated yield and percent purity. Theo Goh excised silver stained bands (Figure 1-2A) under my direction. I worked with him to destain gel bands and prepare tryptic peptides for analysis. Other than the image acquisition and band assignments for Figure 1-2A, I prepared the Figures.

Figures 1-2B-C. Theo Goh operated HPLC and mass spectrometry instruments and acquired data. He (or other staff) matched peptides to protein databases. I prepared the Figure using information that Theo provided.

Figure 1-4A. Le Zheng purified Trx-HIS6-hTERT(1-200). I performed SDS-PAGE and Deep Purple staining. I prepared the Figure. (This protein material was also used in Figures 1-5A and 1-6A.)

Figures 1-8, 1-9, 1-10. While I performed the in vitro transcription and gel-purification of hTR during my studies, the hTR used in these telomerase reconstitution experiments was produced by Dr. Linda Holland.

Figure 1-10B. Jennifer Cruickshank performed the standard elongation assays of wild-type and mutant telomerases that I reconstituted in vitro. We both analyzed the data. I prepared the Figure.

Figure 1-11A-C. Le Zheng produced wild-type and mutant Trx-HIS6-hTERT(1-200). I analyzed the protein by SDS-PAGE followed by Deep Purple staining, Coomassie staining, and western blotting. I prepared the Figure and calculated yield and percent purity.
Figures 1-12, 1-13, 1-14. I designed the experiments to analyze the functionality of hTERT mutants in HA5 cells. Michael Taboski linearized and gel-purified plasmids, transfected cells, selected cells in hygromycin, passaged and counted cells in culture, and determined the number of population doublings. He and I tracked results. He prepared and analyzed telomerase activity in cell lysates (Figure 1-13B), and designed primers for, and performed the RT-PCR analysis (Figures 1-13, 1-14). Michael prepared cell pellets from which I extracted genomic DNA. I performed restriction digests and analyzed telomere lengths by Southern blotting (Figure 1-13D). Michael monitored apoptosis of HA5 cells by visual inspection under microscopy. We both analyzed the data and I prepared the Figures.

Materials and Methods. Le Zheng drafted the first paragraph of ‘Expression and purification of recombinant hTERT’, which I subsequently modified. I wrote ‘Mass spectrometry analysis of recombinant Trx-HIS6-hTERT(1-200)’ according to experimental details provided by Theo Goh. Jennifer Cruickshank drafted the section of ‘Telomerase activity assays’ pertaining to standard elongation assays, and I subsequently modified the text. Michael Taboski drafted ‘Generation and passaging of stable cell lines’, ‘Analysis of mRNA by RT-PCR’, and ‘Protein extraction.’ I subsequently modified these sections.

Of the DNA vectors used for experiments in this Chapter, I designed and constructed the following:


- pET32a-Trx-HIS6-hTERT(1-200) mutants: Y18A, G100V, T116A/T117A/T118A and Q169A.

CHAPTER 2

Functional dimerization of human telomerase via TERT-TERT interactions

The experiments described in this Chapter were designed, executed and interpreted by me in the laboratory of Lea Harrington.
INTRODUCTION

The interaction of telomerase with itself (i.e., as a dimer or multimer) has been investigated in several systems. Based on studies of the composition and behaviour of the recombinant or native enzyme, *T. thermophila* telomerase appears to be monomeric (Bryan *et al.*, 2003; Cunningham and Collins, 2005; Witkin and Collins, 2004). However, both physical and functional studies suggest that the telomerases of several other organisms are dimeric. For example, partially purified, *S. cerevisiae* telomerase, which tends to remain bound to its substrates post-elongation, can be captured onto streptavidin in complex with the elongation products of both biotinylated and non-biotinylated primers (Prescott and Blackburn, 1997). This result, together with the observation that that partially purified telomerase sediments to a single position in a glycerol gradient, suggests that *S. cerevisiae* telomerase contains two active sites and is dimeric (Prescott and Blackburn, 1997). *E. crassus* telomerase partially purified from mating or vegetative cells captures biotinylated and non-biotinylated elongation products in a similar manner (Wang *et al.*, 2002). Also, defective hTERT (Beattie *et al.*, 2001; Moriarty *et al.*, 2002; Moriarty *et al.*, 2004) or hTR (Ly *et al.*, 2003; Rivera and Blackburn, 2004) mutants, when extracted from cell lysates or produced *in vitro*, complement each other in trans to restore telomerase activity.

Additional physical evidence supporting that telomerase dimerization occurs is as follows. Full-length, or N- and C-terminal fragments of human or *E. crassus* TERT can be co-immunoprecipitated, suggesting that telomerase dimerization may involve TERT-TERT interactions (Arai *et al.*, 2002; Armbruster *et al.*, 2001; Beattie *et al.*, 2001; Moriarty *et al.*, 2004; Chapter 1; Wang *et al.*, 2002). Also, in the context of recombinant human telomerase, one hTR variant can be purified upon selection for a second hTR variant, and vice versa, implying that telomerase contains two hTR molecules (Wenz *et al.*, 2001). As determined by glycerol gradient sedimentation or gel filtration, telomerasess extracted from *E. aediculatus*¹ and human

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¹ A preliminary study reported that telomerase extracted from *E. aediculatus* nuclei sediments in a glycerol gradient to a position indicative of monomeric composition (Lingner and Cech, 1996). As reported by the authors, the reason for the discrepancy with their later report is unclear.
cell nuclei appear as dimers (Aigner et al., 2003; Fouche et al., 2006; Schnapp et al., 1998; Wenz et al., 2001). As determined by electron microscopy, E. aediculatus telomerase extracted from nucleic and selected using a TR-antisense oligonucleotide appears dimeric in complex with a model telomere substrate (Fouche et al., 2006). Mass spectrometry analysis of immuno-purified, telomeric substrate-selected, active cellular telomerase reveals a complex whose mass can be accounted for by two molecules each of hTERT, hTR, and dyskerin (Cohen et al., 2007). Despite the apparent existence of human telomerase dimers in vitro and in cell extracts, evidence of the function of telomerase as a dimer inside cells has not been described. We hypothesized that human telomerase functions as a dimer. This Chapter describes physical and functional interactions between hTERT fragments and/or inactive mutants which provide evidence of telomerase dimerization in vitro.

The experimental system described in this Chapter, which utilizes Q169A and DD868-9AA mutants of hTERT as tools, exploits the cooperation between TEN and RT domains of hTERT (Chapter 1). The following information summarizes the biochemical activities of the TEN and RT domains. Mutation of conserved aspartic acid residues in the active site of TERT (hTERT-D712, D868, D869) eliminates catalytic activity and the ability of telomerase to immortalize cells (Beattie et al., 1998; Harrington et al., 1997; Kim et al., 2003; Zhu et al., 1999). The N-terminus of TERT, otherwise known as the Telomerase Essential N-terminal domain (TEN), is also required for normal telomerase function in vitro and in cells (Armbruster et al., 2001; Beattie et al., 2000; Jacobs et al., 2006; Lue and Li, 2007; Moriarty et al., 2004; Moriarty et al., 2005; Wyatt et al., 2007; Wyatt et al., 2009; Chapter 1). The TEN domain interacts with telomeric DNA (Finger and Bryan, 2008; Jacobs et al., 2006; Lue, 2005; Romi et al., 2007; Wyatt et al., 2007; Wyatt et al., 2009; Chapter 1), the telomerase RNA (Jacobs et al., 2006; Moriarty et al., 2002; O’Connor et al., 2005), and may also interact with other factors that modulate telomerase function in cells (Armbruster et al., 2001; Moriarty et al., 2004; Chapters 1 and 3, Appendix A). Mutation of a conserved

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1 Some protocols employed TR-antisense affinity selection (Fouche et al., 2006; Schnapp et al., 1998; Wenz et al., 2001).
glutamine in the TEN domain (hTERT-Q169, *T. thermophila* TERT-Q168, *S. cerevisiae* Est2p-Q146) impairs catalytic activity (*T. thermophila*, *S. cerevisiae*, *H. sapiens*), telomere DNA affinity/crosslinking (*T. thermophila*, *H. sapiens*), and the ability of telomerase to maintain telomeres (*S. cerevisiae*, *H. sapiens*) and immortalize cells (*H. sapiens*) (Jacobs et al., 2006; Lue and Li, 2007; Romi et al., 2007; Wyatt et al., 2009; Chapter 1). Interestingly, hTEN can interact with the inactive ΔTEN truncation mutant of hTERT as a separable domain, and can restore functionality to this mutant in a Y18- and Q169-dependent manner (Chapter 1). Therefore, we sought evidence of telomerase dimerization by probing the functional complementation of inactivating mutations within the TEN and RT domains.

**MATERIALS AND METHODS**

**Reconstitution of telomerase activity in reticulocyte lysates**

Telomerase activity was reconstituted in rabbit reticulocyte lysates (RRL) as described (Beattie et al., 1998; Weinrich et al., 1997), with modifications. Human telomerase RNA (hTR) was transcribed *in vitro* using the RiboMax kit (Promega) and gel-purified. Coupled *in vitro* transcription-translation reactions (Promega Corp.) were prepared with 0.01 µg/µL hTR RNA, 0.4 µCi/µL [³⁵S]methionine, and 0.01 µg/µL of the following cDNAs: pCR3-FLAG-hTERT(201-1132), pcDNA3.1(*HYG*)-hTERT wild-type or mutant, as indicated. Mock reactions contained no cDNA. Reactions were incubated separately for 60 min at 30°C, and then 20 µL of each reaction was mixed (as indicated) and incubated for an additional 60 min. RRL mixtures were then diluted to 500 µL with cold CHAPS buffer (0.5% w/v CHAPS, 10 mM Tris-Cl pH 7.5, 10% v/v glycerol, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT, Roche EDTA-free Complete protease inhibitor cocktail) and rocked for 1 hr at 4°C. FLAG-tagged complexes were immunoprecipitated with pre-equilibrated anti-FLAG M2 affinity resin (Sigma) and washed three times with 1 mL cold CHAPS buffer. For PCR-based analysis of telomerase activity, 1 µL of the immunoprecipitate was assayed by the TRAP assay (described below). The remainder of the pellet was

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¹ Mutation of Q169 reduces the affinity of hTERT(1-300) for short telomeric primers (Wyatt et al., 2009).
boiled in SDS-PAGE loading dye and proteins were resolved through a 4-20% w/v Tris-Glycine Novex gel. The gel was dried at 80°C for 1 hr and exposed to a phosphorimager screen. Radioactive bands were identified using a Typhoon Trio variable mode imager (GE Healthcare).

**TRAP assay**

TRAP assays were performed using the TRAPEze kit (Millipore) with modifications to the manufacturer’s protocol. Cell lysate or in vitro-reconstituted telomerase was incubated with the TS primer at 30°C for 30 min prior to heat inactivation at 95°C for 2 min. Reactions were held at 95°C while Taq DNA polymerase (New England Biolabs) was added. Primer extension products were amplified through 25 cycles of 30 s at 94°C, 30 s at 50°C and 90 s at 72°C. 20 µL of each 50 µL reaction was combined with DNA loading dye and resolved through a 10% w/v 19:1 acrylamide:bisacrylamide gel in 0.6X TBE at 15-20 V/cm. DNA was stained with SYBR Green I (Sigma) and fluorescent signals were scanned on a Typhoon Trio variable mode imager.

**Co-immunoprecipitation of hTERT fragments and micrococcal nuclease treatment**

TERT(1-200)-S-HIS8 and FLAG-tagged hTERT truncations were synthesized in RRL according to the manufacturer’s instructions (Promega Corp.) in the absence of hTR. Mock reactions contained no cDNA. Reactions were incubated separately for 60 min at 30°C, and then 20 µL of each reaction was mixed (as indicated) and incubated for an additional 60 min. RRL mixtures were then diluted to 500 µL with cold CHAPS buffer (0.5% w/v CHAPS, 10 mM Tris-Cl pH 7.5, 10% v/v glycerol, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT, Roche EDTA-free Complete protease inhibitor cocktail) and rocked for 1 hr at 4°C. FLAG-tagged complexes were immunoprecipitated with pre-equilibrated anti-FLAG M2 affinity resin (Sigma). In Figures 2-2 and 2-3, immunoprecipitates were washed three times in cold CHAPS buffer. In Figure 2-4, immunoprecipitates were washed two times with cold CHAPS buffer and then treated with 40 U/µL micrococcal nuclease (New England Biolabs) at 30°C for 45 min in buffer containing 50 mM Tris-Cl pH 7.9, 5 mM CaCl₂ and 1 mg/mL bovine serum albumin, or mock-treated in buffer without
micrococcal nuclease. Reactions were stopped by the addition of excess EGTA, and immunoprecipitates were twice in CHAPS buffer. In Figures 2-2 to 2-4, immunoprecipitates were boiled in SDS-PAGE loading dye. Proteins were resolved through 4-20% w/v Tris-Glycine Novex gels (Invitrogen), transferred to PVDF membranes, and subjected to immunoblotting with S protein-HRP (Novagen), followed by mouse anti-FLAG M2 antibody (Sigma) and sheep anti-mouse IgG-HRP (GE Healthcare). Reactive bands were detected with ECL Plus reagents (GE Healthcare) and fluorescent signals were captured using a Typhoon Trio variable mode imager. To analyze co-purifying nucleic acids, duplicate immunoprecipitates were extracted twice with phenol:chloroform:isoamyl alcohol. Material in the aqueous layer was subjected to ethanol precipitation, resuspended in TE pH 8.0, resolved through a 2% agarose gel containing 0.5 µg/mL ethidium bromide, and visualized using a Typhoon Trio variable mode imager.

RESULTS

Trans-complementation of inactive hTERT mutants in vitro

To better understand the interaction and functional complementation of inactive hTERT mutants, I reconstituted human telomerase in reticulocyte lysates (Beattie et al., 1998; Weinrich et al., 1997). I mixed FLAG-tagged hTERT(201-1132) with wild-type or mutant hTERT, and assayed anti-FLAG immunoprecipitates for telomerase activity. Removal of the TEN domain eliminated the ability of telomerase to elongate a DNA oligonucleotide primer, as described previously (Beattie et al., 2000; Chapter 1, Figure 2-1A, lane 1). Activity was restored upon co-purification with full-length hTERT (Figure 2-1A, lane 2). This restoration of activity was dependent on Q169 (lane 4) but not D868/D869 (lane 5) in the co-purifying hTERT (n=3). Notably, each of the wild-type and mutant full-length variants of hTERT were expressed (Figure 2-1B), and no residual activity was captured onto on anti-FLAG resin in the absence of FLAG-hTERT(201-1132) (Figure 2-1A, lanes 6-9). Therefore, telomerase requires at least one functional TEN domain and one functional reverse transcriptase domain, and these domains can
be provided by different TERT molecules.

**Interaction of hTERT fragments in vitro**

The *trans*-complementation of TERT mutants may reflect an ability of TERT to dimerize. To identify TERT-TERT interaction sites, I assayed hTEN (amino acids 1-200) for precipitation onto anti-FLAG resin in the presence of FLAG-tagged hTERT fragments and absence of hTR. hTEN co-immunoprecipitated with full-length hTERT, the TEN domain itself, as well as several non-contiguous fragments of hTERT spanning the entire length of the protein (Figure 2-2A-D; Figure 1-8, lanes 5,13,14). Interestingly, the hTERT(92NAAIRS) DAT mutation (Armbruster et al., 2001) in the context of hTERT(1-350) did not interfere with the hTEN interaction (Figure 2-2B). Attempts to delineate smaller interacting regions within hTEN were unsuccessful; smaller fragments of hTEN immunoprecipitated nonspecifically, suggesting that the domain was soluble only in its entirety (Figure 2-3). Given that several domains in TERT bind DNA and/or RNA (Finger and Bryan, 2008; Hossain et al., 2002; Jacobs et al., 2006; Moriarty et al., 2002; O'Connor et al., 2005; Romi et al., 2007; Wyatt et al., 2007; Wyatt et al., 2009; Chapter 1; Xia et al., 2000), I evaluated the dependence of these TERT-TERT interactions on nucleic acids. The interactions between hTERT(1-200) and 1-1132, 1-200, 201-560, 601-928 and 928-1132 fragments were maintained even after co-purifying nucleic acids were degraded with micrococcal nuclease (Figure 2-4). Interestingly, hTEN interacted with 201-560, but not the smaller fragments of 201-350 or 350-560, suggesting that division of this region disrupted critical structural elements (Figures 2-2, 2-4). Indeed, the overlapping region of *T. thermophila* and *T. castaneum* TERTs contains a largely alpha-helical RNA binding domain (Gillis et al., 2008; Rouda and Skordalakes, 2007). Taken together, these results indicate that head-to-head, head-to-middle, and/or head-to-tail TERT interactions may facilitate human telomerase dimerization.
DISCUSSION

We found that two inactive hTERT mutants can cooperate to regenerate telomerase activity in vitro (Figures 2-1). In addition to trans hTR interactions (Ly et al., 2003; Rivera and Blackburn, 2004), this functional dimerization may be coordinated by DNA- and RNA-independent contacts between various domains of hTERT (Figures 2-2, 2-4).

That essential regions of hTERT can regenerate activity in trans (Beattie et al., 2001; Moriarty et al., 2002; Moriarty et al., 2004; Chapter 1, Figure 2-1) suggests that the telomerase holoenzyme complex may contain two (or more) hTERT polypeptides. I found that essential residues in the TEN and RT domains – Q169 and D868/D869, respectively – could be provided by different hTERT polypeptides (Figure 2-1). This result is reminiscent of the previous finding that the activity defects of hTERT N-terminal truncation mutants can be rescued by co-purification with full-length hTERT, even when the catalytic site of full-length TERT is inactivated by mutation of D712 (Beattie et al., 2001). Collectively, these observations underscore the essential contribution of the TEN domain to telomerase function.

Several observations suggest that the TEN domain may function as an accessory domain in telomerase. Firstly, the TEN domain is separated from CP/QFP/T domains (encompass RID2/hTR interaction domain 2/major hTR interaction domain) by a non-conserved region that varies in length among various organisms (reviewed in Kelleher et al., 2002). Also, the TEN domain is soluble as an independent domain (Jacobs et al., 2006; Jacobs et al., 2005; Chapter 1, Figure 2-3), and can interact with DNA, other domains in TERT, and other proteins independently of the rest of TERT (Finger and Bryan, 2008; Jacobs et al., 2006; Chapters 1 and 3, Appendix A). Finally, the TEN domain can confer telomerase function as a separable domain to hTERT truncation mutants containing an intact linkage between RID2 and RT domains (Beattie et al., 2001; Chapter 1; Moriarty et al., 2002). In the context of a dimeric telomerase, the TEN domain of one TERT molecule may support the reverse transcriptase function of the RNA binding and reverse transcriptase domains of a second TERT molecule.
This functional interaction between N- and C-terminal segments of hTERT may be supported by multiple intra- and/or intermolecular hTERT-hTR (Bachand and Autexier, 2001; Bachand et al., 2001; Beattie et al., 1998; Beattie et al., 2000; Mitchell and Collins, 2000; Moriarty et al., 2002; Moriarty et al., 2004), hTR-hTR (Chen and Greider, 2005; Keppler and Jarstfer, 2004; Ly et al., 2003; Moriarty et al., 2004; Ren et al., 2003; Theimer et al., 2005) and hTERT-hTERT contacts (as follows). Differentially tagged hTERT molecules generated in vitro co-purify in the absence of hTR (Armbruster et al., 2001; Moriarty et al., 2002), and multiple domains have been implicated in the hTERT-hTERT interaction (summarized in Figure 2-5). For example, recombinant fragments of hTERT, namely (201-538), (301-538) and (914-1132), interact with full-length hTERT (Arai et al., 2002). hTERT(914-1132), but not (928-1132), co-purifies with hTERT(301-534) from transfected COS-1 cell lysates, suggesting that hTERT(914-927) is required for the interaction between middle and C-terminal fragments of hTERT (Arai et al., 2002). Validation of these biochemical data was provided by the crystal structure of T. castaneum TERT which revealed an interface between C-terminal and RID2 regions through 1,677 Å² of shared, hydrophobic surface (Gillis et al., 2008).¹ According to experiments using in vitro-generated proteins, hTERT(1-200) interacts with hTERT(1-595), (595-946), and (946-1132) (Moriarty et al., 2004). In a similar experiment, I observed interactions between hTERT(1-200) and (1-200), (201-560), (601-928), and (928-1132) (Figures 2-2, 2-4). Notably, hTERT(914-928) was not required for the interaction between the hTERT C-terminus and hTERT(1-200). Beyond what has been described in the literature, I identified hTERT(201-560) as an RNA-independent interaction site with the hTERT N-terminus (Figure 2-5). I also presented inaugural evidence of a homotypic TEN domain interaction. In addition, my observation that the +92NAAIRS “DAT” mutation in the context of hTERT(1-350) did not disrupt the interaction with hTERT(1-200) (Figure 2-2), which is consistent with the sustained interaction between hTERT(+92NAAIRS) and a second full-length hTERT molecule (Armbruster et al., 2001), suggests that the in vivo defect of the +92 DAT mutant does not stem from an inability of the N-terminus to engage in

¹ Notably, T. castaneum TERT lacks a TEN domain, and is monomeric in solution (Gillis et al., 2008).
homotypic interactions. Collectively, my results suggest that, like *E. crassus* TERT (Wang *et al.*, 2002), hTERT is capable of N-terminal/N-terminal, N-terminal/RID2, and N-terminal/C-terminal interactions. *E. crassus* hTERT C-terminal/C-terminal interactions have also been described (Wang *et al.*, 2002).

