EXPLORING THE ASSEMBLY AND FUNCTION OF THE TELOMERASE ACCESSORY PROTEINS EST1 AND EST3 IN *SACCHAROMYCES CEREVISIAE*

By

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A	Adenosine
A	Alanine
aa	Amino acid
AAA	Acquired aplastic anemia
ANOVA	Analysis of variance
APC	Anaphase promoting complex
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
Bar1	Barrier to the alpha factor response
ß-ME	Beta-mercaptoethanol
bp	Base pair
BRCA1	Breast cancer gene 1
BRCT	BRCA1 C-terminus
BSA	Bovine serum albumin
C	Cytidine
C	Celsius
Ccq1	Coiled coil protein quantitatively enriched
CD	Circular dichroism
Cdc13/16/20	Cell division cycle 13/20
Cdh1	Cdc20 homolog
CHIP	C-terminal heat shock protein

ChIP	Chromatin immunoprecipitation
cm	Centimeter
CST	Cdc13/Stn1/Ten1
CTE	C-terminal extension
D	Aspartate
DAT	Dissociates activities of telomerase
D-box	Destruction box
DC	Dyskeratosis congenital
dCTP	Deoxycytosine tri-phosphate
deg	degrees
dGTP	Deoxyguanosine tri-phosphate
dmol	Decimole
DNA	Deoxyribonucleic acid
ds	double-stranded
DTT	Dithiothreitol
dTTP	Deoxythymidine tri-phosphate
E	Glutamate
EDTA	Ethylenediaminetetraacetic acid
Erg6	Ergosterol biosynthesis
Esc1	Establishes silent chromatin
EST	Ever shorter telomere
Exo1	Exonuclease
G	Guanosine

G1	Gap phase 1
G2	Gap phase 2
Gal1/4	Galactose metabolism 1/4
h	Hour
H3/4	Histone 3/4
HA	Hemagglutinin
HCl	Hydrogen chloride
HDAC8	Histone deacetylase
HDR	Homology directed repair
His	Histidine
HIV	Human immunodeficiency viruses
hnRNP D	Heterogeneous ribonucleoprotein particle D
HOAP	HP1/ORC-associated protein
HP1	Heterochromatin protein
HRP	Horseradish peroxidase
Hsp23/90	Heat-shock protein 23/90
HSQC	Heteronuclear single quantum correlation
IPF	Idiopathic pulmonary fibrosis
IPTG	Isopropyl ≤-D-1-thiogalactopyranoside
K	Lysine
KAN	Kanamycin
kb	Kilobase
kDa	Kilo Dalton

L	Leucine
LB	Luria broth
Leu2	Leucine biosynthesis 2
M phase	Mitosis phase
Mad1	Mitotic arrest deficient protein 1
Max	Myc associated factor X
MBP	Maltose binding protein
mg	Milligram
MgCl2	Magnesium Chloride
MHz	Mega Hertz
µL	Microliter
µM	Micromolar
min	Minute
MKRN1	Makorin ring finger protein 1
mL	Milliliter
mM	Millimolar
Moi	Modigliani
Mre11	Meiotic recombination
mRNA	Messenger ribonucleic acid
MRX	Mre11/Rad50/Xrs2
Msp3	Monopolar spindle
Myb	Myeloblastosis
Myc	Myelocytomatosis

N	Asparagine
NaCl	Sodium chloride
NAD+	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
Ndj1	Non-disjunction 1
NE	Nuclear envelope
NEB	New England Biolabs
NHEJ	Non-homologous end joining
NHP2	Non-histone protein 2
nm	Nanometer
NMR	Nuclear magnetic resonance
NOP10	Nucleolar protein 10
NTE	N-terminal extension
OB-fold	Oligonucleotide/oligosaccharide Binding fold
OD	Optical Density
ORC	Origin of replication
PBS-T	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein Kinase C
PNK	T4 Polynucleotide Kinase
POT1	Protection of telomeres
Poz1	Pot1 associated protein in *Schizosaccharomyces pombe*

psi	Pounds per square inch
Q	Glutamine
R	Arginine
Rad50/52	Radiation sensitive
Rap1	Repressor activator protein
Rb	Retinoblastoma
rDNA	Ribosomal deoxyribonucleic acid
Rif1/2	Rap1 interacting factor 1/2
RNA	Ribonucleic acid
RNaseA	Ribonuclease A
RPA	Replication protein A
RT	Reverse transcription
S phase	Synthesis phase
scaRNA	Cajal body ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sir 2/3/4	Silent information regulator
SMN	Survivor of motor neuron
snoRNA	Small nucleolar ribonucleic acid
SPB	Spindle pole body
ss	Single-stranded
Stn1	Suppressor of Cdc13
T	Thymidine
T	Threonine