The preferred orientation of TERT molecules in native telomerase is unknown. The plasticity and exclusivity of TERT inter-domain interactions are also unknown. For example, hTEN can interact with more than one domain in TERT (including another entity of itself) (discussed above), but whether these interactions can occur simultaneously or independently in different contexts is not known. Also, some of the inter-domain interactions identified *in vitro* may not be utilized *in vivo*. Adding another stratum of complexity, hTEN and other hTERT domains interact with DNA and other proteins (discussed above). Even though these interactions are not required for TERT-TERT dimerization *in vitro*, their effect on the formation and function of dimeric telomerase *in vivo* has not been investigated.

Not every study of human and *S. cerevisiae* telomerase has found evidence of dimerization. For example, two differentially tagged hTERT variants failed to co-purify from cells post-transfection (Errington *et al.*, 2008). Similarly, two hTR variants that could be discriminated by the addition of PP7 hairpins to the 5′ end failed to co-purify (Errington *et al.*, 2008). From an *S. cerevisiae* strain expressing similar levels of wild-type TLC1 and TLC1 containing a streptavidin aptamer insertion, wild-type TLC1 was not co-purified onto streptavidin with the aptamer-bearing TLC1 (Shcherbakova *et al.*, 2009). The reason for the incongruence of these findings with evidence of telomerase dimerization, as discussed above, is unclear; however, an absence of evidence for dimerization cannot necessarily be interpreted as evidence for the absence of dimerization. In any case, one investigation of telomerase utilizing two-colour coincidence detection presented strong evidence for the absence of dimerization, at least *in vitro*. The authors of this study reported that hTERT expressed and purified from reticulocyte lysates is monomeric in solution, and forms an active complex with hTR with an absolute stoichiometry of 1:1. Further, in complex with a telomeric primer, the absolute stoichiometry remains 1:1:1 (Alves *et al.*, 2008).
Why telomerase is observed as a dimer under some conditions but not others is unclear. Dimeric telomerase may not be detected upon disruption of intermolecular complexes during cellular fractionation or reconstitution of the enzyme in vitro in the absence of co-factors or enzyme biogenesis pathways. On the other hand, dimeric telomerase may be more prevalent upon over-expression in cells or when the enzyme is present in solution beyond a threshold concentration. While higher order telomerase interactions are not necessarily obligatory for function, the data presented in this Chapter demonstrate that TERT-TERT interactions can enable the formation and function of a telomerase dimer in vitro.

Several models of dimeric telomerase function have been proposed: the “parallel extension” of two telomeres by two active sites; the simultaneous binding of one telomere by the “anchor site” of one TERT and the active site of the second TERT; and the consecutive “switching” of two active sites – each containing its own TR/RNA template – at a given telomere (Kelleher et al., 2002; Prescott and Blackburn, 1997; Wenz et al., 2001). Human and S. cerevisiae telomerases do not appear to utilize template switching (Chang et al., 2007; Rivera and Blackburn, 2004). Our demonstration that telomerase is active when the sole, essential TEN (the putative anchor site) and reverse transcriptase domains are contributed by two different TERTs argues that parallel extension is not an obligatory mechanism of action. Alternatively, primer binding by the TEN domain (anchor site) of one TERT and the RT domain of a second TERT are compatible with the behaviour of telomerase in our model system. Given the potential utility of targeting telomerase in cancer, further study of the form and function of dimeric human telomerase in vivo is warranted.

**FUTURE DIRECTIONS**

**Defining the physical requirements for TERT dimerization**

Several domains in hTERT interact with one or more distinct domains. For example, the hTEN domain can interact with a second entity of itself, the RID2 domain, the RT domain, and the C-terminal extension (Figures 2-4, 2-5). Whether these interactions are mutually exclusive is not known. *In vitro* mixing
studies of saturating amounts of differentially tagged domains may resolve which inter-domain interactions are permitted to occur simultaneously. Also, the identification and artificial disruption of inter-domain contact surfaces will facilitate the investigation of whether these interactions, and telomerase dimerization in general, are required for function in vitro and in cells. The selection of potentially surface-exposed residues in hTERT for mutagenesis and phenotypic analysis may be guided by the structures of telomerase proteins/domains that have been determined to date (Gillis et al., 2008; Jacobs et al., 2006; Rouda and Skordalakes, 2007). Notably, none of the mutations in hTEN that I generated disrupted the interaction of hTEN with hTERT(201-1132) in the presence of hTR (Chapter 1).

**Functional complementation of inactive hTERT mutants in cells**

Despite the identification of an active, apparently dimeric, human telomerase complex isolated from various cell types (Cohen et al., 2007), as well as our demonstration that inactive hTERT mutants dimerize and exhibit functional complementation in vitro, evidence of the activity of a dimeric human telomerase in vivo is lacking. The dimerization of human telomerase in vivo could be inferred by the functional complementation of two inactive hTERT mutants in telomerase-negative cell lines. For example, whereas neither hTERT(Q169A) or hTERT(DD868-9AA) are active in telomerase-negative cells (Kim et al., 2003; Wyatt et al., 2009; Zhu et al., 1999; Chapter 1), when expressed in the same cell, these mutants may combine to regenerate telomerase activity, maintain telomeres, and immortalize cells.

To investigate this possibility, we transfected an HA5(H-Ras<sup>G12V</sup>) cell line<sup>1</sup> with combinations of pcDNA3.1(HYG) and pcDNA3.1(ZEO) vectors expressing wild-type or mutant hTERT variants (or empty vector controls), and monitored population doubling levels in culture under selection with hygromycin and zeocin (David Sealey and Michael Taboski). A polyclonal population of cells receiving both hTERT mutants – HYG/hTERT(Q169A) + ZEO/hTERT(DD868-9AA) – exhibited telomerase activity, telomere maintenance and colony forming potential, and continued to divide beyond cells

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<sup>1</sup>Michael Taboski generated the HA5(H-Ras<sup>G12V</sup>) cell line by retroviral infection of HA5 cells in a similar manner to the infection of HA1 HEK cells as described previously (Hahn et al., 1999a).
transduced with hTERT(Q169A) or hTERT/DD868-9AA (David Sealey, Michael Taboski, Natalie Erdmann). However, post-hoc RT-PCR analysis of mRNA transcripts suggested that wild-type hTERT had been re-generated at the exogenous locus (or loci) in these cells (i.e., at pcDNA3.1 integration sites) (Bryan Snow, Michael Taboski, David Sealey). While this apparent recombination between hTERT alleles precluded an examination of hTERT dimerization in this experiment, I propose that the functional complementation strategy may be utilized to query telomerase dimerization in vivo. In future experiments, telomerase-negative, diploid fibroblasts could be transduced with hTERT mutants via retroviral infection. Transduced cells could be selected using antibiotics that do not cause DNA damage (as zeocin does), or via fluorescence-activated sorting of cells co-expressing fluorescent proteins (Michael Taboski, David Sealey).

**Direct observation of telomerase dimers in vivo**

The supposition that human telomerase dimerizes in vivo is, thus far, based on analysis of the size and composition of active telomerase purified from cells (Cohen et al., 2007). Direct observation of dimeric telomerase inside cells has not yet been described. To this end, dual-label fluorescence techniques (e.g., fluorescence resonance energy transfer) may be suitable tools. These techniques may also be used to study inhibitors or endogenous modulators of telomerase dimerization.
Figure 2-1. Telomerase requires at least one N-terminus and one reverse transcriptase domain for activity, and these regions can be provided by different hTERT molecules. (A) FLAG-hTERT(201-1132)-FLAG, and wild-type (WT) or mutant full-length hTERT were synthesized in rabbit reticulocyte lysates (RRL) in the presence of [35S]methionine and hTR, mixed together as indicated, and immunoprecipitated onto anti-FLAG resin. Mock RRL reactions contained no DNA template. Top: IPs were subjected to SDS-PAGE and autoradiography. Bottom: Telomerase activity of the IPs was examined by TRAP assay. IC, internal control TRAP product. (B) 20% of input protein analyzed in (A) was subjected to SDS-PAGE and autoradiography. (C) Schematic representation of hTERT variants assayed in the indicated lane numbers. For example, lane 3 contains FLAG-hTERT(201-1132)-FLAG + hTR + hTERT(Q169A,DD868-9AA). Checkmarks indicate combinations that regenerated telomerase activity. Arrowheads indicate position of mutations. Conserved regions are indicated by white and black boxes.
Figure 2-2. N-terminus of hTERT interacts with several regions of hTERT, including the N-terminus itself. 
(A-B) Fragments of hTERT were synthesized in rabbit reticulocyte lysates (RRL), mixed together as indicated, and 
immunoprecipitated onto anti-FLAG resin. Immunoprecipitates (IP) were analyzed by western blotting using S 
protein-HRP (WB1), followed by anti-FLAG M2 and anti-mouse IgG-HRP antibodies (WB2). Arrows indicate 
proteins of interest. Molecular mass indicated at left (kDa).
Figure 2-2. N-terminus of hTERT interacts with several regions of hTERT, including the N-terminus itself.  
(C) Experiment performed as in (A-B). (D) Schematic representation of hTERT fragments that interacted (solid line) or did not interact (dashed line) with hTERT(1-200) as shown in (A-C).
Figure 2-3. Truncation of hTERT(1-200) results in non-specific immunoprecipitation. (A) Fragments of hTERT were synthesized in rabbit reticulocyte lysates (RRL), mixed together as indicated, and immunoprecipitated onto anti-FLAG resin. Immunoprecipitates (IP) were analyzed by western blotting using S protein-HRP (WB1), followed by anti-FLAG M2 and anti-mouse IgG-HRP antibodies (WB2). Arrows indicate proteins of interest. (B) Schematic representation of hTERT-S-HIS8 truncations that were immunoprecipitated specifically (solid line) or non-specifically (dashed line) onto FLAG-hTERT-FLAG truncations.
Figure 2-4. N-terminus of hTERT interacts with several regions of hTERT, including the N-terminus itself, independently of nucleic acids. (A) Fragments of hTERT were synthesized in rabbit reticulocyte lysates (RRL), mixed together as indicated, and immunoprecipitated onto anti-FLAG resin. Immunoprecipitates were subjected to incubation with micrococcal nuclease (MNase) or buffer, and analyzed by western blotting using S protein-HRP (WB1) followed by anti-FLAG/anti-mouse IgG-HRP antibodies (WB2). 10% of input (In) hTERT(1-200)-S-HIS8 was analyzed in the first lane. Arrows indicate proteins of interest. (B) Nucleic acid components of parallel immunoprecipitates were analyzed by electrophoresis through 2% agarose containing etidium bromide. Last lane, 1 kb+ DNA ladder (Invitrogen). (C) Schematic representation of hTERT fragments that interacted with hTERT(1-200) independently of nucleic acids (solid line), or did not interact with hTERT(1-200) (dashed line).
Figure 2-5. Schematic representation of interacting hTERT fragments described in this work and other studies. For each pair of interacting fragments, the following features are noted: amino acid boundaries, conserved domains, literature source. Interactions involving longer fragments that overlap with fragments depicted here are not shown.
STATEMENT OF CONTRIBUTIONS

Figures 2-1. Dr. Lea Harrington and I developed the strategy to examine the *trans*-complementation of hTERT(201-1132) by full-length hTERT mutants. While I had generated hTR by *in vitro* transcription during my studies, the hTR used in this experiment was produced by Dr. Linda Holland.

Of the DNA vectors used for experiments in this Chapter, I designed and constructed the following:

- pBiEx3-hTERT-S-HIS8 truncations: 1-200, 1-200(P2V), 1-57(P2V), 58-200.
- pcDNA3.1(*HYG*)-hTERT mutants: Q169A, Q169A/DD868-9AA, DD868-9AA.
- pcDNA3.1(*ZEO*)-hTERT mutants: DD8689-AA.

(Counter et al., 1992; Fouche et al., 2006; Gillis et al., 2008; Lingner and Cech, 1996; Schnapp et al., 1998; Wenz et al., 2001)
CHAPTER 3

The TPR domain of EST1 is a species-specific telomerase interaction module that regulates telomere length.

The experiments described in this Chapter were designed, executed and interpreted by me in the laboratory of Lea Harrington, with the following exceptions, for which credit is shared as indicated. **Figure 3-2.** David Sealey, Catherine LeBel, Alex Kostic. **Figure 3-3 and 3-4.** (A-C,E) Alex Kostic, David Sealey, Catherine LeBel. (D) David Sealey, Alex Kostic, Catherine LeBel. **Figure 3-5.** David Sealey, Catherine LeBel, Alex Kostic. **Figure 3-6.** Alex Kostic, David Sealey, Catherine LeBel. **Figure 3-7.** (A-D) Alex Kostic, David Sealey, Catherine LeBel. (E) David Sealey, Alex Kostic, Catherine LeBel. **Figure 3-8.** (A-D) David Sealey, Alex Kostic, Catherine LeBel. (E-F) David Sealey, Alex Kostic, Catherine LeBel. For additional details, refer to Statement of Contributions.

Portions of this Chapter are in preparation for publication. Lea Harrington and I have agreed that I will be first author, and Alex Kostic and Catherine LeBel will be middle authors.
INTRODUCTION

In *S. cerevisiae*, telomeres can be maintained by telomerase or *RAD52*-dependent recombination.\(^1\) *EST1*, *EST2* (TERT), *EST3*, and *TLC1* are essential for telomerase function, because loss of any of these genes results in progressive telomere shortening and senescence (Lendvay *et al.*, 1996; Lundblad and Szostak, 1989). Mutation of *CDC13* – an essential gene – elicits similar consequences (Lendvay *et al.*, 1996; Weinert and Hartwell, 1993). Rare “survivors” can bypass this senescence by maintaining telomeres through recombination. *RAD52*, while not absolutely essential for survival in the absence of telomerase in some genetic backgrounds, plays a critical role in the generation of telomerase-independent Type I and Type II survivors (Grandin and Charbonneau, 2009; Lebel *et al.*, 2009; Lee *et al.*, 2008b; Lundblad and Blackburn, 1993; Marinegele and Lydall, 2004b). Type I survivors maintain telomeres by amplification of Y’ elements (Lundblad and Blackburn, 1993) whereas Type II survivors maintain telomeres by amplification of telomeric repeats (Le *et al.*, 1999; Teng and Zakian, 1999).

Est1 recruits Est2 to the telomere in S phase via interactions with telomere DNA, TLC1, and Cdc13 (refer to Thesis Introduction) (reviewed in Evans and Lundblad, 2000; Smogorzewska and de Lange, 2004; Taggart and Zakian, 2003; Vega *et al.*, 2003). Est1 homologs in other organisms associate with telomerase and regulate telomere length and stability, although their roles are not completely understood. For example, *S. pombe* Est1 associates with active telomerase in cells, and *est1A* cells exhibit telomere shortening, senescence, and defects in chromosome end protection (Beernink *et al.*, 2003). In *C. albicans*, telomere lengths in *est1A* cells fluctuate over serial passages, suggesting that Est1 may repress homologous recombination at telomeres (Singh *et al.*, 2002). In humans, three Est1 homologs, hEST1A/SMG6, hEST1B/SMG5 and hEST1C/SMG7 (Figure 3-1), interact with chromatin and bind preferentially at telomeres (Azzalin *et al.*, 2007; Reichenbach *et al.*, 2003; Snow *et al.*, 2003). (Hereafter, the following nomenclature will be used: hEST1A, hEST1B, hEST1C.) hEST1A and hEST1B associate

\(^{1}\) Other pathways, which are beyond the scope of this Chapter, can be invoked in specific mutant backgrounds.
with active telomerase in cell lysates (Reichenbach et al., 2003; Snow et al., 2003), and these interactions can be recapitulated in vitro; in a reticulocyte lysate expression system, hEST1A and hEST1B interact with hTERT in the absence of hTR (Snow et al., 2003). Like ScEst1, hEST1A binds single-stranded telomeric DNA (Snow et al., 2003). Transient over-expression of hEST1A causes telomere uncapping/end-to-end fusion and apoptosis, and telomerase-positive cell lines over-expressing hEST1A exhibit short telomeres (Reichenbach et al., 2003; Snow et al., 2003). These effects suggest that excess hEST1A may titrate limiting amounts of telomerase away from the telomere. Indeed, concurrent over-expression of hTERT reverses the effect, causing telomere elongation (Snow et al., 2003). (hEST1B does not elicit the same changes in telomere length/stability.) Also, depletion of hEST1A results in the loss of telomere DNA at chromosome ends, suggesting that hEST1A may play a role in chromosome end protection (Azzalin et al., 2007).

EST1 homologs vary in size and structure. The region of highest homology among EST1 proteins includes tetratricopeptide repeat (TPR) consensus sequences (Beernink et al., 2003; Reichenbach et al., 2003; Singh et al., 2002; Snow et al., 2003; Figure 3-1). Typically, TPR domains mediate protein-protein interactions (Blatch and Lassle, 1999). The structure of hEST1C reveals a bona fide 14-3-3-like TPR domain of alpha helices, and several contiguous, upstream alpha helices fold into a TPR-like structure even though they lack the TPR repeat consensus sequence (Fukuhara et al., 2005). A DNA binding domain in hEST1A is localized to an N-terminal region that other EST1 proteins lack (Snow et al., 2003; Jen Cruickshank and Lea Harrington, unpublished). hEST1A interacts with hTR and other types of RNA through a so-called hTR-interaction domain (TRID) immediately downstream of the DNA binding domain (Redon et al., 2007). A region downstream of the TPR domain in hEST1B bears resemblance to the DNA binding domain of ScEst1 (Lundblad, 2003), although an ability of hEST1B to bind directly to DNA has not been described. hEST1A and hEST1B each contain a C-terminal ‘Pil N-terminal’ (PIN) domain (Glavan et al., 2006). The PIN domain of hEST1A provides a single-stranded RNA endonuclease activity and degrades PTC-containing mRNA (see below) (Eberle et al., 2009; Glavan et al., 2006). The
nuclease activity of the hEST1B PIN domain is greatly reduced compared to that of the hEST1A PIN domain, likely due to the absence of critical residues in the active site (Glavan et al., 2006).

Human EST1A, EST1B, and EST1C were identified not only by homology to *S. cerevisiae* Est1 in the TPR domain, but also by homology to the *C. elegans* nonsense-mediated mRNA decay (NMD) factors SMG-6, SMG-5, and SMG-7, respectively, in the TPR and PIN domains (Chiu et al., 2003; Clissold and Ponting, 2000; Gatfield *et al.*, 2003; Ohnishi *et al.*, 2003; Reichenbach *et al.*, 2003; Snow *et al.*, 2003). mRNA transcripts containing premature termination codons (PTC) a certain distance upstream of a terminal exon-exon junction are degraded by nonsense-mediated mRNA decay (NMD) – a process involving the phosphorylation and dephosphorylation of UPF1 by SMG1 and PP2A, respectively (reviewed in Behm-Ansmant *et al.*, 2007; Maquat, 2004). Depletion of hEST1A, hEST1B or hEST1C by RNA interference results in stabilization of PTC-containing mRNA (Gatfield *et al.*, 2003; Luke *et al.*, 2007; Paillusson *et al.*, 2005), indicating that each EST1 protein performs an essential role in NMD. hEST1A, hEST1B and hEST1C form complexes with SMG1, UPF1, PP2A, and other components of the NMD pathway (Chiu *et al.*, 2003; Ohnishi *et al.*, 2003), and are thought to mediate the dephosphorylation of UPF1 by recruiting PP2A (Chiu *et al.*, 2003; Ohnishi *et al.*, 2003). As described above, the hEST1A endonuclease also degrades NMD targets (Eberle *et al.*, 2009). Interestingly, levels of *S. cerevisiae* Est1, Est2, Est3, Stn1, Ten1 are regulated by NMD (Dahlseid *et al.*, 2003), but ScEst1 has no known role in NMD (Luke *et al.*, 2007).

Despite the structural and functional differences between human and *S. cerevisiae* EST1 proteins, we hypothesized that the functions and interactions of ScEST1 with respect to telomere length maintenance are conserved in the human EST1 proteins. This Chapter describes an investigation of the functional conservation of EST1 proteins in a model yeast system (Results: Part 1), as well as the determination of domains and residues in ScEst1 (Part 1) and hEST1A (Part 2) that are critical for function.
MATERIALS AND METHODS

Cloning of hEST1C

The cDNA corresponding to hEST1C/SMG7 transcript variant 2 (nucleotide accession no. NM_201568.1; protein accession no. NP_963862.1) was assembled from two I.M.A.G.E. clones. The NotI/BglII fragment of I.M.A.G.E. clone 4829267 (American Type Culture Collection 6821854) containing the first 2700 nt of hEST1C was inserted into NotI/BglII acceptor sites in I.M.A.G.E. clone 6044605 (available from Open Biosystems, EHS1001-6395538) in-frame with the remaining 3′ hEST1C sequence to yield pSport6-hEST1Cv2. Hereafter, hEST1Cv2 will be referred to as hEST1C. hEST1C was amplified by PCR using forward (5′-CCGATATCATGAGCCTGCAGAGCGC-3′) and reverse (5′-GCCTCGAGTCAGTGTGGAGGGTTCATGG-3′) primers containing EcoRV and XhoI sites, respectively. The PCR product was digested with EcoRV and XhoI, and subcloned into EcoRV/XhoI sites of pCDNA3.1(ZEO) in-frame with an HA2 tag to yield pcDNA3.1(ZEO)-HA2-hEST1C.STOP. The construct was verified by DNA sequencing.

Generation of the starting diploid yeast strain

The diploid S288C est1Δ::KAN/EST1 strain (MATα his3Δ ura3Δ leu2Δ met15Δ LYS2+ TRP1+) was a gift from Brenda Andrews (University of Toronto). The diploid was sporulated to obtain a haploid S288C est1Δ::KAN strain (MATa). The KAN gene replacement cassette was substituted with a NAT cassette by transformation and appropriate selection to obtain S288C est1Δ::NAT. This strain was crossed with a haploid S288C strain (MATa) of the same genetic background to create a diploid S288C est1Δ::NAT/EST1 strain. RAD52 was deleted by transformation with a KAN gene replacement cassette followed by appropriate selection to obtain S288C est1Δ::NAT/EST1 rad52Δ::KAN/RAD52 (Figure 3-2A). Gene deletions were verified by PCR and restriction enzyme digestion.
Yeast expression vectors and manipulation

Open reading frames were cloned into the multiple cloning sites of low-copy, pRS316 (CEN6, ARS4, URA3) (Sikorski and Hieter, 1989) and high-copy, pRS426 (2µ ori, REP3, FRT, URA3) (Christianson et al., 1992) expression plasmids. Plasmids were obtained from Raymund Wellinger (University of Sherbrooke). Diploid yeast were transformed according to a high-efficiency transformation protocol as described (Amberg et al., 2005) and adapted from (Gietz, 1995). Diploids were sporulated on solid media, asci were digested with zymolase, and haploid spores were dissected under a microscope using a microneedle.

Propagation of yeast on solid media

Haploid spores of the desired genotype were streaked onto plates containing synthetic dropout media lacking uracil (SD-URA) to select for maintenance of plasmids expressing the URA3 gene (refer to Figure 3-2A). After two or four days of growth at 30°C (P1), allowing for 20 generations, a single colony was re-streaked onto a second plate and incubated as described above (P2). Meanwhile, the P1 plate was stored at 4°C. This scheme was continued until P4 or P5 plates were obtained. To summarize growth from P1-6, one colony from each plate stored at 4°C (including the starting haploid spore from the tetrad dissection plate) was re-streaked onto a sector of a “summary plate” which was then incubated at 30°C for a final two or four days, as indicated. Plates were imaged on a Bio-Rad Molecular Imager Gel Doc XR System under white light. Images were captured below the point of signal saturation.

Yeast protein extraction and western blotting

Protein extracts were prepared according to a protocol as described (Amberg et al., 2005) and adapted from (Kushnirov, 2000). Briefly, single colonies were inoculated into 5 mL SD-URA media and grown overnight in a roller drum at 26°C. Cells were pelleted and resuspended in 100 µL distilled water. Cells were incubated at room temperature in 100 µL 0.2 M NaOH for 5 min. Cells were pelleted and resuspended in 50 µL PAGE buffer (120 mM Tris-Cl pH 6.8, 10% v/v glycerol, 4% w/v SDS, 8% v/v
β-mercaptoethanol, 0.004% w/v bromophenol blue) and boiled for 3 min. Proteins were resolved through 5% w/v 29:1 acrylamide:bisacrylamide gels, transferred to PVDF membranes, and subjected to western blotting using the following antibodies: anti-HA-HRP (3F10; Roche), rabbit anti-actin (Sigma), or mouse anti-FLAG M2 antibody (Sigma). Secondary antibodies used were sheep anti-mouse IgG-HRP or donkey anti-rabbit IgG-HRP (GE Healthcare), as appropriate. Reactive bands were detected with ECL Plus reagents, and fluorescent signals were captured using a Typhoon Trio variable mode imager (GE Healthcare).

Isolation of yeast genomic DNA

A single colony growing on solid media was inoculated into 5 mL SD-URA and grown overnight in a roller drum at 26°C. Cells were pelleted, transferred to 2 mL sterile tubes, washed with sterile water, and resuspended in 500 µL lysis buffer (0.1 M Tris-Cl pH 8.0, 50 mM EDTA, 1% w/v SDS). Glass beads (diameter = 425-600 µm; Sigma) were added up to two-thirds of the volume of the tube, followed by the addition of 25 µL 5 M NaCl. The bead preparation was vortexed for four intervals of 30 s. Soluble material was transferred to a clean tube, and extracted once with phenol and once with 24:1 chloroform:isoamyl alcohol. DNA was precipitated by the addition of 1 mL cold ethanol and incubation at -20°C for at least 1 hr. DNA was pelleted by centrifugation at 13,200 rpm at 4°C, and washed in 70% v/v ethanol. DNA was resuspended in 50 µL TE pH 8.0 and incubated with RNase A (Sigma) at 37°C for 30 min. 200 µL TE, 25 µL EDTA-Sarkosyl and 5 µL Proteinase K (10 mg/mL, Roche) were added, and the mixture was incubated at 37°C for 1 hr. Following the addition of 250 µL 5 M ammonium acetate, DNA was extracted, precipitated, and washed as described above.

Telomere length analysis by Southern blot

Genomic DNA (5 µg) was digested with XhoI overnight. Restriction fragments were resolved through a 0.7% w/v agarose gel at 45 V (2 V/cm). DNA was denatured in the gel by incubation in buffer containing 0.5 M NaOH and 1.5 mM NaCl for 30 min, and neutralized in buffer containing 1.5 M NaCl and 0.5 mM
Tris-Cl pH 7.5 for 30 min at room temperature. DNA was transferred to Hybond-N+ membrane in 20X SSC overnight. Following transfer, the membrane was rinsed in 2X SSC. Telomeric DNA was hybridized to a $^{32}$P 5’-end-labeled (CACACCCA)$_2$CC probe in Church buffer (0.5 M NaPO$_4$ pH 7.2, 1% w/v BSA, 7% w/v SDS, 1 mM EDTA), then washed with 1X SSC/0.1% w/v SDS. The membrane was exposed to a phosphorimager screen which was then scanned using a Typhoon Trio variable mode imager.

**Mutagenesis of hEST1A and ScEst1 TPR domains**

The structure-based sequence alignment of the TPR-like/TPR domain of hEST1C/SMG7 and other EST1 proteins (Fukuhara *et al.*, 2005) was used as a guide to select residues in hEST1A(551-785) and ScEst1(14-289) that may be surface-exposed. The design of PCR primers for site-directed mutagenesis of hEST1A was aided by The Primer Generator (Turchin and Lawler, 1999), and primers were obtained from Operon. The design of PCR primers for site-directed mutagenesis of ScEst1 was aided by SiteFind (Evans and Liu, 2005), and primers were obtained from IDT. The incorporation of the intended mutations into the respective cDNAs (and the absence of unwanted mutations) was confirmed by DNA sequencing.

**Generation of human/yeast EST1 TPR domain hybrids**

The boundaries of the TPR domain were selected according to the structure-based alignment of EST1 sequences (Fukuhara *et al.*, 2005; refer to Figure 3-5A). Plasmids encoding hybrid EST1 proteins in which the TPR domain of ScEst1 was replaced with the TPR domain of hEST1A, hEST1B, or hEST1C were generated by the *in vivo* gap-repair cloning method (Rothstein, 1991). The cloning scheme is depicted in Figure 3-5B. In the first step, pRS316*(URA3)*-Est1-FLAG was digested with NruI and PflMI which each cleave the TPR domain coding sequence at unique restriction sites in the plasmid. The digested plasmid was treated with alkaline phosphatase to remove phosphate moieties at the DNA ends. In parallel, the sequence encoding the TPR domain of hEST1 was amplified by PCR using hybrid primers. The 5’ ends of the forward primers contained 45 nt of sequence identical to sense strand immediately upstream of ScEst1 codon 14 (the TPR sequence starts at codon 14). The 3’ ends of the
Primers contained 30 nt of sequence identical to the sense strand of the TPR coding sequence of hEST1A (starting at codon 551), hEST1B (codon 25), or hEST1C (codon 2). GFP(S65T) (starting at codon 1) was also engineered in place of the TPR domain of ScEst1 as a control for disruption of the domain. The forward primers were as follows: Est1p13/hEST1A551, 5′- (PO₄)₂-GGCTTAATGGATAATGAAAGGTTAACGAAGAATGTATGAGAT-3′; Est1p13/hEST1B25, 5′- (PO₄)₂-GGCTTAATGGATAATGAAAGGTTAACGAAGAATGTATGAGAT-TACGGGCTGTGGAGGCTGTGCATCGACTT-3′; Est1p13/hEST1C2, 5′- (PO₄)₂-GGCTTAATGGATAATGAAAGGTTAACGAAGAATGTATGAGAT-TACGGGCTGTGGAGGCTGTGCATCGACTT-3′; Est1p13/GFP, 5′- (PO₄)₂-GGCTTAATGGATAATGAAAGGTTAACGAAGAATGTATGAGAT-TACGGGCTGTGGAGGCTGTGCATCGACTT-3′. The 5′ ends of the reverse primers contained 45 nt of sequence identical to the antisense strand of ScEst1 immediately downstream of codon 289 (the TPR sequence ends at codon 289). The 3′ ends of the primers contained 30 nt of sequence identical to the antisense strand at the end of the TPR encoding sequence of hEST1A (upstream of, and including codon 785), hEST1B (codon 262), or hEST1C (codon 236). The reverse primers used were as follows: Est1p290/hEST1A785, 5′- (PO₄)₂- TTTTGACACAAGAATTGCCAATTTTTTCATCAGACGTC-TTCTTTCTCCGCTGTCTCTCTAAACAAAGCTCAT-3′; Est1p290/hEST1B262, 5′- (PO₄)₂- TTTT-GACACAAGAATTGCCAATTTTTTCATCAGACGTC-TTCTTTCTCCGCTGTCTCTCTAAACAAAGCTCAT-3′; Est1p290/hEST1C236, 5′- (PO₄)₂- TTTTGACACAAGAATTGCCAATTTTTTCATCAGACGTC-TTCTTTCTCCGCTGTCTCTCTAAACAAAGCTCAT-3′; Est1p290/GFP, 5′- (PO₄)₂- TTTT-GACACAAGAATTGCCAATTTTTTCATCAGACGTC-TTCTTTCTCCGCTGTCTCTCTAAACAAAGCTCAT-3′. Primers were obtained from Invitrogen. Linearized plasmid DNA and PCR products were resolved through an agarose gel, excised, and purified. In step two, the gel-purified linearized plasmid DNA and individual PCR products were transformed into S. cerevisiae (haploid S288C est1Δ::NAT RAD52) and grown on solid SD media containing clonNAT (nourseothricin) and lacking uracil to select for the est1Δ genotype as well as for regeneration of the circular plasmid via homologous recombination between the 45 nt flanking sequences of the PCR products matching ScEst1 and plasmid-
borne Est1. The yeast contained intact RAD52 in order to allow recombination, and lacked EST1 to prevent recombination between the transformed PCR product and the endogenous locus. In step three, the resulting plasmids, in which the TPR domain sequence of ScEst1 was replaced with that of hEST1A, hEST1B, or hEST1C (or GFP(S65T)), were recovered using the Zymoprep II Yeast Plasmid Miniprep kit (Zymo Research) and amplified in E. coli via transformation and maxiprep (Qiagen). The coding sequences of the hybrid genes, including the junctions between human and yeast EST1 sequences, were verified by DNA sequencing. To create a set of pRS426 vectors, NotI/Sall fragments from the pRS316 vectors were ligated into the NotI/Sall sites of pRS426. The following plasmids were constructed:
pRS316(URA3)- and pRS426(URA3)-Est1(1-13)/hEST1A(551-785)/Est1(290-699)-FLAG;
pRS316(URA3)- and pRS426(URA3)-Est1(1-13)/hEST1B(25-262)/Est1(290-699)-FLAG;
pRS316(URA3)- and pRS426(URA3)-Est1(1-13)/hEST1C(2-236)/Est1(290-699)-FLAG;
pRS316(URA3)- and pRS426(URA3)-Est1(1-13)/GFP(S65T)/Est1(290-699)-FLAG.¹

Co-immunoprecipitation of hTERT and hEST1A fragments and micrococcal nuclease treatment

pCR3-FLAG-hTERT-FLAG and pcDNA3.1-HA2-hEST1A truncations were expressed in reticulocyte lysate in vitro transcription-translation reactions according to the manufacturer’s instructions (Promega Corp.) in the presence or absence of hTR. Mock reactions contained no cDNA. pcDNA3.0-FLAG-AKT (obtained from Jim Woodgett care of Lynn Wong, University of Toronto) was expressed as a negative control. Reactions were incubated separately for 90 min at 30°C. 15-20 µL of each reaction was mixed in a total volume of 500 µL with cold CHAPS buffer (0.5% w/v CHAPS, 10 mM Tris-Cl pH 7.5, 10% v/v glycerol, 100 mM NaCl, 1 mM MgCl₂ 1 mM DTT, Roche EDTA-free Complete protease inhibitor cocktail) and rocked for 1 hr at 4°C. FLAG-tagged complexes were immunoprecipitated with pre-equilibrated anti-FLAG M2 agarose resin (Sigma). Protein G sepharose (GE Healthcare), anti-c-Myc agarose (BD Biosciences), and glutathione sepharose (GE Healthcare) were used as negative control

¹ GFP(S65T) contains 237 amino acids.
For micrococcal nuclease digestions, immunoprecipitates were washed two times with cold CHAPS buffer and treated with 40 U/µL micrococcal nuclease (New England Biolabs) at 30°C for 45 min in buffer containing 50 mM Tris-Cl pH 7.9, 5 mM CaCl₂ and 1 mg/mL bovine serum albumin, or mock-treated in buffer without micrococcal nuclease. Reactions were stopped by the addition of excess EGTA, and immunoprecipitates were washed an additional two times in cold CHAPS buffer. For experiments not involving micrococcal nuclease, immunoprecipitates were washed three times in cold CHAPS buffer.

Immunoprecipitates were boiled in SDS-PAGE loading dye. Proteins were resolved through 4-20% w/v Tris-Glycine Novex gels (Invitrogen), transferred to PVDF membranes (Invitrogen), and subjected to immunoblotting with anti-HA-HRP (3F10; Roche). Antibodies were removed (where indicated) by incubation in stripping buffer (100 mM β-mercaptoethanol, 62.5 mM Tris-Cl pH 6.8, 2% w/v SDS) for 10 min at 50°C. Membranes were washed four times in 1X TBS-0.05% v/v tween20 (BioRad), and re-blotted with mouse anti-FLAG M2 (Sigma) and sheep anti-mouse IgG-HRP (GE Healthcare), or anti-hTERT(RT domain) (Oulton and Harrington, 2004) and donkey anti-rabbit IgG-HRP (GE Healthcare). Reactive bands were detected with ECL reagents (GE Healthcare) and exposure to film (Kodak). In later experiments, reactive bands were detected with ECL Plus reagents, and fluorescent signals were captured using a Typhoon Trio variable mode imager.

To analyze the presence of nucleic acids following micrococcal nuclease digestion, duplicate immunoprecipitates were extracted twice with 25:24:1 phenol:chloroform:isoamyl alcohol. Material in the aqueous layer was subjected to ethanol precipitation, resuspended in TE pH 8.0, resolved through a 1% agarose gel containing 0.5 µg/mL ethidium bromide, and visualized using a Typhoon Trio variable mode imager.
RESULTS: Part 1 of 2

Expression of hEST1A, hEST1B and hEST1C in S. cerevisiae

To learn more about the role of the human EST1 proteins in the telomerase pathway, we developed a system to assay human EST1 function in est1Δ S. cerevisiae, and to examine the interaction of human EST1 proteins with yeast telomerase components. In order to mitigate the long-term survival of cells via recombination-based telomere maintenance, we generated an est1Δ rad52Δ starting yeast strain. We first generated a diploid S288C strain that was heterozygous for EST1 and RAD52 (EST1/est1Δ::NAT RAD52/rad52Δ::KAN) (Figure 3-2A; refer to Materials and Methods). To validate our starting strain with respect to the previously characterized longevity of this genetic background, we sporulated the diploid to obtain four haploids, and assayed the spores for their replicative growth potential (Figure 3-2B). As expected, wild-type (EST1 RAD52) spores grew normally through six passages (representing 120 generations), likely due to the maintenance of telomeres by telomerase. This viability did not require RAD52, since est1Δ RAD52 cells exhibited decreased colony growth and small colony sizes beyond 40 generations – a senescence phenotype associated with the inability of telomerase to maintain telomeres (Lundblad and Szostak, 1989). Colonies exhibited weak growth potential over several more generations, but suddenly resumed proliferation in a manner typically associated with the activation of RAD52-dependent telomere recombination (Lundblad and Blackburn, 1993). In contrast, est1Δ rad52Δ spores became senescent after 40 generations and failed to recover. Therefore, the haploid progeny of the starting heterozygous diploid strain behaved as expected with respect to the onset of senescence and dependence of long-term proliferation on EST1 and RAD52.

We used this system to determine whether hEST1A, hEST1B or hEST1C could confer S. cerevisiae with the ability to proliferate in the absence of EST1 and RAD52. The diploid EST1/est1Δ RAD52/rad52Δ starting strain was transformed with pRS316(URA3) plasmids bearing hEST1A, hEST1B, or hEST1C,
selected on media lacking uracil, and sporulated (as above) to obtain haploid spores of all possible
genotypes with or without the plasmid. Two independent spores bearing the genotype of interest were
assayed in parallel (Figure 3-3). Haploid est1A rad52A spores expressing ScEst1, but not an empty vector
control, exhibited telomere maintenance and robust growth over many generations (Figure 3-3A,C,D).
Est1 fused at the C-terminus to a double HA tag (Est1-HA2) maintained telomeres at a shorter, stable
length (Figure 3-3D), and also allowed cells to avert senescence (Figure 3-3A,C). est1A rad52A cells
bearing HA2-hEST1B or HA2-hEST1C expression vectors exhibited telomere shortening and senescence
(Figure 3-3A,C,D). Interestingly, beyond a period of weak growth at P2, one of two isolates expressing
HA2-hEST1A exhibited telomere maintenance and extended longevity (Figure 3-3A,C,D).

Provided that telomeres are sufficiently long, S. cerevisiae can emerge from senescence at a low
frequency in the absence of both EST2 and RAD52 (or TLC1 and RAD52) when grown on solid media for
four days instead of two (Lebel et al., 2009). The longer growth interval may allow telomeres to activate a
telomerase- and RAD52-independent telomere maintenance mechanism. To query whether a longer
growth interval might facilitate the function of human EST1 proteins in yeast, we assayed the growth of the same est1A rad52A spores described above when passaged every four days. None of the spores
containing HA2-hEST1A, hEST1B, or hEST1C expression plasmids bypassed senescence when passaged
every four days (Figure 3-3A). That we did not observe RAD52-independent survivors (that exhibit a
Type II-like TRF pattern of telomeric repeat amplification) was not surprising given the limited number
of spores analyzed and the low frequency at which these survivors arise (Lebel et al., 2009).

To further investigate the ability of human EST1 proteins to function in place of ScEst1, the experiment
was repeated using pRS426 plasmids. pRS426 contains the 2µ origin of replication, and thus is expected
to be retained at a higher copy number (approximately 20 copies per cell; Christianson et al., 1992) than
the single-copy pRS316 plasmid which contains the ARS4 origin of replication (Sikorski and Hieter,
1989). As expected, est1A rad52A cells transformed with the pRS426 vector did not maintain their
telomeres or avert senescence (Figure 3-4A,C,D). Cells transformed with pRS426-Est1 or pRS426-Est1-
HA2 exhibited robust growth and telomere maintenance, suggesting that the HA tag did not diminish Est1 function (Figure 3-4A,C,D). Neither HA2-hEST1A or HA2-hEST1B allowed est1Δ rad52Δ spores to proliferate beyond P1 when passaged every two days or four days (Figure 3-4A,C). Telomere lengths could not be queried in these cells due to the inability of colonies to saturate a liquid culture for the purpose of extracting genomic DNA. Interestingly, like the surviving est1Δ rad52Δ spore containing pRS316-HA2-hEST1A, one of two spores containing pRS426-HA2-hEST1C exhibited telomere length maintenance and robust growth over several passages when re-streaked every two days, but not every four days (Figure 3-4A,C,D). Collectively, these results suggest that unlike ScEst1, human EST1 proteins do not have a generalized ability to complement the lack of est1Δ in the telomerase pathway; however, a minority of spores containing hEST1A or hEST1C continued to proliferate, indicating that these proteins may complement the absence of ScEst1 at a stochastic frequency.

That hEST1A, hEST1B and hEST1C did not universally function in place of ScEst1 suggests that the human proteins fail to engage in some or all of the interactions of Est1 with essential components of the telomerase pathway such as the telomere and/or Cdc13. As described above, rad52Δ cells survive due to the Est1-dependent maintenance of telomeres by telomerase (Figure 3-2B). We hypothesized that if human EST1 proteins were to interact with some, but not all of the in vivo targets of ScEst1, then human EST1 may interfere with the function of endogenous ScEst1 and cause telomere shortening and senescence. In effect, we sought indirect evidence of the interactions of human EST1 proteins with cellular targets by monitoring the effect on longevity of expressing the human EST1 proteins in the rad52Δ EST1 background. rad52Δ EST1 haploid isolates containing pRS316-HA2-hEST1A, hEST1B or hEST1C continued to survive beyond four/five passages, as did isolates expressing exogenous, untagged or HA-tagged EST1 or empty vector (Figure 3-3B-C). The expression of HA2-hEST1A and Est1-HA2 was confirmed by western blotting, but HA2-hEST1B and hEST1C were not detected (Figure 3-3E).

To confirm these results, we repeated the experiment using pRS426 plasmids. As before, none of the vectors shortened the proliferative lifespan of rad52Δ cells or interfered with telomere length maintenance
(Figure 3-4B-D). In fact, telomeres in the rad52Δ EST1 background became slightly longer with an increasing number of passages (Figure 3-4D; lanes 14-31, compare P1 to P3/P5), which is in accord with previous observations of increased telomere length in the S288C rad52Δ background (Chang et al., 2007; Lebel et al., 2009). Expression of HA2-hEST1A and Est1-HA2 from pRS426 appeared to be more robust compared to expression from pRS316 (Figure 3-4E versus Figure 3-3E). HA2-hEST1B and hEST1C were not detected, suggesting that these proteins were unstable, expressed below the level of detection, or not expressed (Figure 3-4E). Taken together, these results indicate that hEST1A did not competitively inhibit endogenous ScEst1 function, suggesting that the molecular determinants of the interaction of Est1 with cellular targets in S. cerevisiae may not be conserved in hEST1A. Due to an inability to detect hEST1B and hEST1C expression (Figure 3-4E), we reserve judgement on the ability of these proteins to competitively inhibit endogenous ScEst1.

**Expression of human/yeast EST1 hybrids in S. cerevisiae**

Given that the TPR domain stimulated the interactions between hEST1A and N-terminal fragments of hTERT (Results: Part 2 of 2), and that the TPR domain is contained within the region of highest homology among EST1 proteins, we investigated the functional conservation of the TPR domain between species. Using an *in vivo* gap-repair cloning strategy in S. cerevisiae (Rothstein, 1991), we generated hybrid human/yeast EST1 expression constructs in which the TPR domain of ScEst1 was replaced with the TPR domain of hEST1A, hEST1B or hEST1C (or GFP(S65T) – a similarly-sized control for disruption of the TPR domain) (Figure 3-5). The boundaries of the TPR domain were set according to the structure-based sequence alignment of EST1 proteins (Fukuhara et al., 2005). Specifically, the first nine alpha helices of hEST1C, or the corresponding regions of hEST1A or hEST1B, were integrated into the corresponding region of ScEst1.

We first determined whether the yeast/human hybrid pRS316-EST1 vectors could complement the senescence phenotype of the est1Δ rad52Δ strain. Replacement of the TPR domain of Est1 with
GFP(S65T) rendered Est1-FLAG incapable of rescuing senescence when spores were passaged every two or four days (Figure 3-6A,C). Similarly, none of the yeast/human hybrid Est1-FLAG constructs rescued senescence (Figure 3-6A,C). To verify these results, we repeated the experiment using pRS426 vectors. Even though the hybrid Est1-FLAG proteins were expressed (Figure 3-7D), they failed to facilitate telomere maintenance or rescue the senescence phenotype (Figure 3-7A,C,E). Therefore, the cognate TPR domain of *S. cerevisiae* Est1 is essential for telomere length maintenance.

We reasoned that if the TPR domain is essential for Est1 function, then co-expression of an EST1 variant containing a related, but non-functional TPR domain (*i.e.*, that of human EST1) may interfere with the normal operation of telomerase by interacting with some, but not all components of the telomerase pathway and/or titrating essential factors away from the telomere. Contrary to our prediction, *rad52Δ* cells expressing the hybrid EST1 pRS316 constructs continued to grow for many generations (Figure 3-6B-C). We repeated the experiment using pRS426 constructs. The hybrid Est1-FLAG proteins were expressed (Figure 3-7D), but had no effect on telomere length or proliferative lifespan (Figure 3-7B,C,E).

There are at least two possible explanations for this result. The hybrid EST1 proteins may have failed to interact with endogenous telomerase components, suggesting that the ScEst1 TPR domain is essential for these interactions. Alternatively, the hybrid EST1 proteins may have interacted with endogenous telomerase components (through the human TPR domain or other domains in Est1) but not out-competed ScEst1 at the telomere. These possibilities are not mutually exclusive.

**Function of the Est1 TPR domain**

To identify residues in the ScEst1 TPR domain that may be important for function, I generated point mutants at positions that I predicted to be exposed to the concave surface of the binding cleft based on the alignment of the ScEst1 sequence to the hEST1C TPR domain structure (Fukuhara et al., 2005; Table 3-1). I targeted acidic, basic, large and/or polar residues, and, in contrast to the hEST1A mutagenesis strategy described below (Results: Part 2 of 2), I changed all candidate residues to alanine. Whereas *est1Δ*
rad52Δ cells containing the empty pRS426 vector became senescent, all of the pRS426-Est1-FLAG mutants conferred viability over multiple generations (Figure 3-8,A,C). These Est1 mutants were assayed alongside Est1(F511S), which confers viability in the absence of endogenous Est1 but cannot prevent telomere shortening (Evans and Lundblad, 2002; Virta-Pearlman et al., 1996). As expected, telomeres in est1Δ rad52Δ cells containing Est1-FLAG were stable, but telomeres in cells containing Est1(F511S)-FLAG were shorter, and continued to shorten with increasing passages (Figure 3-8E, lanes 13-15, 28-33). Similarly, cells expressing Est1(K84A/W87A/Q89A)-FLAG exhibited telomere shortening (lanes 19-21) and microcolony formation (data not shown), suggesting that the mutated residues perform an important, but non-essential role in the telomerase pathway. Surprisingly, est1Δ rad52Δ cells containing any of the other Est1-FLAG mutants – namely E92A/Q96A/W97A, R193A/N197A, S200A/F203A/Y204A, F243A/Q244A/K247A, N271A/N278A, or D281A/T285A – exhibited longer telomeres than cells containing wild-type Est1-FLAG (Figure 3-8E). For some mutants, telomeres were long at an early passage and became longer and more heterogeneous with increasing passages.

The TPR domain mutants were also examined for dominant effects on telomere length in the rad52Δ background. None of the Est1-FLAG mutants interfered with the growth or viability of rad52Δ cells over several passages (Figure 3-8B,D). Est1(F511S) has been described to cause telomere shortening in a wild-type background (Evans and Lundblad, 2002). Interestingly, whereas both F511S and K84A/W87A/Q89A Est1 mutants did not prevent telomere shortening in est1Δ rad52Δ cells, Est1(F511S)-FLAG caused telomere shortening in rad52Δ cells while Est1(K84A/W87A/Q89A)-FLAG caused telomere elongation (Figure 3-8F, lanes 19-21, 28-33). Therefore, K84/W87/Q89 and F511 residues likely participate in different aspects of Est1 function. Expression of the other Est1-FLAG mutants in rad52Δ cells resulted in telomere elongation. Collectively, these results indicate that TPR domain of Est1 is required for telomere length maintenance, and also negatively regulates telomere length.
RESULTS: Part 2 of 2

Mapping the interaction between hEST1A and hTERT

The limited ability or inability of human EST1 proteins to complement the lack of ScEst1, and their apparent inability to interfere with endogenous Est1 via competition for binding partners (Figures 3-3, 3-4) are perhaps not surprising given that human and S. cerevisiae EST1 proteins share limited homology (Reichenbach et al., 2003; Snow et al., 2003; Figure 3-1). Notably, the region of highest homology contains a TPR domain that may be key to EST1 function. Thus, I investigated the function of the TPR domain of hEST1A.

Based on previous findings that hEST1A (and hEST1B) interacts with telomerase in vitro independently of hTR (Snow et al., 2003), and that that TPR domains typically mediate protein-protein interactions (Blatch and Lassle, 1999), I investigated whether the TPR domain of hEST1A interacts with hTERT. A TPR domain-containing, HA2-tagged fragment of hEST1A (HA2-hEST1A(502-824)) was expressed in reticulocyte lysates, mixed with FLAG-tagged full-length hTERT, or fragments of hTERT, and immunoprecipitated onto anti-FLAG agarose (Figure 3-9). hEST1A(502-824) precipitated onto the resin in the presence of full-length hTERT, hTERT(1-350) or hTERT(601-1132) (Figure 3-9, lanes 8, 9, 13). Curiously, hEST1A(502-824) also precipitated onto protein G sepharose and glutathione sepharose when hTERT was added to the mixture, even though hTERT was not also precipitated (lanes 17, 21). I confirmed this non-specific precipitation in a second experiment (Figure 3-10A). HA2-hEST1A(502-824) precipitated onto anti-FLAG agarose when mixed with FLAG-hTERT, FLAG-AKT1, or even mock RRL (lanes 5-7). Further, hEST1A(502-824) precipitated onto protein G sepharose when mixed with FLAG-hTERT, FLAG-AKT, or mock RRL, even though these proteins were not also precipitated (lanes 12-14). Notably, the binding of hEST1A(502-824) to resins without the addition of other factors was low or undetectable (Figure 3-9, lanes 7, 12, 16, 20; Figure 3-10A, lanes 4, 11). Even though, the TPR domain of

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1 An interaction between hEST1A and AKT has not been documented. AKT was intended as a negative control.
hEST1A may be predicted to have a defined structure by analogy to hEST1C (Fukuhara et al., 2005), these results suggest that the isolated domain is unstable and/or prone to non-specific precipitation, and, therefore, is not amenable to protein-protein interaction studies in vitro.

In an attempt to identify a soluble TPR domain-containing fragment of hEST1A, I assayed the interaction of HA2-hEST1A(114-824) with hTERT. hEST1A(114-824) precipitated onto anti-FLAG resin in the presence of FLAG-hTERT, but not FLAG-AKT or mock RRL (Figure 3-10A, lanes 19-21). hEST1A(114-824) did not precipitate non-specifically onto protein G sepharose (lanes 25-28). The interaction of hEST1A(114-824) with hTERT was re-capitulated with both hTERT(1-350) and hTERT(601-1132) (Figure 3-10B, lanes 7-9). hTERT and/or hEST1A(114-824) did not precipitate onto anti-Myc agarose (a matrix containing the same IgG subtype as anti-FLAG agarose; lanes 12-14), confirming the specificity of the co-immunoprecipitation of hEST1A and hTERT. Therefore, hEST1A(114-824) is sufficiently stable to be studied in vitro.

To isolate the region of hEST1A that interacts with hTERT, I assayed N- and C-terminal truncations of hEST1A(114-824). The level of hEST1A(114-631) immunoprecipitated by anti-FLAG resin in the presence of hTERT was barely detectable, suggesting that the TPR domain of hEST1A mediates the hTERT interaction (Figure 3-11A, lanes 7-9). Interestingly, truncating the protein at, or near the TPR domain boundary (114-780 and 114-749, respectively) resulted in non-specific precipitation (Figure 3-11B). Trimming the N-terminus of the stable hEST1(114-824) fragment to residue 334 also resulted in non-specific precipitation (Figure 3-11A, lanes 26-28). hEST1A(334-824) lacks the DNA binding domain (Snow et al., 2003; Jen Cruickshank and Lea Harrington, unpublished) and part of the RNA binding domain (Redon et al., 2007), suggesting that these domains may stabilize the hEST1A(114-824) fragment.

The C-terminus of the hEST1A(114-824) fragment assayed in Figure 3-10 contained an additional 24 amino acids resulting from translation beyond amino acid 824 up to the stop codon encoded by the vector.
To verify the C-terminal boundary of the hTERT-interacting fragment of hEST1A, I modified the vector to include a stop codon (*** immediately following residue 824. hEST1A(114-824)*** immunoprecipitated specifically with full-length hTERT, hTERT(1-350) and hTERT(601-1132), verifying the initial result (compare Figure 3-12 to Figure 3-10).

The hEST1A TPR domain sequence contains two KpnI restriction sites. Codons 708-724 were removed by digestion of the hEST1A(114-824) vector with KpnI. Subsequent ligation resulted in the in-frame abutment of amino acids 707 and 725. Loss of amino acids 708-724 did not impair the interaction with hTERT (Figure 3-12, lanes 13-15). The corresponding portion of hEST1C was not present in the crystal structure, leading Fukuhara et al. to propose that the region is a disordered loop (Fukuhara et al., 2005). Thus, while this segment of hEST1A was not required for the interaction with hTERT, it may not be a key structural component of the domain. The hEST1A-hTERT interactions described up to this point are summarized in Figure 3-13.

To identify residues in the TPR domain of hEST1A that mediate the hTERT interaction, I employed a mutagenesis strategy. Guided by the alignment of the hEST1A sequence to the hEST1C TPR domain structure (Fukuhara et al., 2005), I generated point mutations in acidic, basic, large and/or polar residues in hEST1A that I predicted to be exposed to the concave surface of the putative binding cleft (Table 3-1). In designing mutations, I attempted to match the approximate size of the side chains in the native amino acids while swapping the chemical nature (i.e., from acidic to basic) and avoiding moieties matching the corresponding amino acids in hEST1B and hEST1C. I assayed the mutations in the context of hEST1A(114-824)*** for an inability to interact with hTERT(1-350) or hTERT(601-1132). All of the mutants were expressed in reticulocyte lysates (Figure 3-14A-D). The extent to which any of the mutants co-immunoprecipitated with hTERT(1-350) or hTERT(601-1132) could not be reliably distinguished from that of wild-type hEST1A(114-824)*** (Figure 3-14A-D). Therefore, amino acids in the TPR

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1 This construct encoded the 24 C-terminal amino acids derived from the vector backbone, as described above.
domain required for the interaction with hTERT were not identified.

“DAT” mutations in hTERT have modest effects on activity \textit{in vitro}, yet render telomerase unable to maintain telomeres and immortalize cells (Armbruster \textit{et al.}, 2001; Banik \textit{et al.}, 2002; Moriarty \textit{et al.}, 2005; Chapter 1, Appendix A). The cellular defects of some hTERT-DAT mutants can be rescued by fusion to the telomere binding proteins TRF2 or POT1, suggesting that the DAT phenotype may stem from an inability of telomerase to become recruited to the telomere. ScEst1 is thought to recruit telomerase to the telomere during S phase by bridging Cdc13 and TLC1 (refer to Introduction). hEST1A interacts with telomerase and telomeres, and causes telomere lengthening when over-expressed with hTERT (Azzalin \textit{et al.}, 2007; Reichenbach \textit{et al.}, 2003; Snow \textit{et al.}, 2003); however, whether hEST1A recruits telomerase to the telomere in a similar manner to ScEst1 is not known. Using the \textit{in vitro} co-immunoprecipitation assay, I explored the possibility that hEST1A may be a telomerase recruitment factor. The +92 or +122 DAT mutations (NAAIRS substitutions starting at codons 92 or 122, respectively) in the context of full-length hTERT or hTERT(1-350) did not impair the interaction with hEST1A(114-824) (Figure 3-15), suggesting that the \textit{in vivo} defect of these mutants does not stem from an inability to interact with hEST1A, at least at a gross level.

During this work, Redon \textit{et al.} reported that a region upstream of the TPR domain in hEST1A interacts with a portion of the N-terminus of hTERT in an RNA-dependent manner, and that both regions of hEST1A and hTERT interact with hTR (Figure 3-18). They also provided evidence of an hTR-independent interaction between hEST1A and hTERT, but did not identify the interaction site in hEST1A (Redon \textit{et al.}, 2007). Meanwhile, I gained access to western blotting tools that lowered the threshold of detection compared to the detection method I had been using (ECL Plus and the Typhoon Trio variable mode imager versus ECL and exposure to film). Using these tools, I re-evaluated the requirement of the TPR domain in hEST1A for interaction with hTERT, both in the presence and absence of hTR.

Using the more sensitive co-immunoprecipitation assay, I observed interactions between
hEST1A(114-631) and hTERT(201-560), 1-350, and 601-1132 at a level above background in the presence of hTR (Figure 3-16A, compare lane 2 to lanes 6-8). An interaction of hEST1A(114-631) with hTERT(1-200) was barely detectable at a level above background (lane 3). To determine the dependence of these interactions on hTR and other nucleic acids, hTR was omitted from the reticulocyte lysate reactions, and the immunoprecipitation samples were treated with micrococcal nuclease (MNase). To verify MNase activity, nucleic acid components of the immunoprecipitates were purified, resolved by gel electrophoresis and stained with ethidium bromide (Figure 3-16B). MNase treatment increased the background level of hEST1A(114-631) immunoprecipitated onto the resin (Figure 3-16A, compare lanes 1, 9), but a clear signal above background was observed in the presence of hTERT(1-200), 201-560, 1-350, and 601-1132. Therefore, the hEST1A TPR domain, hTR, or other nucleic acids present in the reticulocyte lysates were not required for the interaction of hEST1A with several regions of hTERT. I also re-evaluated hEST1A(114-824)*** which previously exhibited a robust interaction with hTERT N- and C-termini (Figure 3-12). Like hEST1A(114-631), hEST1A(114-824)*** interacted with hTERT(1-200), 201-560, 201-560, 1-350, and 601-1132, but not hTERT(201-350) or 350-560, in the presence and absence of hTR (Figure 3-16A, lanes 16-30).

The interaction of hEST1A(114-824)*** with hTERT appeared to be more robust than that of hEST1A(114-631) (Figure 3-16); however, a direct comparison could not be made because the fragments were analyzed on separate gels. In order to evaluate the contribution of the hEST1A TPR domain to the interaction with hTERT, I repeated the experiment and compared both hEST1A fragments on the same gel (Figure 3-17A). Even though hEST1A(114-631) was not limiting (compare lanes 1, 14), the level of hEST1A(114-824) that co-immunoprecipitated with hTERT(1-200), 1-350, and 201-560 following treatment with MNase was greater than that of hEST1A(114-631) (compare lanes 8-10 to 5-7). hEST1A(114-631) and hEST1A(114-824)*** co-immunoprecipitated with C-terminal fragments of hTERT to a similar extent post-MNase treatment, as determined by comparing the fold-enrichment of signal in the presence versus in the absence of hTERT (Figure 3-17B; for example, compare lanes 3 and 4
versus lanes 9 and 10). Notably, the interaction of hEST1A with hTERT(601-1132) was recapitulated with hTERT(601-928) and 928-1132 (Figure 3-17B, lanes 4-9). Taken together, these data indicate that the TPR domain stimulated the interaction of hEST1A with multiple N-terminal fragments of hTERT, but had no effect on the interaction with C-terminal fragments (summarized in Figure 3-17C). (Refer to Discussion for further comparison with the findings of Redon et al.)

**DISCUSSION**

The original motivation for this study was to determine whether the functions and inter-molecular interactions of human EST1A, EST1B and EST1C were conserved with those of the telomerase recruitment protein ScEst1. hEST1B did not function in place of Est1, and the complementation of the telomere maintenance function of Est1 by hEST1A and hEST1C appeared to be stochastic. hEST1A interacted with multiple domains of hTERT independently of hTR, and the TPR domain of hEST1A engaged in a specific interaction with the N-terminus of hTERT. The TPR domain of ScEst1, but not that of hEST1A, hEST1B or hEST1C, afforded yeast the ability to maintain telomeres and divide indefinitely. This telomere maintenance function was mapped to specific residues in the TPR domain. We also identified residues in the Est1 TPR domain that, paradoxically, negatively regulated telomere length.

The lack of a universal ability of hEST1A, hEST1B or hEST1C to function in place of Est1 in the telomere maintenance pathway is not surprising given the distinct molecular features of each protein (Figure 3-1) and the divergence of other aspects of telomere biology. For example, the sequence of the telomere differs between humans and *S. cerevisiae*; hPOT1 protects human telomeres whereas Cdc13 protects yeast telomeres (through its binding partners); and the length and structure of the human and *S. cerevisiae* telomerase RNAs are quite different (refer to Thesis Introduction; Chen et al., 2000; Dandjinou et al., 2004; Zappulla and Cech, 2004). One of four est1Δ rad52Δ spores expressing hEST1A that we examined survived beyond 100 generations when passaged every two days, but not every four days (Figures 3-3, 3-4). One of four spores expressing hEST1C displayed a similar ability to survive. S288C
Est2Δ rad52Δ and tlc1Δ rad52Δ yeast can bypass senescence at a low frequency by activating a telomerase- and RAD52-independent telomere maintenance pathway (Lebel et al., 2009). The telomere restriction fragment pattern in the surviving est1Δ rad52Δ spores containing hEST1A or hEST1C did not resemble the Type II-like telomeric repeat amplification pattern that is characteristic of telomerase- and RAD52-independent survivors (Lebel et al., 2009; Teng et al., 2000; Figures 3-3D, 3-4D). Rather, telomeres resembled those in est1Δ rad52Δ cells expressing exogenous Est1 (Figures 3-3D, 3-4D). Therefore, telomeres in these cells were likely maintained by telomerase. The apparently stochastic nature of the hEST1-dependent survival suggests that hEST1A and hEST1C did not engage in the full array of canonical Est1 interactions. Rather, hEST1A and hEST1C may have enabled the action of telomerase at the telomere by binding to enough Est1 targets. However, such interactions, had they occurred, did not interfere with Est1 and telomerase in the rad52Δ background (Figures 3-3, 3-4).

We were unable to confirm the expression of HA2-hEST1A, HA2-hEST1B, HA2-hEST1C, or Est1-HA2 in the est1Δ rad52Δ background by western blotting (data not shown). Nevertheless, cells transformed with Est1-HA2 survived, suggesting that pRS316 and pRS426 were competent vectors. Expression of HA2-hEST1A and Est1-HA2 but not HA2-hEST1B was evident in the rad52Δ background; therefore, we cannot formally exclude an ability of hEST1B to function in yeast under conditions when it is expressed.

There is no evidence to suggest that hEST1B was overtly toxic to cells. While harboring pRS316/426-HA2-hEST1B, diploid transformants and haploid spores survived, and haploid est1Δ rad52Δ spores did not exhibit accelerated senescence compared to cells transduced with empty vectors (Figures 3-3, 3-4).

The yeast experiments did not further elucidate the roles of human EST1 proteins – only that there exist inter-species incompatibilities in the telomere maintenance pathway. Even in humans, the three EST1 proteins perform unique roles. For example, each of hEST1A, hEST1B, and hEST1C performs a non-redundant role in the NMD pathway (Gatfield et al., 2003; Luke et al., 2007; Paillusson et al., 2005).

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1 That Type II-like survivors did not emerge in the est1Δ rad52Δ background was not surprising given that we analyzed a limited number of spores and colonies.
hEST1A contains an active PIN endonuclease domain, whereas hEST1B and hEST1C do not (Glavan et al., 2006). hEST1B and hEST1C localize to cytoplasmic foci that exclude hEST1A (Unterholzner and Izaurralde, 2004). Lastly, hEST1A and hEST1B (but apparently not hEST1C) interact with telomerase (Redon et al., 2007; Reichenbach et al., 2003; Snow et al., 2003).\(^1\) If ScEst1 functions have been segregated to individual human EST1 proteins, co-expression of human hEST1 proteins (for example, hEST1B and hEST1C, or hEST1A, B and C) may complement the loss of ScEST1.

A recent report suggested that hEST1C resembles ScEbs1 – a protein which also shares homology with Est1 in the EST1 homology domain (Luke et al., 2007). hEST1C and ScEbs1 share a second region of homology in their C-termini (amino acids 883-963 in hEST1C). Similar to the effect of depleting hEST1C (or hEST1A or hEST1B) in human cells, deleting EBS1 (but not EST1) in yeast impairs NMD (Gatfield et al., 2003; Luke et al., 2007). Also, over-expression of hEST1C or Ebs1 in S. cerevisiae disrupts the NMD pathway (Luke et al., 2007). Whether hEST1C can complement the NMD defect of ebs1Δ cells has not been described. In any case, these findings suggest that hEST1C may be the functional homolog of ScEbs1 rather than ScEst1.

An in vitro approach yielded more information about functional domains in hEST1A. By co-immunoprecipitation of proteins expressed in reticulocyte lysates, I identified the determinants of the previously described, RNA-independent interaction between hEST1A and hTERT (Snow et al., 2003). N- and C-terminal regions of hTERT interacted with hEST1A(114-631). The interaction of N-terminal regions of hTERT (1-200, 1-350, 201-560), but not C-terminal regions (601-928, 928-1132), with hEST1A became more robust when the hEST1A fragment included the TPR domain (Figures 3-13, 3-17). These findings are partially complementary with those of a previous study (Redon et al., 2007; compare Figures 3-17 and 3-18). hEST1A(212-381) and hTERT(147-311) were identified as hTR-interaction

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\(^1\) We investigated the interaction of hEST1C with telomerase by transfecting telomerase-positive cells with pcDNA3.1(ZEO)-HA2-hEST1C and assaying anti-HA immunoprecipitates for telomerase activity. Due to non-specific precipitation of telomerase activity, we were unable to resolve an interaction between hEST1C and telomerase (David Sealey, Michael Taboski and Alex Kostic).
domains. hTERT(147-311) co-immunoprecipitated hEST1A from co-transfected 293T cell lysates, and hEST1A(212-381) immunoprecipitated telomerase activity from 293T lysates, suggesting that these regions of hEST1A and hTERT interact through hTR (Redon et al., 2007). hEST1A fragments beyond 212-502 did not associate with telomerase activity, implying that these fragments do not interact with telomerase (Redon et al., 2007); however, positive detection in the assay depended on hTR and telomerase activity. If co-precipitating EST1 fragments interfered with telomerase activity, interactions would not have been detected. The finding that hTERT(147-311) interacts with hEST1A is compatible with my finding that hTERT(1-200), 1-350, and 201-560 interacted with hEST1A in vitro, although the interactions that I observed were RNA-independent. Redon et al. also reported that hTERT(1-144) and 1-78 failed to co-immunoprecipitate hEST1A from cell lysates, perhaps suggesting that interaction between hEST1A and hTERT(1-200) that I observed occurred through hTERT(145-200). While C-terminal fragments of hTERT beyond amino acid 347 were not tested by Redon et al., I observed interactions between such fragments and hEST1A. Finally, Redon et al. pointed to an RNA-independent interaction between hTERT(147-311) and hEST1A outside of (212-502) on the basis that full-length hEST1A, but not hEST1A(212-502) remained bound to hTERT(147-311) following treatment with RNaseA, and that hEST1A and TERT co-purified from VA13 cells which do not express hTR. Collectively, our results suggest that an RNA-independent hTERT binding site exists in hEST1A(502-824) (Figure 3-17). This region contains the TPR domain, yet is unstable in vitro when isolated from the remainder of the protein (Figures 3-9, 3-10A). While hTR may bind hEST1A and hTERT simultaneously, hTR is clearly not required for the interaction of these two proteins in vitro or in cells (Redon et al., 2007; this work). The reason for the apparent discrepancy in the hTR-dependence of some hEST1A/hTERT interactions between our two studies is unclear, but may stem from the use of different expression systems (i.e., 293T cells versus reticulocyte lysates) and fragments with different N- and C-terminal boundaries.

The role of the TPR domain of hEST1A in the hTERT interaction was determined by assaying the
co-precipitation of hEST1A(114-631) and 114-824 fragments, because was 502-824 was unstable on its own (Figures 3-9, 3-10A). Therefore, to identify critical residues in the TPR domain, the longer fragment (114-824) was subjected to mutagenesis. None of the mutants that we assayed disrupted the hTR-independent interaction with hTERT(1-350) or 601-1132 (Figure 3-14). The ability to detect interaction defects among the hEST1A(114-824) mutants may have been hampered by confounding interactions between residues 114-631 and hTERT. Also, in designing the mutations, I purposely avoided amino acids predicted to reside in the interior of the domain so as to not disrupt overall folding. Instead, I attempted to change the chemical nature of the surface of the putative concave binding cleft (by analogy to hEST1C). Each set of mutations altered only a localized area of the predicted protein-protein interaction interface. Future studies may utilize combinations of mutations across different regions of the predicted surface. Also, since all of the mutations were designed to target the predicted concave surface of the TPR domain of hEST1A, defects in hTERT binding would not have been observed if hTERT interacts with the opposite, convex surface.

The TPR domains of EST1 proteins also interact with other proteins. For example, GST-hEST1A(545-785) interacts with in vitro-synthesized UPF1 (Fukuhara et al., 2005). The TPR domain of hEST1C binds a phosphoserine-containing UPF1 peptide in a similar manner to the binding of phosphopeptides by the TPR domain of 14-3-3. The interactions of hEST1C(1-497) and hEST1A(545-785) with UPF1 are reduced by K66E/R163E and R625E/R706E mutations, respectively, as well as by treatment with phosphatase (Fukuhara et al., 2005). Therefore, the aforementioned residues in the TPR binding cleft are thought to coordinate a phosphate moiety of a target protein. Notably, I observed that the R625E/R706E double-mutation in hEST1A(114-824) did not disrupt the interaction with RRL-expressed hTERT(1-350) or 601-1132 (Figure 3-14D). The interactions also remained intact upon disruption of neighbouring amino acids (D703/R706/Y707/Y724) corresponding to residues of prominence in hEST1C (Figure 3-14D). While hTERT can be phosphorylated by Akt and c-Abl (Kang et al., 1999; Kharbanda et al., 2000), the phosphorylation sites have not been determined. Also, whether hEST1A binds a phosphopeptide motif in
hTERT is not known. These results suggest that UPF1 and hTERT interact with distinct sites within the TPR domain of hEST1A.

The sequence conservation among EST1 proteins in the TPR domain suggests a conserved role for the domain. The loss of ScEst1 function upon replacement of the TPR domain with GFP or the corresponding regions of hEST1A, hEST1B or hEST1C indicates that the cognate TPR domain of ScEst1 is required for telomere length maintenance (Figures 3-5 to 3-7). The cross-species incompatibility of EST1 TPR domains suggests that even though they may perform analogous functions, their molecular surfaces and/or those of their binding partners are not conserved. Indeed, hTERT interacts with the TPR domain and upstream region of hEST1A independently of nucleic acids (Figure 3-17). In contrast, Est1 and Est2 interact through TLC1. Est1 and Est2 interact with separate regions of TLC1 (Hughes et al., 2000; Livengood et al., 2002; Seto et al., 2002; Zhou et al., 2000), and Est1 does not co-purify with Est2 in a tlc1Δ strain (Evans and Lundblad, 2002). Also, mutations in the TPR domain of ScEst1 reduce TLC1 binding (Evans and Lundblad, 2002) (see below), suggesting that the modes of EST1 interaction with telomerase in human and S. cerevisiae are different. Notably, while hTR may bridge hTERT and hEST1A (as described above), the interaction of hTR with hEST1A does not appear to involve the TPR domain (Redon et al., 2007).

Previous mutagenesis of Est1 (Evans and Lundblad, 2002) was performed before the structure of a sequence homolog (hEST1C) was available. According to the hEST1C TPR structure, and if the Est1 structure is conserved, some of the previously characterized mutants may involve residues that are not exposed to either surface of the domain (Est1-K24, K50, E56, E57, K61) or that are exposed to the convex surface of the domain (R19, D23, R49, R53). Mutation of these residues resulted in a wild-type phenotype. Further, some mutations were made at residues that may reside in a turn between two helices.

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1 Treatment of hTERT(1-350), 1-200, 201-560, 601-928, and 928-1132 with lambda phosphatase did not reduce the interaction with hEST1A(114-824) (data not shown). However, because I did not have an assay to verify phosphatase activity, this result is subject to confirmation.
The short telomere phenotype associated with mutation of these residues may have resulted from misfolding of the domain. Interestingly, two of the mutants that exhibited short telomeres and a reduced association with TLC1 involved residues that may be exposed to the convex surface of the domain (118/122/123, 222/223/226), suggesting that, like hEST1A (as discussed above), Est1 may interact with telomerase through the convex surface and not the concave surface. Future mutagenesis of the convex surfaces of hEST1A and Est1 may identify a conserved, species-specific telomerase interaction site.

My strategy to mutagenize ScEst1 targeted the residues in the TPR domain that align to the concave surface of the TPR domain of hEST1C (Table 3-1). The K84A/W87A/Q89A mutant had a telomere shortening phenotype, but continued to grow beyond 100 generations (Figure 3-8). In contrast, six of the mutants caused telomeres to become longer than telomeres in cells expressing wild-type Est1 (Figure 3-8). This telomere lengthening was unexpected given that telomeres became shorter upon complete disruption of the domain or deletion of the entire gene (Figure 3-7; Lundblad and Szostak, 1989). Taken together, these results indicate that the Est1 TPR domain both positively and negatively regulates telomere length, and that both surfaces of the domain are required for proper regulation.

The concave surface of the Est1 TPR domain may negatively regulate telomere length through an interaction with Ies3—a component the INO80 chromatin remodeling complex (Yu et al., 2007). Est1 and Ies3 interact by yeast two-hybrid and co-immunoprecipitation criteria, and deletion of Ies3 results in telomere lengthening (Yu et al., 2007). Interestingly, Est1(G199A/P236A/N278A) fails to interact with Ies3 (Yu et al., 2007), and we observed that expression of Est1(N277A/N278A) in est1Δ rad52Δ or rad52Δ backgrounds caused telomere elongation (Figure 3-8). Yu et al. proposed that INO80 may negatively regulate telomere length directly by altering telomeric chromatin, or indirectly by regulating

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1 Four mutants that I designed have not yet been constructed or characterized: H21A/H25A, S28A/R29A, N68A/N69A/N70A, E99A/H100A/H103A.

2 By sequence correlation with the structure of hEST1C (Fukuhara et al., 2005), Est1-N278 may reside on the concave surface of the TPR domain.
the transcription of telomere-related genes. Further studies are needed to elucidate the interactions of the Est1 TPR domain with TLC1, Ies3, and potentially other factors. The interaction of INO80 with human EST1 proteins is another area for investigation.

Several cellular pathways and processes, including those that become deregulated in cancer such as the cell cycle and the DNA damage checkpoint response have been elucidated in yeast (e.g., Hartwell et al., 1970; Weinert and Hartwell, 1988). Many of the genes and proteins involved in these pathways are evolutionarily conserved. Thus, yeast species, with genomes that are easier to manipulate than those of many higher eukaryotes, continue to serve as informative hosts for the study of these pathways and the structure and function of human protein counterparts (Nitiss et al., 2007; Resnick and Cox, 2000). For example, hRAD54 partially rescues the sensitivity of rad54Δ yeast to methyl methanesulfonate (Kanaar et al., 1996), and the core domain of the transcriptional silencing protein hSIR2A can substitute for the core domain of ScSir2 to without disrupting cell viability (Sherman et al., 1999). Of course, many genetic pathways, developmental programs, homeostatic controls, protein-protein interactions, telomere-related interactions, and so on are not ubiquitously conserved. For example, the TP53 tumor suppressor and POT1 telomere protection genes are absent from yeast. In any case, we turned to a Saccharomyces cerevisiae system to learn more about the functions of hEST1A, hEST1B, and hEST1C. The incompatibility of the human EST1 proteins in the yeast telomerase pathway, and the interaction between the N-terminus of hTERT and the TPR domain of hEST1A in vitro led us to uncover positive and negative regulatory roles for the TPR domain of ScEst1 in telomere length control.

**FUTURE DIRECTIONS**

**Frequency of complementation of est1A rad52A yeast by human EST1**

A subset of est1A rad52A spores containing hEST1A and hEST1C, but not hEST1B, exhibited telomere maintenance and an ability to proliferate when passaged every two days but not every four days (Figures 3-3, 3-4). By assaying an increased number of spores, the frequency of complementation may be
determined.

Mapping the interactions of hEST1A, hEST1B, and hEST1C with telomerase

hEST1A(114-631) and hEST1A(114-824) interacted with multiple regions of hTERT, including 1-200 (Figure 3-17). To isolate residues in the N-terminus of hTERT that mediate these interactions, the panel of hTERT(1-200) mutants that I generated (Table 1-1) may be employed. An inability to interact with hEST1A may reveal the molecular defect of one or more of the hTERT N-terminal DAT mutants (refer to Chapter 1, Appendix A). Stated in another way, these experiments may identify a role for hEST1A in facilitating the action of human telomerase in vivo, perhaps akin to the role of ScEst1 in recruiting yeast telomerase to the telomere.

Mutations on the (predicted) concave surface of the TPR-domain of hEST1A did not disrupt the interaction with the N- or C-termini of hTERT. Mutagenesis of the opposite, convex surface may identify residues involved in the hTERT interaction. These residues may perform an analogous role to those on the putative convex surface of Est1 that are involved in the TLC1 interaction (Evans and Lundblad, 2002; refer to Discussion).

The molecular determinants of the RNA-independent interaction between hEST1B and hTERT (Snow et al., 2003) have not been described. Co-immunoprecipitation assays involving fragments of hEST1B and hTERT may define the interaction interface. The hTERT-interacting regions of hEST1A and hEST1B can then be compared.

An interaction of hEST1C with hTERT in vitro or telomerase activity in cellular lysates has not been described. The molecular differences between hEST1C, hEST1A and hEST1B that underlie this apparent specificity are unclear. For example, the TPR domain of hEST1A (and perhaps hEST1B) may be unique in its ability to interact with hTERT. Further TPR domain swapping experiments involving the human EST1 proteins may reveal these differences.
Elucidation of the positive regulatory role of the Est1 TPR domain in telomere length control

Disruption of the TPR domain of Est1 by replacement with the TPR domains of hEST1A/B/C, or mutation of K84/W87/Q89 resulted in senescence and/or telomere shortening in the est1Δ rad52Δ background (Figures 3-6, 3-7, 3-8), indicating that the wild-type domain can positively regulate telomere length. This function may involve binding partners in the cell. For example, previous studies have suggested that the TPR domain interacts with TLC1 (Evans and Lundblad, 2002; Yu et al., 2007). To verify this interaction, anti-FLAG immunoprecipitates of human/yeast hybrid EST1 proteins expressed in est1Δ rad52Δ cells (refer to Figure 3-7) could be examined by northern blotting for the presence of TLC1. This experiment would also reveal the ability or inability of the TPR domains of human EST1 proteins to interact with TLC1. Conservation of the TLC1 interaction is not expected, given the different mode by which the TPR domain of hEST1A interacts with hTERT (i.e., through an RNA-independent protein-protein interaction; Figure 3-17). Chromatin immunoprecipitation and co-immunoprecipitation assays may reveal whether the interactions of Est1 with the telomere and Cdc13, the interaction of Est2 with the telomere, and/or the kinetics of those interactions are compromised by disruption of the TPR domain. The TPR domain mutants could also be examined for an inability to stimulate telomerase activity in vitro (DeZwaan and Freeman, 2009).

Elucidation of the negative regulatory role of the Est1 TPR domain in telomere length control

Several mutations in the TPR domain of Est1 caused telomere lengthening in est1Δ rad52Δ and rad52Δ backgrounds (Figure 3-8), suggesting that the domain, while required for telomere maintenance, can also restrict telomerase action at the telomere. Firstly, the telomerase-dependence of the telomere lengthening we observed could be confirmed by re-evaluating telomere length changes in est1Δ rad52Δ est2Δ or est1Δ rad52Δ tlc1Δ backgrounds. To determine whether Est1 negatively regulates telomere length via association with INO80 (refer to Discussion), defects in the recruitment of Ies3 to the telomere (Yu et al., 2007) or changes in telomeric chromatin caused by mutation of the Est1 TPR domain could be evaluated.
by chromatin immunoprecipitation. There is currently no evidence to suggest that Est1 can recruit the lagging strand replication machinery, but a defect in recruiting this complex (and subsequent loss of negative feedback on telomerase) would be evidenced by an increased G-strand overhang and an epistatic relationship with other lagging strand replication mutants (refer to Thesis Introduction). Also, if residues in the TPR domain function to dissociate Est1 and TLC1, Cdc13 or the telomere, defects in these potential ‘telomerase-off pathways’, which could cause telomere elongation, could be observed by increased protein signals in immunoprecipitation assays. Also, if the TPR domain normally limits the levels telomerase catalytic activity or recruitment of Est2 to the telomere, defects could be observed by assaying the telomerase activity of cell lysates, or association of Est2 with the telomere by chromatin immunoprecipitation.

**Complementation of yeast with human telomerase – potential use of the human TPR domain**

Attempts have been made to humanize yeast telomeres via expression of hTR and/or hTERT. Human sequences become incorporated onto telomeres upon substitution of the template region of TLC1 with the template for the human telomeric sequence (Henning et al., 1998), and telomerase activity can be detected in lysates of yeast expressing hTERT and hTR (Bachand and Autexier, 1999). However, hTERT and hTR cannot complement the senescence that occurs upon the loss of EST2 and TLC1, even upon co-expression of hEST1A or hEST1A/hEST1B (Bah et al., 2004). Given that the TPR domain of hEST1A interacts with hTERT (Figure 3-17), the human/yeast hybrid EST1 protein bearing the TPR domain of hEST1A (*i.e.*, Est1(ΔTPR::hEST1A.TPR); Figure 3-5) may allow hTERT and hTR to add human telomeric repeats onto yeast telomeres and complement *est2Δ tle1A* yeast, provided that the hybrid protein retains the ability to interact with Cdc13 and the telomere.
Table 3-1. Comparative sequence analysis of EST1 at positions selected for mutation.

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Sequence alignment adapted from Fukuhara et al., 2005. Numbers indicate amino acid positions. Boxes delineate multi-point mutants. Conserved/semi-conserved residues are shaded. Refer to Figures 3-14<sup>1</sup>, 3-8<sup>2</sup>. Accession nos. NP_001164428.1<sup>a</sup>, AAO17582<sup>b</sup>, NP_963862<sup>c</sup>. 
Figure 3-1. **Schematic representation of human and *S. cerevisiae* EST1 homologs.** Numbers indicate amino acids. White boxes indicate featured domains. Structural data are available for regions represented by dark lines. The region of highest homology among EST1 proteins (EST1 homology domain) is indicated by the grey shaded area. In this region, TPR domains are common to EST1 proteins. **(hEST1A / SMG6)** Amino acid numbers and domains pertain to Accession no. NP_001164428.1. DBD, N-terminal DNA binding domain (Snow *et al.*, 2003; Cruickshank and Harrington, unpublished). TRID, hTR-interaction domain (Redon *et al.*, 2006). PIN, nuclease domain; PDB code 2HWW (Glavan *et al.*, 2006). **(hEST1B / SMG5)** Refer to Accession no. AA017582. Est1-like DBD resembles the DNA binding domain (DBD) of Est1 (Lundblad, 2003). PIN, nuclease domain; PDB code 2HWY (Glavan *et al.*, 2006). **(hEST1C / SMG7)** Splice variant 2 is depicted. Refer to Accession no. NM_201568. TPR-like and TPR domains assume a TPR-fold architecture upstream of an alpha-helical domain; PDB code 1YAO (Fukuhara *et al.*, 2005).
Figure 3-2. Generation of the *S. cerevisiae* starting strain, and validation of the strain by replicative growth assay. (A) Diploid S288C *est1Δ::NAT/EST1* *S. cerevisiae* were transformed with a RAD52 replacement cassette (*KAN*). Stable transformants were selected for growth in media containing clonNAT (noursethricin) and G418. Proper integration of each cassette was verified by PCR and restriction enzyme digestion. Diploids were sporulated and tetrads were dissected to isolate spores. Spore genotypes were confirmed by growth on media containing/lacking selective agents, as appropriate. Each spore was streaked onto plates and incubated at 30°C (P1). After two days (allowing for approximately 20 generations of growth), one colony was re-streaked onto another plate, and incubated as before. To summarize the growth of cells from P1-6, one colony from each plate was re-streaked onto a sector of a “summary plate.” (B) Cells lacking both *EST1* and *RAD52* become senescent and do not recover. Summary plates of each genotype represent approximately 20 generations of growth per passage.
Heterozygous diploid est1∆::NAT rad52∆::KAN yeast were transformed with pRS316(URA+) plasmids expressing human or yeast EST1 genes (or empty vector), as indicated. est1∆::NAT rad52∆::KAN URA+ (A) and rad52∆::KAN URA+ (B) spores were isolated and passaged every two or four days on plates containing synthetic dropout media lacking uracil. Cell growth was scored as follows: robust (black bars; large, plentiful colonies), weak (grey bars; small, isolated colonies), no growth (gradient bars; no colonies), N/A (white bars; not assayed). * growth of cells every two days depicted in (C).
Figure 3-3. (C) Summary plate of growth of spores as indicated in (A-B). (D) Telomere DNA Southern blot. Genomic DNA isolated from est1Δ rad52Δ cells at the indicated passage (P) was digested with XhoI to release Y'-containing telomeres. Terminal restriction fragments were analyzed by Southern blotting using a (CACACCCA)2CC DNA probe. (E) Western blot of rad52Δ total cell lysates using anti-HA-HRP antibody. Molecular mass is indicated at left (kDa).
Figure 3-4. pRS426 senescence assays: human EST1 in *S. cerevisiae*. Heterozygous diploid est1Δ::NAT rad52Δ::KAN yeast were transformed with pRS316(*URA*+) plasmids expressing human or yeast EST1 genes (or empty vector), as indicated. *est1Δ::NAT rad52Δ::KAN URA*+ (A) and *rad52Δ::KAN URA*+ (B) spores were isolated and passaged every two or four days on plates containing synthetic dropout media lacking uracil. Cell growth was scored as follows: robust (black bars; large, plentiful colonies), weak (grey bars; small, isolated colonies), no growth (gradient bars; no colonies), N/A (white bars; not assayed). * growth of cells every two days depicted in (C).
Figure 3-4. (C) Summary plate of growth of spores as indicated in (A-B). (D) Telomere DNA Southern blot. Genomic DNA isolated from est1Δ rad52Δ cells at the indicated passage (P) was digested with XhoI to release Y'-containing telomeres. Terminal restriction fragments were analyzed by Southern blotting using a (CACACCCA)2CC DNA probe.
Figure 3-4. (E) Western blot of rad52Δ total cell lysates using anti-HA-HRP antibody (upper). Blot was reprobed using anti-actin antibody (lower). A non-specific cross-reacting band (*) serves as a loading control. Molecular mass is indicated at left (kDa).
Figure 3-5. Generation of human/yeast EST1 TPR domain hybrids. (A) Shaded boxes indicate the TPR domain of hEST1C and the homologous regions in hEST1A, hEST1B and ScEst1 (according to the structure-based alignment of EST1 sequences; Fukuhara et al., 2005). Numbers indicate amino acid boundaries. (B) In vivo gap-repair cloning strategy. Shaded boxes correspond to the shaded boxes in (A). (I) ScEst1 cDNA (dashed line) was digested in the TPR sequence at two sites that were unique in the plasmid (only one required), and treated with phosphatase. The TPR sequence of hEST1 (solid line) was amplified using primers bearing 30-mer hEST1 sequences flanked by 45-mer ScEst1 sequences. (II) DNA was gel-purified and transformed into est1∆::NAT RAD52 S. cerevisiae (to prevent recombination into the endogenous EST1 locus). Cells were grown in media containing nourseothricin and lacking uracil to select for the est1∆ genotype and for regeneration of the URA+ plasmid by homologous recombination. (III) The plasmid, in which the TPR domain sequence of ScEst1 was replaced with that of hEST1, was recovered.
Figure 3-6. pRS316 senescence assays: human/yeast EST1 hybrids (TPR domain swap) in *S. cerevisiae*. Heterozygous diploid est1Δ::NAT rad52Δ::KAN yeast were transformed with pRS316(URA+) plasmids expressing human/yeast EST1 hybrids in which the TPR domain of ScEst1 was replaced with the TPR domain of human EST1 or GFP(S65T), as indicated. (A) est1Δ::NAT rad52Δ::KAN URA+ and (B) rad52Δ::KAN URA+ spores were isolated and passaged every two or four days on plates containing synthetic dropout media lacking uracil. Cell growth was scored as follows: robust (black bars; large, plentiful colonies), weak (grey bars; small, isolated colonies), no growth (gradient bars; no colonies), N/A (white bars; not assayed). * growth of cells every two days depicted in (C).
Figure 3-6. (C) Summary plate of growth of spores as indicated in (A-B).
Figure 3-7. prs426 senescence assays: human/yeast EST1 hybrids (TPR domain swap) in S. cerevisiae. Heterozygous diploid est1Δ::NAT rad52Δ::KAN yeast were transformed with prs426(Ura4+) plasmids expressing human/yeast EST1 hybrids in which the TPR domain of ScEst1 was replaced with the TPR domain of human EST1 or GFP(S65T), as indicated. (A) est1Δ::NAT rad52Δ::KAN URA4+ and (B) rad52Δ::KAN URA4+ spores were isolated and passaged every two or four days on plates containing synthetic dropout media lacking uracil. Cell growth was scored as follows: robust (black bars; large, plentiful colonies), weak (grey bars; small, isolated colonies), no growth (gradient bars; no colonies), N/A (white bars; not assayed). * growth of cells every two days depicted in (C).
Figure 3-7. (C) Summary plate of growth of spores as indicated in (A-B). (D) Western blot of cell lysates using anti-FLAG and anti-mouse IgG-HRP antibodies. A cross-reacting band (*) serves as a loading control. Molecular mass is indicated at left (kDa).
Figure 3-7. (E) Telomere DNA Southern blot. Genomic DNA isolated from cells at the indicated passage (P) was digested with Xhol to release Y'-containing telomeres. Terminal restriction fragments were analyzed by Southern blotting using a (CACACCCA)₉CC DNA probe.
Figure 3-8. pRS426 senescence assays: Est1 TPR domain mutants in \textit{S. cerevisiae}. Heterozygous diploid \textit{est1\Delta::NAT rad52\Delta::KAN} yeast were transformed with pRS426(\textit{URA+}) plasmids expressing ScEst1 TPR domain mutants, as indicated. (A) \textit{est1\Delta::NAT rad52\Delta::KAN URA+} and (B) \textit{rad52\Delta::KAN URA+} spores were isolated and passaged every two or four days on plates containing synthetic dropout media lacking uracil. Cell growth was scored as follows: robust (black bars; large, plentiful colonies), weak (grey bars; small, isolated colonies), no growth (gradient bars; no colonies), N/A (white bars; not assayed). * growth of cells every two days depicted in (C,D). ^ grown from same spore as in Figure 3-7.
Figure 3-8. (C-D) Summary plate of growth of spores as indicated in (A-B).
Figure 3-8. (E) Telomere DNA Southern blot. Genomic DNA isolated from est1Δ rad52Δ cells (refer to A, C) at the indicated passage (P) was digested with XhoI to release Y'-containing telomeres. Terminal restriction fragments were analyzed by Southern blotting using a (CACACCCA)\(_2\)CC DNA probe.
Figure 3-8. (F) Telomere DNA Southern blot. Genomic DNA isolated from rad52Δ cells (refer to B, D) at the indicated passage (P) was digested with Xhol to release Y'-containing telomeres. Terminal restriction fragments were analyzed by Southern blotting using a (CACACCCA)₂CC DNA probe.
Figure 3-9. TPR domain of hEST1A precipitates non-specifically in vitro. Proteins were expressed in reticulocyte lysates. (Left) FLAG-tagged hTERT was immunoprecipitated (IP) onto anti-FLAG agarose (F) with or without prior mixing with HA-tagged hEST1A(502-824) (lanes 3, 5, 8, 9). 25% of input protein was analyzed in lanes 1, 2, 4, 6. Mock reaction contained no cDNA. (Right) Experiment was performed as in the left panel. Proteins were immunoprecipitated onto anti-FLAG agarose (F; lanes 12-14), protein G sepharose (P; lanes 16-18), or glutathione sepharose (G; lanes 20-22). 25% of input protein was analyzed in lanes 11, 15, 19. (Left, Right) Proteins were resolved through a 4-20% Tris-Glycine Novex gel, and subjected to western blotting using anti-HA-HRP (WB1). Blots were stripped and reprobed using anti-FLAG M2 and anti-mouse IgG-HRP (WB2; left) or anti-hTERT (RT domain) and anti-rabbit IgG-HRP (WB2; right). Reactive bands were detected on film using ECL reagent. Molecular mass is indicated at the left (kDa).
Figure 3-10. hEST1A(114-824) precipitates specifically with N- and C-termini of hTERT in vitro. Proteins were expressed in reticulocyte lysates. (A) HA-tagged hEST1A(502-824) (left) or (114-824) (right) was assayed for immunoprecipitation (IP) onto anti-FLAG agarose or Protein G sepharose with or without prior mixing with FLAG-tagged hTERT or AKT. 25% of input protein (In) was analyzed (lanes 1, 8, 15, 22). Mock reaction contained no cDNA. Proteins were resolved through 4-20% Tris-Glycine Novex gels, and subjected to western blotting using anti-HA-HRP (WB1). Blots were stripped and reprobed using anti-FLAG M2 and anti-mouse IgG-HRP (WB2). Reactive bands were detected on film using ECL reagent. Molecular mass is indicated at the left (kDa).
HA2-hEST1A(114-824) was assayed for immunoprecipitation (IP) onto anti-FLAG agarose or anti-MYC agarose with or without prior mixing with FLAG-tagged hTERT or AKT. 25% of input protein (In) was analyzed (lane 1). Proteins were resolved through a 4-20% Tris-Glycine Novex gel, and subjected to western blotting using anti-HA-HRP (WB1). Blot was stripped and reprobed using anti-FLAG M2 and anti-mouse IgG-HRP (WB2). Reactive bands were detected on film using ECL reagent. Molecular mass is indicated at the left (kDa).
Figure 3-11. N- and C-terminal boundaries of hEST1A(114-824) are required for specific interaction with hTERT in vitro. Proteins were expressed in reticulocyte lysates. (A) HA-tagged hEST1A(114-631) (left) or (334-824) (right) was assayed for immunoprecipitation (IP) onto anti-FLAG agarose or anti-MYC agarose with or without prior mixing with FLAG-tagged hTERT or AKT. 25% of input protein (In) was analyzed (lanes 1, 15). Proteins were resolved through 4-20% Tris-Glycine Novex gels, and subjected to western blotting using anti-HA-HRP (WB1). Blots were stripped and reprobed using anti-FLAG M2 and anti-mouse IgG-HRP (WB2). Reactive bands were detected on film using ECL reagent. Molecular mass is indicated at the left (kDa).
Figure 3-11. (B) HA-tagged hEST1A(114-631) (left) or (334-824) (right) was assayed for immunoprecipitation (IP) onto anti-FLAG agarose or anti-MYC agarose with or without prior mixing with FLAG-tagged hTERT or AKT. 25% of input protein (In) was analyzed (lanes 1, 15). Proteins were resolved through 4-20% Tris-Glycine Novex gels, and subjected to western blotting using anti-HA-HRP (WB1). Blots were stripped and reprobed using anti-FLAG M2 and anti-mouse IgG-HRP (WB2). Reactive bands were detected on film using ECL reagent. Molecular mass is indicated at the left (kDa).
Figure 3-12. Confirmation that hEST1A(114-824) interacts with N- and C-termini of hTERT \textit{in vitro}; interactions occur independently of a putative disordered loop. Proteins were expressed in reticulocyte lysates. HA-tagged hEST1A(114-824) was engineered to contain a stop codon after codon 824 (***) or a Δ708-724 deletion, and was assayed for immunoprecipitation (IP) onto anti-FLAG agarose or anti-MYC agarose with or without prior mixing with FLAG-tagged hTERT or AKT. 25\% of input protein (In) was analyzed (lanes 1, 11). Proteins were resolved through 4-20\% Tris-Glycine Novex gels, and subjected to western blotting using anti-HA-HRP (WB1). Blots were stripped and reprobed using anti-FLAG M2 and anti-mouse IgG-HRP (WB2). Reactive bands were detected on film using ECL reagent. Molecular mass is indicated at the left (kDa).
Figure 3-13. Schematic representation of interacting fragments of hEST1A and hTERT; TPR domain of hEST1A is required for robust interaction with hTERT. Black line, specific interaction with hTERT. Grey line, weak specific interaction with hTERT. Dotted line, non-specific precipitation (unable to determine interaction with hTERT). hEST1C is shown for reference. Numbers indicate amino acids. White boxes indicate featured domains. Structural data are available for regions represented by dark lines (refer to Figure 3-1).
Figure 3-14. Mutations in the TPR domain of hEST1A do not disrupt interactions with hTERT N- and C-termini in vitro. (A) Proteins were expressed in reticulocyte lysates. HA-tagged hEST1A(114-824)*** was engineered to contain mutations at residues predicted to be surface-exposed (refer to Results), and assayed for immunoprecipitation (IP) onto anti-FLAG agarose (F) after mixing with FLAG-tagged hTERT. 25% of input protein (I) was also analyzed. Proteins were resolved through 4-20% Tris-Glycine Novex gels, and subjected to western blotting using anti-HA-HRP (WB1). Blots were reprobed using anti-FLAG M2 and anti-mouse IgG-HRP (WB2). Reactive bands were detected using ECL+ reagent and scanned in the fluorescent channel on a Typhoon Trio variable mode imager. Molecular mass is indicated at the left (kDa).
Figure 3-14. Mutations in the TPR domain of hEST1A do not disrupt the interaction with N- and C-termini of hTERT in vitro. (B) Proteins were expressed in reticulocyte lysates. HA-tagged hEST1A(114-824)** was engineered to contain mutations at residues predicted to be surface-exposed (refer to Results), and assayed for immunoprecipitation (IP) onto anti-FLAG agarose (F) after mixing with FLAG-tagged hTERT. 25% of input protein (I) was also analyzed. Proteins were resolved through 4-20% Tris-Glycine Novex gels, and subjected to western blotting using anti-HA-HRP (WB1). Blots were reprobed using anti-FLAG M2 and anti-mouse IgG-HRP (WB2). Reactive bands were detected using ECL+ reagent and scanned in the fluorescent channel on a Typhoon Trio variable mode imager. Molecular mass is indicated at the left (kDa).
Figure 3-14. Mutations in the TPR domain of hEST1A do not disrupt the interaction with N- and C-termini of hTERT in vitro. (C) Proteins were expressed in reticulocyte lysates. HA-tagged hEST1A(114-824)*** was engineered to contain mutations at residues predicted to be surface-exposed (refer to Results), and assayed for immunoprecipitation (IP) onto anti-FLAG agarose (F) after mixing with FLAG-tagged hTERT. 25% of input protein (I) was also analyzed. Proteins were resolved through 4-20% Tris-Glycine Novex gels, and subjected to western blotting (WB) using anti-HA-HRP, anti-FLAG M2 and anti-mouse IgG-HRP. Reactive bands were detected using ECL+ reagent and scanned in the fluorescent channel on a Typhoon Trio variable mode imager. Molecular mass is indicated at the left (kDa).
Figure 3-14. Mutations in the TPR domain of hEST1A do not disrupt the interaction with N- and C-termini of hTERT in vitro. (D) Proteins were expressed in reticulocyte lysates. HA-tagged hEST1A(114-824)*** was engineered to contain mutations at residues predicted to be surface-exposed (refer to Results), and assayed for immunoprecipitation (IP) onto anti-FLAG agarose (F) after mixing with FLAG-tagged hTERT. 25% of input protein (I) was also analyzed. Proteins were resolved through a 4-20% Tris-Glycine Novex gel, and subjected to western blotting (WB) using anti-HA-HRP, anti-FLAG M2 and anti-mouse IgG-HRP. Reactive bands were detected using ECL+ reagent and scanned in the fluorescent channel on a Typhoon Trio variable mode imager.
Figure 3-15. hTERT “DAT” mutations do not disrupt interaction with hEST1A(114-824). Proteins were expressed in reticulocyte lysates. HA-tagged hEST1A(114-824) was assayed for immunoprecipitation (IP) onto anti-FLAG agarose or anti-MYC agarose with or without prior mixing with FLAG-tagged hTERT or AKT. TERT proteins were engineered to contain “DAT” mutations (replacement of amino acids starting at 92 or 122 with the NAAIRS sequence). 25% of input protein (In) was analyzed (lane 1). Proteins were resolved through a 4-20% Tris-Glycine Novex gel and subjected to western blotting using anti-HA-HRP (WB1). Blot was stripped and reprobed using anti-FLAG M2 and anti-mouse IgG-HRP (WB2). Reactive bands were detected on film using ECL reagent. Molecular mass is indicated at the left (kDa).
Figure 3-16. hEST1A(114-824) and (114-631) interact with several regions of hTERT independently of hTR. (A-B) HA-tagged hEST1A fragments were synthesized in RRL with or without hTR, mixed with FLAG-tagged hTERT fragments as indicated, and immunoprecipitated (IP) onto anti-FLAG resin. Precipitates were subjected to incubation with micrococcal nuclease (MNase) or buffer. 20% of input hEST1A (In) was analyzed in lanes 1, 16. Mock reactions contained no cDNA. *** indicates a stop codon. (A) Proteins retained in the pellet were resolved through 4-20% Tris-Glycine Novex gels and analyzed by western blotting using anti-HA-HRP (top) and anti-FLAG and anti-mouse IgG-HRP antibodies (bottom). Reactive bands were detected using ECL+ reagent and scanned in the fluorescent channel on a Typhoon Trio variable mode imager. Arrows indicate proteins of interest. (B) Nucleic acid components of duplicate immunoprecipitates were analyzed by electrophoresis through 1% agarose containing ethidium bromide. DNA lengths are indicated at right (kbp). Fluorescent signals were detected using a Typhoon Trio variable mode imager.
Figure 3-17. hEST1A(114-824) and (114-631) interact with several regions of hTERT independently of hTR; the TPR domain of hEST1A contributes to the interaction with the N-terminus but not the C-terminus of hTERT. (A-B) HA-tagged hEST1A fragments were synthesized in RRL without hTR, mixed with FLAG-tagged hTERT fragments as indicated, and immunoprecipitated (IP) onto anti-FLAG resin. Precipitates were subjected to incubation with micrococcal nuclease (MNase). 20% of input hEST1A protein (In) was analyzed, as indicated. Mock reactions contained no cDNA. *** indicates a stop codon. Proteins retained in the pellet were resolved through 4-20% Tris-Glycine Novex gels and analyzed by western blotting using anti-HA-HRP (top) and anti-FLAG and anti-mouse IgG-HRP (bottom). Reactive bands were detected using ECL+ reagent and scanned in the fluorescent channel using a Typhoon Trio variable mode imager. Arrows indicate proteins of interest. Data in (A) are representative of three experiments. Molecular mass is indicated at the left (kDa).
Figure 3-17. (C) Summary of hTERT/hEST1A co-IP experiments; TPR domain of hEST1A is required for robust interaction with the N-terminus but not the C-terminus of hTERT. Line representations of hTERT reflect robust (black line), weak (grey line), or no interaction (dashed line) with the indicated fragment of hEST1A. hEST1C is shown for reference. Numbers indicate amino acids. White/black boxes indicate featured domains. Structural data are available for regions represented by dark lines (refer to Figure 3-1).

Figure 3-18. Summary of interactions between hEST1A and hTERT according to Redon et al. Redon et al. (2007) reported an RNA-dependent interaction between an hTR-interaction domain (TRID) of hEST1A and an EST1-interaction domain (E1D) of hTERT. These regions also bind hTR. The authors provided preliminary evidence of an RNA-independent interaction of E1D with hEST1A downstream of amino acid 502, although the boundaries of the region were not identified ("?"). hEST1A amino acid numbers correspond to Accession no. NP_001164428.1.
STATEMENT OF CONTRIBUTIONS

Experiments involving *S. cerevisiae* were designed by me and Dr. Catherine LeBel. We worked with and supervised Alex Kostic. Together, we performed experiments, and interpreted and documented results.

**Cloning of hEST1C.** I constructed pSport6-hEST1Cv2 following a cloning strategy designed by Bryan Snow. I designed and constructed pcDNA3.1(ZEO)-HA2-hEST1Cv2.STOP.

**S. cerevisiae experiments.** The S288C *est1Δ::KAN/EST1* strain was a gift from Dr. Brenda Andrews. The S288C *est1Δ::NAT/EST1* strain was generated by Dawn Edmonds. The S288C *est1Δ::NAT/EST1 rad52Δ::KAN/RAD52* strain was generated by Dr. Catherine LeBel. Attributions for plasmid construction are listed below. Alex Kostic performed most of the yeast transformations with pRS expression plasmids; I performed some of the transformations. Catherine sporulated diploid transformants, dissected tetrads, and genotyped haploid spores. Alex passaged most of the haploid spores; I passaged some of the spores containing Est1 TPR domain point mutants. Alex and I prepared yeast protein extracts and performed western blotting. All of the western blots shown were performed by Alex Kostic. Alex and I prepared genomic DNA. I digested gDNA using restriction enzymes and analyzed telomere length by Southern blotting.

**Generation of human/yeast EST1 TPR domain hybrids.** I instigated the plan to construct human/yeast EST1 hybrids containing swapped TPR domains. Dr. Lea Harrington suggested the gap-repair cloning strategy. Following Dr. LeBel’s advice, I designed human/yeast hybrid 75-mer PCR primers. I determined the appropriate genetic background for the cloning, which Catherine prepared. Alex Kostic amplified EST1 TPR domain sequences by PCR, designed and performed pRS316-Est1 digestions, and performed *in vitro* manipulation of DNA. He transformed yeast and genotyped transformants by colony PCR. I isolated the target plasmids by yeast miniprep, and shuttled the DNA inserts into the pRS426 backbone.

In addition to the preceding description of contributions, the following notes indicate which data were
generated by whom when the data were not generated solely by me.

Alex Kostic performed the senescence assays in the following Figures: 3-2B, 3-3A-C, 3-4A-C, 3-6A-C, 3-7A-C. Alex Kostic and I performed the senescence assays in Figure 3-8A-D.

Alex Kostic generated the western blots in the following Figures: 3-3E, 3-4E, 3-7D.

Alex Kostic and I prepared genomic DNA from S. cerevisiae. I generated the Southern blots in the following Figures: 3-3D, 3-4D, 3-7E, 3-8E-F.

With the exception of the acquisition of some images, I prepared all of the Figures. I wrote the text. Of the DNA vectors used for experiments in this Chapter, and that have not been described elsewhere in this thesis, I designed and constructed the following (also refer to contributions listed in footnotes):

- pSport6-hEST1Cv2\textsuperscript{a}; pcDNA3.1(ZEO)-HA2-hEST1Cv2.
- pRS316\textsuperscript{b}: HA2-hEST1A, HA2-hEST1B, HA2-hEST1Cv2, Est1-HA2\textsuperscript{γ}, Est1.
- pRS316-Est1-FLAG.\textsuperscript{γ}
- pRS316-Est1(ΔTPR::___)-FLAG: hEST1A.TPR, hEST1B.TPR, hEST1Cv2.TPR, GFP(S65T).\textsuperscript{δ}
- pRS426\textsuperscript{b}: HA2-hEST1A, HA2-hEST1B, HA2-hEST1Cv2, Est1-HA2\textsuperscript{γ}, Est1.
- pRS426-Est1(ΔTPR::___)-FLAG: hEST1A.TPR, hEST1B.TPR, hEST1Cv2.TPR, GFP(S65T).
- pCR3-FLAG-hTERT-FLAG: 1-350(+92NAAIRS), 1-350(+122NAAIRS), 1-1132(+92NAAIRS), 1-1132(+122NAAIRS).

\textsuperscript{a}Designed by Bryan Snow. \textsuperscript{b}Designed by me and Dr. Catherine LeBel. \textsuperscript{γ} Constructed by Alex Kostic. \textsuperscript{δ} Constructed with assistance from Alex Kostic. \textsuperscript{ε}F511S was generated for use as a control based on a previous study (Evans and Lundblad, 2002).
APPENDIX A

Physical and functional interactions between hTERT and hPOT1/hTPP1

This work involved contributions from the laboratories of Lea Harrington, Tom Cech, and Mitsu Ikura. Credit for the design, execution, and interpretation of results is shared as follows. Figure A-1. David Sealey, Elaine Podell, Le Zheng. Figure A-2. (A) Michael Taboski, David Sealey. (B) David Sealey, Michael Taboski. Figure A-3. (A-C) Michael Taboski, David Sealey. (D) David Sealey, Michael Taboski.

Portions of this Appendix were cited as personal communications (David Sealey, Michael Taboski, and Lea Harrington) in a manuscript submitted by the laboratory of Tom Cech.
INTRODUCTION

hPOT1 – a component of the shelterin complex – interacts with the telomere and functions in chromosome end protection (refer to Introduction). The minimum sequence of telomeric DNA that POT1 can bind with high-affinity is 5′-TAGGGTTAG-3′\(^1\) (Loayza et al., 2004) or 5′-TTAGGGTTAG-3′\(^2\) (Lei et al., 2004). According to the crystal structure of the OB-fold domains of POT1 in complex with telomeric DNA, the OB-fold domains accommodate the longer 5′-TTAGGGTTAG-3′ sequence (Lei et al., 2004). Approximately 40% of telomeres in cells expressing telomerase terminate in 5′-TTAG-3′ (Sfeir et al., 2005). Interestingly, this sequence corresponds to the 5′ end of the template in hTR (Feng et al., 1995), suggesting that POT1 may protect telomere integrity by binding to the end of the telomere.

In addition to protecting chromosomes, POT1 and TPP1, as “terminal transducers” of the shelterin complex, can negatively regulate telomere length (Loayza and De Lange, 2003; Takai et al., 2010). Several manipulations of the shelterin complex have revealed this function. For example, reducing levels of TPP1 or POT1 by RNA interference results in telomere lengthening in cells over time in culture (Kendellen et al., 2009; Veldman et al., 2004; Ye et al., 2004b). Reciprocally, over-expression of hTPP1 can cause telomere shortening (Houghtaling et al., 2004).\(^3\) Over-expression of a TPP1 mutant lacking the TIN2 binding domain results in telomere lengthening, possibly due to uncoupling of the connection between POT1 and the duplex telomere and/or titration of POT1 away from the telomere (Houghtaling et al., 2004; O’Connor et al., 2006). Similarly, over-expressing the POT1-interacting domain of TPP1 or the TPP1-interacting domain of POT1 causes telomere lengthening (Liu et al., 2004b). Over-expression of a POT1 mutant lacking the N-terminal OB-fold domain causes telomere lengthening, demonstrating the critical role of the interaction of POT1 with DNA in controlling telomere length (Armbruster et al., 2004;

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\(^1\) Determined by competition experiments involving 50 nt-long oligonucleotides containing 3′ telomeric ends of varying length.

\(^2\) Determined by direct binding to telomeric oligonucleotides of varying length.

\(^3\) Some studies report no change in telomere length upon over-expression of hTPP1 (Liu et al., 2004b; O’Connor et al., 2006).
Liu et al., 2004b; Loayza and De Lange, 2003). This effect is dependent on the interaction of POT1 with TRF2, but not TPP1 (Kendellen et al., 2009). Further illustrating the importance of the connection between POT1 and TRF2 in regulating telomere length, over-expression of a POT1 mutant that does not interact with TRF2 causes telomere elongation (Kendellen et al., 2009).

The coverage of the terminal TTAG of a single-stranded telomeric DNA by the OB-fold domains of POT1 suggested a mechanism by which POT1 restricts telomerase (Lei et al., 2004). Indeed, recombinant POT1 can reduce the activity of telomerase on telomeric primers (Kelleher et al., 2005; Lei et al., 2005; Wang et al., 2007). Therefore, POT1 may negatively regulate telomere length by binding to the 3′ end of the telomere and blocking access by telomerase. However, the impact of POT1 on telomere length control may depend on where on the telomere the protein is bound. For example, POT1 can stimulate telomerase activity and processivity in vitro when it is positioned several nucleotides upstream of the 3′ end (Lei et al., 2005; Wang et al., 2007). This stimulatory effect is enhanced in combination with TPP1 (Wang et al., 2007). Interestingly, TPP1 stimulates telomerase processivity in the absence of hPOT1 (Wang et al., 2007). Wang et al. have proposed that a shift of POT1 from the 3′ end to an upstream region of the G-overhang switches its role, in concert with TPP1, from one of negative regulation, to one of positive regulation of telomerase (Wang et al., 2007).

Several observations exemplify a role for POT1 and TPP1 in positively regulating telomere length. Clonal or polyclonal populations of cells expressing exogenous hPOT1 exhibit telomere elongation over time in culture (Armbuster et al., 2004; Colgin et al., 2003; Kendellen et al., 2009; Liu et al., 2004b). Over-expression of the OB-fold domain of TPP1 (which interacts with telomerase; refer to Discussion) induces slight telomere shortening (Xin et al., 2007), suggesting that excess TPP1 can titrate telomerase away from the telomere.

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1 The titration of negative telomere length regulators away from the telomere could be a confounding effect of POT1 over-expression. Stimulation of the helicase activity of WRN and BLM by POT1 at telomeres may also be implicated (Opresko et al., 2005).
The exact mechanism by which POT1 and TPP1 can induce telomere lengthening is unclear. Interestingly, the stimulation of telomerase processivity by TPP1 or POT1/TPP1 in vitro is dependent on hTERT-G100 (Art Zaug and Tom Cech, personal communication). Therefore, we hypothesized that the stimulatory effects of POT1/TPP1 on telomerase activity/processivity and telomere length are mediated via direct interactions with hTERT.

MATERIALS AND METHODS

Expression and purification of recombinant hTERT

Refer to Chapter 1.

DNA oligonucleotide electrophoretic mobility shift assay

A single-stranded DNA oligonucleotide bearing the telomeric sequence (TTAGGG)$_3$ was labeled at the 5’ end with $^{32}$P. Residual $\gamma$-$^{32}$P-ATP was removed by centrifugation through a G25 spin column (GE Healthcare). DNA was boiled for 5 min and cooled on ice to minimize secondary structures. Each 10 μL binding reaction contained 11.2 nM $^{32}$P-(TTAGGG)$_3$, 50 mM Tris-Cl pH 8.0, 8.75% v/v glycerol, 205 mM NaCl, 5 mM DTT, and recombinant Trx-HIS6-hTERT(1-200), hPOT1, and/or HIS-hTPP1(89-334) proteins. Prior to incubation with other components, HIS-hTPP1(89-334) was incubated at 94°C for 10 min followed by 5 min on ice.$^1$ All components were assembled at room temperature, incubated for 60 min at room temperature, and resolved at 125 V at 4°C in cold 0.5X TBE through a pre-cooled gel containing 5% w/v 29:1 acrylamide:bisacrylamide, 0.5X TBE and 5% v/v glycerol. Gels were dried at 80°C for 1 hr and exposed to a phosphorimager screen which was then scanned on a Typhoon Trio variable mode imager. DNA oligonucleotides were obtained from Integrated DNA Technologies after polyacrylamide gel electrophoresis (PAGE) purification.

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$^1$ hTPP1 is stable under these conditions (Elaine Podell, personal communication).
Generation and passaging of stable cell lines

Refer to Chapter 1. The experiment was performed with late-passage HA5 cells approaching crisis (Figure A-3), and also with cells at an earlier passage (Figure A-2).

Analysis of telomerase activity by TRAP, mRNA by RT-PCR, and telomere length by Southern blotting

Refer to Chapter 1.

RESULTS

Physical interaction between hPOT1 and hTERT(1-200)

To investigate potential interactions between hTERT(1-200) (hTEN) and hPOT1/hTPP1, we employed a native EMSA approach. As described previously, the interaction of hPOT1 with a radiolabeled single-stranded telomeric DNA oligonucleotide was evidenced by the reduced migration of a radiolabeled single-stranded telomeric probe (Kelleher et al., 2005; Figure A-1, lanes 2, 9, position iv; Lei et al., 2004; Loayza et al., 2004). Whereas hTPP1(89-334) did not display DNA binding activity (Figure A-1B, lane 7), the combination of hTPP1(89-334) and hPOT1 further slowed migration of the probe, implying the formation of a ternary oligonucleotide-hPOT1-hTPP1(89-334) complex (Wang et al., 2007; Xin et al., 2007; Figure A-1B, lane 3, position ii). We reasoned that adding hTEN to the mixture might further shift the hPOT1/hTPP1-dependent migration of probe, or, that POT1/TPP1 might further shift the hTEN-dependent migration of probe (refer to Chapter 1). In contrast to the experiments described in Chapter 1 (Figure 1-3), hTEN did not alter the electrophoretic mobility of the telomeric oligonucleotide probe under conditions suitable for the analysis of the POT1-DNA interaction (Figure A-1B, lane 5).1 However,

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1 In order to detect the interaction of hTERT(1-200) with the telomeric DNA oligonucleotide (Chapter 1), a greater amount of protein was mixed with oligonucleotide in 20 µL containing 50 mM Tris-Cl pH 7.5, 450 mM NaCl, and >10% glycerol. Complexes were resolved, using a discontinuous buffer system, in a medium-format, 1.5 mm-thick, ... (continued on next page)
mixing Trx-HIS6-hTERT(1-200) with hPOT1 and HIS-hTPP1(89-334) increased the intensity of the hPOT1/hTPP1-dependent supershift signal, suggesting that hTEN may increase affinity of the hPOT1/hTPP1 complex for DNA (Figure A-1B, lane 4, position ii). Notably, Trx-HIS6-hTERT(1-200) supershifted the hPOT1-dependent complex suggesting that the two proteins interacted (Figure A-1B, lane 8, position iii).¹ HIS-hTPP1(89-334) and Trx-HIS6-hTERT(1-200) supershifted the hPOT1-dependent complex to different positions, which would be expected given the unique characteristics of each protein (Figure A-1B, compare position ii in lane 3 to position iii in lane 8).

**hTERT-G100 is not required for the interaction of hTEN with DNA**

An inability of hTEN to interact with DNA may cause a loss of telomerase activity/processivity (refer to Chapter 1). Based on the observation that mutation of hTERT-G100 blocks the stimulation of telomerase processivity by hPOT1/hTPP1 (Art Zaug and Tom Cech, personal communication), we explored the effect of this mutation on the interaction of hTEN with DNA. I observed a robust interaction of limited quantities of Trx-HIS6-hTERT(1-200,G100V) with a telomeric DNA oligonucleotide by EMSA (Figure 1-11A-D, lanes 14, 15). Therefore, the inability of hPOT1/hTPP1 to stimulate the processivity of hTERT(G100V) does not stem from an inability of hTEN(G100V) to bind DNA.

5% w/v 19:1 acrylamide:bisacrylamide gel containing 5% glycerol. I was unable to detect an interaction between hPOT1 and the telomeric DNA oligonucleotide under these conditions. With advice from Elaine Podell on EMSA conditions for POT1, I switched to conditions that were more likely to support a POT1-DNA interaction. The 10 µL gel shift mixtures described here included 50 mM Tris-Cl pH 8, 205 mM NaCl and 8.75% w/v glycerol. Complexes were resolved in a mini-format, 1.0 mm-thick, 5% 29:1 acrylamide:bisacrylamide gel containing 5% w/v glycerol. The gel was submerged in buffer. I could add only a limited volume of our dilute Trx-HIS6-hTERT(1-200) while leaving sufficient volume for the other protein components, and minimizing the final concentrations of salt (which could potentially disrupt hPOT1-hTPP1 interactions; not tested) and glycerol (which, at concentrations that exceed the resolving capabilities of the mini-gel, can trail radioactivity through the lane). Refer to Materials and Methods for further information on conditions.

¹ hPOT1 and HIS-hTPP1(89-334), supplied in concentrated form, displayed robust gel shift/supershift activity; however, no changes in probe migration were observed upon adding limiting amounts of Trx-HIS6-hTERT(1-200). In order to match the molar amount of Trx-HIS6-hTERT(1-200), I diluted hPOT1 and HIS-hTPP1(89-334) into their respective storage buffers. When mixed with diluted hPOT1, the telomeric DNA oligonucleotide migrated to position iv (Figure A-1B) and was also retained in the well (position i). Therefore, hPOT1 may have become partially misfolded/aggregated upon dilution.
To determine whether the inability of hPOT1/hTPP1 to stimulate the processivity of hTERT(G100V) could be attributed to the loss of a direct interaction between hPOT1 and hTEN, I attempted EMSA experiments with hPOT1 and Trx-HIS6-hTERT(G100V). Unfortunately, once the recombinant hTEN mutant became available, hPOT1 (which had been stored at -80°C in a diluted form for approximately one year\(^1\)) had lost DNA binding activity (data not shown). Because my graduate studies were drawing to a close, the experiment was not pursued further.

**hTERT-G100 is required for the maintenance of telomeres and extension of cellular replicative potential by telomerase**

To determine whether hTERT-G100 is required for telomerase function in cells, we performed cellular immortalization experiments. We created polyclonal HA5 cell lines stably expressing wild-type or mutant hTERT, and monitored population doubling levels at regular intervals. Cells that received wild-type hTERT cDNA continued to divide through the duration of the experiment, whereas cells receiving empty vector (pcDNA3.1) or hTERT(D868A/D869A) (mutations that inactivate reverse transcriptase activity) succumbed to apoptosis within approximately 10 (or fewer) population doublings (Figure A-2A; see also Figure 1-12), which is consistent with previous observations (Harrington et al., 1997; Kim et al., 2003; Zhu et al., 1999). Cells receiving hTERT(G100V) were also unable to divide beyond 10 population doublings. To determine whether mutation of hTERT-G100 interfered with the ability of telomerase to maintain telomeres, we performed terminal restriction fragment (TRF) analysis. Telomeres lengths in cells expressing wild-type hTERT were maintained; however, telomeres in cells expressing hTERT(G100V) or hTERT(D868A/D869A) mutants became shorter as the populations reached crisis (Figure A-2B).

To confirm these results, we derived a second set of stable lines from HA5 cells at an earlier passage. As before, cells receiving wild-type hTERT survived through the duration of the experiment (Figure A-3A; \(^1\)Refer to the previous note regarding the effect of dilution of POT1 on DNA binding activity.)
see also Figure 1-13). In contrast, cells receiving hTERT(G100V) or hTERT(D868A,D869A) mutant cDNAs failed to survive beyond 12 or 17 population doublings, respectively (Figure A-3A). Lysates prepared from cells containing G100V or D868A/D869A mutants did not display telomerase activity (Figure A-3B). Analysis of mRNA transcript levels confirmed expression of the vector-encoded hygromycin-resistance gene at early and late time-points in the experiment (Figure A-3C). hTERT(WT), G100V, and D868A/D869A mutants were also expressed. Average telomere lengths in cells expressing wild-type hTERT were maintained up to population doubling 16 (Figure A-3D, lane 4), whereas telomeres in cells expressing the G100V mutant became shorter – at a similar rate to telomeres in cells expressing the vector control – as the populations reached crises (Figure A-3D, lanes 1-2, 8-9). Therefore, the mutation of hTERT-G100 interfered with the ability of telomerase to maintain telomeres and extend cellular replicative lifespan.

**DISCUSSION**

We have found preliminary evidence of a physical interaction between recombinant hTEN and hPOT1 proteins. The experiment involved monitoring changes in the electrophoretic mobility of a single-stranded telomeric DNA oligonucleotide in the presence of hPOT1 with or without Trx-HIS6-hTERT(1-200); hence, the evidence of the interaction between hPOT1 and hTEN is indirect. Our findings are consistent with previous findings that *A. thaliana* POT1A interacts with the N-terminus (amino acids 1-323) of AtTERT *in vitro* and in two-hybrid assays (Rossignol *et al.*, 2007). Because hTPP1 does not bind DNA *in vitro* (Wang *et al.*, 2007; Xin *et al.*, 2007; Figure A-1, lane 7), and hTEN did not bind DNA under conditions suitable for the POT1-DNA EMSA (compare Figure A-1 to Figure 1-3), we were unable to determine whether hTEN and hTPP1 interact. Whether hTEN formed a quaternary complex with hPOT1, hTPP1(89-334) and the telomeric DNA oligonucleotide was unclear. The addition of hTEN to hPOT1 and hTPP1(89-334) did not alter the position to which the probe migrated (Figure A-1, lanes 3, 4), but did increase the intensity of the shifted probe signal. Thus, hTEN appeared to increase the affinity of the
hPOT1/hTPP1 complex for single-stranded DNA. Previous work has shown that the OB-fold of hTPP1 interacts with hTERT/hTR in vitro (Xin et al., 2007).

hPOT1 and hTPP1 may stimulate the activity and/or processivity of telomerase (Lei et al., 2005; Wang et al., 2007) by one or more of the following mechanisms: readying the primer for elongation by telomerase by reducing G-quadruplex (Zaug et al., 2005) and secondary structures; modulating the conformation and/or catalytic efficiency of hTERT (i.e., as an allosteric effector); and/or tethering hTERT to DNA, thereby increasing the affinity for DNA and decreasing the off-rate of the hTERT-primer interaction. That the processivity of hTERT(G100V) is not enhanced by hPOT1/hTPP1 or hTPP1 (Art Zaug and Tom Cech, personal communication) implicates physical contacts between hTEN and hPOT1/hTPP1. The EMSA assay that we described (Figure A-1B) may be used to determine whether mutation of hTERT-G100 interferes with these interactions. In any case, the defect of hTERT(G100V) does not involve an inability to interact with DNA (Figure 1-11D).

Human telomerase is capable of generating long extension products in vitro (Morin, 1989). Whereas yeast telomerase is not so capable (Bosoy and Lue, 2004), once engaged at a cellular telomere, it can generate a substantial number of telomeric repeats (Teixeira et al., 2004). The amount of DNA that human telomerase must generate at a given telomere in order to counteract telomere shortening and stave off telomere dysfunction-induced senescence is not known, but has been estimated to be 50-100 nucleotides per telomere (Zhao et al., 2009). By stimulating telomerase processivity, hPOT1/hTPP1 may increase the efficacy of telomerase at a given telomere. We attempted to address the functional importance of the hTERT-hPOT1/hTPP1 interactions by analyzing the cellular phenotype of the hTERT(100V) mutant. hTERT(G100V) failed to maintain telomerases and immortalize HA5 cells in two independent experiments (Figures A-2, A-3). Supporting the importance of the interaction between hTERT and the OB-fold of hTPP1 for telomerase function, over-expression of an hTPP1 mutant lacking the OB-fold in telomerase-
positive cells causes telomere shortening (Xin et al., 2007). Our investigation revealed that hTERT(G100V) did not reconstitute telomerase activity in cell lysates (Figure A-3B, Lea Harrington, unpublished observations), even though the mRNA encoding hTERT(G100V) was detected (Figure A-3C). Likewise, telomerase activity extracted from pot1 mutant A. thaliana seedlings is reduced (Surovtseva et al., 2007). In contrast, hTERT(G100V) reconstitutes telomerase activity in rabbit reticulocyte lysates, albeit at a reduced level compared to wild-type hTERT (Tom Cech, personal communication; Lea Harrington, unpublished observations). The lack of telomerase activity in cell lysates may reflect a requirement for hPOT1/hTPP1 to support robust telomerase activity in vitro or to stabilize the telomerase complex in vivo. Interactions between telomerase and the POT1/TPP1 complex have been observed. For example, hTPP1 co-immunoprecipitates telomerase activity from cells in a TPP1 OB-fold-dependent manner (Xin et al., 2007). Also, telomerase activity can be immunoprecipitated from A. thaliana with POT1 (Surovtseva et al., 2007). Further study is required to address the importance of hPOT1 and hTPP1 for the constitution of cellular telomerase and the maintenance of telomeres.

Like hTERT(G100V), hTERT(T116S,T117S,T118A) exhibited telomerase activity and interacted with a telomeric DNA oligonucleotide in vitro. Both mutants failed to regenerate telomerase activity in cell lysates and failed to extend the replicative capacity of telomerase-negative human cells (refer to Chapter 1). Interestingly, according to the alignment of the hTEN sequence to the TtTEN structure (refer to Chapter 1), G100 may neighbor T116-T117-S118 on the putative non-DNA binding surface of the domain. Thus, these two mutants may share an inability to interact with hPOT1/hTPP1.

1The effect of over-expressing hTPP1(ΔOB) on the recovery of telomerase activity from cellular extracts was not described (Xin et al., 2007).

2Parallels between the functions of POT1 in divergent organisms must be interpreted carefully, as some organisms have evolved multiple POT proteins with specialized functions (Hockemeyer et al., 2006; Rossingnol et al., 2007). Telomeres in pot1 mutant A. thaliana cells shorten at the same rate as telomeres in tert mutant cells, and AtPOT1 and AtTERT function in the same pathway for telomere maintenance (Surovtseva et al., 2007). In contrast, and as noted above, depletion of hPOT1 by RNA interference induces telomere lengthening (Kendellen et al., 2009; Veldman et al., 2004; Ye et al., 2004b). Also, over-expression of an AtPOT1 mutant lacking N-terminal OB-fold motifs results in telomere shortening (Surovtseva et al., 2007), whereas over-expression of an hPOT1 mutant lacking an N-terminal OB-fold results in telomere lengthening (Armbruster et al., 2004; Liu et al., 2004b; Loayza et al., 2003).
S. cerevisiae Est3 has been modeled as a homolog of mammalian TPP1 (Lee et al., 2008a; Yu et al., 2008). Est3 associates with telomerase and is required for telomere length maintenance and cellular longevity (Friedman et al., 2003; Hughes et al., 2000; Lendvay et al., 1996). Mutation of ScEst2-G85, which is conserved with hTERT-G100V, results in telomere shortening (Friedman et al., 2003; Xia et al., 2000), reduced telomerase activity in purified cellular extracts (Xia et al., 2000), and restricts growth of cells at higher temperatures (Friedman et al., 2003). The temperature sensitivity of this mutant, as well as mutants involving neighbouring residues, can be rescued by over-expression of Est3, suggesting that the N-terminus of Est2 may interact with Est3 (Friedman et al., 2003). Models of S. cerevisiae and C. albicans Est3 reveal structural and sequence similarities to the OB-fold of hTPP1 (Lee et al., 2008a; Yu et al., 2008). Mutation of modeled surface residues in ScEst3 results in telomere shortening and senescence (Lee et al., 2008a), and, like hTPP1, ScEst3 interacts with telomerase through an OB-fold (Lee et al., 2008a; Xin et al., 2007). Whereas ScEst3 is not required for the recovery of enzymatic activity from lysates (Lingner et al., 1997a), telomerase activity purified from C. albicans lacking Est3 is reduced (Hsu et al., 2007). Mutations in CaEst3 residues that are conserved with TPP1 result in telomere shortening, reduced association with telomerase, and reduced telomerase activity in purified cell extracts (Yu et al., 2008). The functions of POT1 and TPP1 in different organisms, namely telomere end protection, telomere length regulation, stabilization of telomerase activity and stimulation of telomerase processivity, are varied; however, the associations of POT1 and TPP1 with telomerase appear to be widely conserved.
FUTURE DIRECTIONS

Clarification of hTEN-hPOT1-hTPP1 interactions

We observed an apparent interaction between recombinant Trx-HIS6-hTERT(1-200) and hPOT1 by native EMSA (Figure A-1B). Additional controls are needed to determine the TERT-specificity of the interaction, such as the inability of Trx-HIS to interact with hPOT1, and the effect of denaturing Trx-HIS6-hTERT(1-200) on the apparent supershift of the hPOT1-DNA complex. To confirm the apparent increase in affinity of hPOT1/hTPP1 for telomeric DNA in the presence of Trx-HIS6-hTERT(1-200) (Figure A-1B, lane 4), an irrelevant protein could be tested as a negative control. Further, an inability of an hTEN(G100V) mutant to alter the migration of the hPOT1-DNA complex or the intensity of the hPOT1-hTPP1-DNA complex, as hypothesized, would demonstrate that the effects are specific to hTEN. To limit the potentially confounding competition of hPOT1 and hTERT for multiple and overlapping DNA binding sites, the experiment could be repeated using oligonucleotides of different length, and oligonucleotides with substitutions designed to restrict hPOT1 binding to the 5′ end (Wang et al., 2007).

Experiments employing protein-based detection methods, such as co-immunoprecipitation/western blotting, could also be used to identify interactions between hPOT1, hTPP1 and hTERT. Further mutagenesis studies may reveal structural elements of these interactions.

Mechanism of telomerase repeat addition processivity

As described in Chapter 1, Zaug et al. proposed models of how telomerase achieves repeat addition processivity. One of these models calls on a putative interaction between the TEN domain and another domain in telomerase (Zaug et al., 2008). While hTERT-L13/L14 are required for telomere repeat addition processivity (Zaug et al., 2008), hTERT-G100 is not so required, although its processivity cannot be stimulated by hPOT1/hTPP1 or hTPP1 (Art Zaug and Tom Cech, personal communication). Intra- or
intermolecular TERT-TERT interactions, and interactions between TERT and other proteins may cooperate to achieve full processivity. Thus, whereas I suggested investigating the effect of mutating L13/L14 on the interaction between the N- and C-terminal domains of hTERT (Chapter 1), I also suggest investigating the effect of mutating G100 on this interaction. Notably, the mutation of T116/T117/T118 – putative neighbours of G100 – did not interfere with the interaction of hTEN with hTERT(201-1132) in the presence of hTR (Table 1-1). Like the processivity of hTERT(G100V), the processivity of hTERT(T116S,T117S,T118A) may be insensitive to stimulation by hPOT1/hTPP1.

Addressing the importance of hTERT/hPOT1/hTPP1 interactions for telomerase function in cells

Both hTERT(G100V) and hTERT(T116S,T117S,T118A) exhibit a DAT phenotype (i.e., they retain partial or normal in vitro function but not in vivo function; Chapter 1). Firstly, the positive identification of these hTERT mutant proteins and their trafficking by western blotting and immunofluorescence, respectively (refer to Chapter 1, Future Directions), may address potential requirements of the hPOT1/hTPP1 interaction for biogenesis and/or stabilization of the enzyme. Secondly, the apparent DAT phenotype of these mutants may involve a defect in recruitment to the telomere. A determination of the ability of these mutants to bind to the telomere by ChIP (refer to Chapter 1, Future Directions) may address whether the defect of these mutants lies downstream of recruitment. Interestingly, the cellular phenotype of certain DAT mutants can be rescued by fusion to hPOT1 (Armbruster et al., 2004). If the hTERT(G100V) and hTERT(T116S,T117S,T118A) mutants do not interact with the telomere, a restoration of function via fusion to hPOT1 may lend further support for the importance of the hTERT-hPOT1 interaction for telomerase function in cells. Notably, hTERT-G100 resides in the DAT domain originally identified by Armbruster et al. (Armbruster et al., 2004).
Figure A-1. Trx-HIS6-hTERT(1-200) interacts with hPOT1. (A) Analysis of recombinant proteins. Trx-HIS6-hTERT(1-200) was expressed and purified as described in Chapter 1. hPOT1 and hTPP1 (HIS-hTPP1(89-334)) were obtained in a concentrated form from Tom Cech and Elaine Podell and diluted to a suitable working concentration. Proteins were boiled in SDS-PAGE loading dye, resolved through a 4-20% w/v Tris-Glycine Novex gel and stained with Deep Purple. The mass (ng) of protein present in the indicated bands (* lane 1, hPOT1; ** lane 2, HIS-hTPP1(89-334); *** lane 3, Trx-HIS6-hTERT(1-200)) was determined by comparing the band signal intensity to the average signal intensity of bands with italicized labels in the RPN5800 molecular size marker (M, n=2, lanes 1 and 5, 30 ng/band). Molecular mass is indicated at the left (kDa). (B) Electrophoretic mobility shift assay of telomeric DNA. 11.2 nM $^{32}$P-(Tel3)=(TTAGGG)$_3$ was mixed with hPOT1, HIS-hTPP1(89-334) (hTPP1), and Trx-HIS-hTERT(1-200) (hTERT) at the indicated molar ratio to probe (refer to Materials and Methods). Lanes 2 and 9 are replicates. Proteins were present at the following molar ratios relative to hTERT: hPOT1, 1; hTPP1, 2.5. Complexes were resolved by native polyacrylamide gel electrophoresis as described in Materials and Methods. The radiolabeled oligonucleotide probe migrated to the following positions: i, gel well; ii, hPOT1/hTPP1 supershift, lanes 3 and 4; iii, hPOT1/hTERT supershift, lane 8; iv, hPOT1 shift, lanes 2 and 9. Data are representative of three experimental replicates.
Figure A-2. hTERT(G100V) cannot maintain telomeres or extend the replicative lifespan of late-passage primary human (HA5) cells. (A) Polyclonal cell lines containing wild-type or mutant hTERT cDNA (or pcDNA3.1 negative control) were derived from HA5 cells (at later passage than cells used in Figure A-3). Cells were passaged under selection with hygromycin. (B) Analysis of the terminal telomere restriction fragments (TRF) at the indicated population doubling (PD) of polyclonal HA5 cell lines receiving wild-type or mutant hTERT. Genomic DNA was isolated, digested with RsaI and HinfI, and subjected to Southern blotting using a (CCCTAA)3 probe (refer to Materials and Methods). Hybridization to detect the 1 kb+ DNA ladder was unsuccessful. Left and right panels were analyzed on the same blot. Irrelevant lanes between 5 and 6 were removed. (A, B) Analysis of hTERT(G100V)-transduced cells was performed in the same experiment as Figure 1-12. For the purposes of displaying this result, pcDNA3.1, hTERT-WT, and hTERT(D868,9AA) controls are duplicated in this Figure.
Figure A-3. hTERT(G100V) cannot maintain telomeres or extend the replicative lifespan of early-passage primary human (HA5) cells. (A) Polyclonal cell lines containing wild-type or mutant hTERT cDNA (or pcDNA3.1 as a negative control) were derived from HA5 cells (at earlier passage than cells used in Figure A-2). Cells were passaged under selection with hygromycin. (B) Lysates of polyclonal cell lines containing wild-type or mutant hTERT cDNA were prepared in CHAPS buffer at day 35 and assayed for telomerase activity by TRAP assay. CHAPS buffer was assayed as a negative control (lane 5). HeLa cell lysate was assayed as a positive control (lane 6). IC indicates TRAP assay internal control product. Lanes containing irrelevant samples were omitted. (C) Analysis of mRNA expression level at early and late passage by RT-PCR. Hygromycin-resistance gene (HYG, upper), hTERT (middle), and GAPDH cDNAs were amplified using gene-specific primers (refer to Materials and Methods). Lanes containing irrelevant samples were omitted. (D) Analysis of the terminal telomere restriction fragments (TRF) at the indicated population doubling (PD) of polyclonal HA5 cell lines receiving wild-type or mutant hTERT. Genomic DNA was isolated, digested with Rsal and HinfI, and subjected to Southern blotting using a (CCCTAA)3 probe. Molecular size (kbp) is shown at left according to the migration of 1 kb+ DNA ladder (not shown). Irrelevant lanes were omitted. The weighted mean telomere length (kbp) is indicated at the bottom of each lane. (A-D) Analysis of hTERT(G100V)-transduced cells was performed in the same experiment as Figure 1-13. For the purposes of displaying this result, pcDNA3.1, hTERT-WT, and hTERT(DD868,9AA) controls are duplicated in this Figure.
STATEMENT OF CONTRIBUTIONS

Figure A-1. The rationale for this experiment was defined by Drs. Lea Harrington and Tom Cech. Le Zheng purified Trx-HIS6-hTERT(1-200). Elaine Podell provided hPOT1 and HIS-hTPP1(89-334). I analyzed the proteins by SDS-PAGE followed by Deep Purple staining (Figure A-1A), and I determined the appropriate quantities to use in the gel shift assay (Figure A-1B). I determined appropriate conditions for the gel shift assay based on Elaine’s experience working with hPOT1 and my experience working with hTERT (Chapter 1). I performed the experiment and I prepared the Figure.

Figures A-2, A-3. I designed the experiments to analyze the functionality of hTERT mutants in HA5 cells. Michael Taboski linearized and gel-purified plasmids, transfected cells, selected cells in hygromycin, passaged and counted cells in culture, and determined the number of population doublings (Figures A-2A, A-3A). He prepared cell lysates and analyzed telomerase activity (Figure A-3B), and designed primers for, and performed the RT-PCR analysis (Figures A-3C). Mike prepared cell pellets from which I extracted genomic DNA. I performed restriction enzyme digests and analyzed telomere length by Southern blotting (Figures A-2B, A-3D). We both analyzed the data and I prepared the Figures.

Of the DNA vectors used for experiments in this Appendix, and that have not been described elsewhere in this thesis, I designed and constructed the following: pcDNA3.1(HYG)-hTERT(G100V).
CONCLUSION

Perspectives on the study of telomerase
Telomerase was discovered in the 1980s (reviewed in Blackburn et al., 2006). A few fundamental observations gave rise to an entire field of study. Notably, the elongation of plasmid-borne *T. thermophila* telomeres in yeast by the addition of yeast telomeric DNA suggested the existence of a DNA template-independent mechanism of generating DNA (Shampay et al., 1984). This RNA-dependent telomerase activity was first identified in *T. thermophila* (Greider and Blackburn, 1985; Greider and Blackburn, 1987). The shortening of telomeres and senescence caused by deletion of *EST1* in *S. cerevisiae* demonstrated the link between telomere maintenance and the ability of cells to divide indefinitely (Lundblad and Szostak, 1989). For this pioneering work, Elizabeth Blackburn, Carol Greider, and Jack Szostak were awarded the 2009 Nobel Prize in physiology or medicine.

Over the past two decades, we have learned how telomeres are differentiated from broken DNA in the nucleus by the binding of specialized proteins, how telomeres are replicated, how telomerase is assembled and recruited to telomeres, and how telomere dysfunction and inhibition of telomerase can lead to senescence or apoptosis, among many other aspects (de Lange et al., 2006). Given the role of telomerase in cancer and the association of telomere shortening with cellular age-associated diseases (Hanahan and Weinberg, 2000; Lansdorp, 2009), we need to learn more.

This thesis describes our investigation into the workings of human and yeast telomerases. We identified interactions between hTERT and DNA, itself and hEST1A that regulate the function of the enzyme. Several domains in hTERT, including the TEN domain, have more than one binding partner. Deciphering the exclusivity of these interactions and contexts in which they occur will be a challenge for the field. Also, addressing the prevalence of human telomerase dimerization in a physiological setting, as well as the importance of dimerization for function, will guide future attempts to manipulate telomerase activity. We also uncovered a pivotal role for the TPR domain of ScEst1 in regulating telomerase. Identifying the binding partners of the TPR domain will likely explain how EST1 accomplishes positive and negative regulation of telomere length.
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