TA	Telomerase activity
Tam1	alias for Ndj1
Taz1	Telomere-associated in *Schizosaccharomyces pombe*
TCA	Trichloroacetic acid
TCAB1	Telomerase Cajal body protein 1
TEBPα/ß	Telomere end binding protein alpha/beta
Tel1	Telomere protein 1
TEN	Telomerase essential N-terminus
Ten1	Telomeric pathways with Stn1
TERT	Telomerase reverse transciptase
TGF-ß	Transforming growth factor beta
TIN2	TRF1 interacting nuclear factor 2
TLC1	Telomerase component
t-loops	Telomere loops
TMG-cap	Trimethyl-guanosine cap
TPE	Telomere Position Effect
TPP1	TINT1-PTOP-PIP1
Tpz1	TPP1 homologue in *Schizosaccharomyces pombe*
TR	Telomerase RNA
TRF1	Telomere repeat binding factor 1
TRF2	Telomere repeat binding factor 2
T-SCE	Telomere-sister chromatid exchange
ug	Microgram

Ura3	Uracil requiring 3
Ver	Verrocchio
WRN	Werner syndrom, ReQ helicase-like
WS	Werner's syndrome
WT	Wild type
WT1	Wilms' tumor 1
Xrs2	X-ray sensitive 2
3'-OH	3'-Hydroxy

CHAPTER I

INTRODUCTION

Telomeres, the protein-DNA complexes found at the chromosome terminus of eukaryotic organisms, and telomerase, the enzyme complex that replicates telomeric DNA, work together to ensure proper chromosome maintenance by distinguishing the normal chromosome end from a double-stranded DNA break and preventing chromosome end-to-end fusions. Telomerase functions by adding a T/G rich repetitive sequence to telomeric DNA. There are a number of mechanisms that regulate the action of telomerase at the telomere. Some of these mechanisms include: temporally regulating the assembly of telomerase, inhibiting access of telomerase to the telomere by changes in DNA structure and competitive exclusion of the enzyme. Improper maintenance of the telomere can lead to chromosomal abnormalities and has been connected to both human aging and cancer. Therefore, understanding the mechanisms that regulate telomerase and telomere maintenance are important to a complete knowledge of these maladies.

The work presented in this thesis focuses on a how the telomerase complex assembles both *in vivo* and *in vitro* and begins to explore how one member of the telomerase enzyme in the budding yeast, *Saccharomyces cerevisiae*, termed Est3, functions within the complex. This chapter begins with a historical overview of the telomere/telomerase field, then moves into an introduction of our current understanding of the importance of the telomere and the telomerase holoenzyme, and concludes with a summary of telomere maintenance in both humans and yeast.

A History: The Beginnings of the Chromosome End From the Discovery of the Telomere to Telomerase

The Telomere: 1920's-1940's

In the 1920's and 1930's two scientists, Hermann Muller and Barbara McClintock, used X-rays to gain insight into the nature of the chromosome and the hereditary unit, the gene. Dr. Muller, a geneticist and former student of Thomas Hunt Morgan, the renowned fly geneticist, mutagenized flies with X-rays and recorded chromosomal abnormalities, classified as inversions, translocations and deficiencies (deletions). He noted that these abnormalities rarely involved the chromosome terminus and that the rejoining of broken ends never occurred between "originally free ends" [1]. It was in this 1938 paper that he termed the "free ends" *telomere*, from the greek nouns *telos*-meaning "end" and *meros*-meaning "part." Simultaneously, Dr. McClintock, a cytogeneticist, was developing maize as a system to study chromosomal inheritance. She too used X-rays to cause alterations within the chromosome and like Dr. Muller noted that never did she observe the natural end of the chromosome being attached to a broken end [2]. Both of these observations revealed that there must be something unique about the telomere because it appeared to be providing a protective "cap" for the chromosome.

In 1939 Dr. McClintock made another important observation regarding the telomere; when she applied X-rays to embryonic maize cells soon after the first zygotic division she found that the broken ends could be "healed" permanently [3]. This was the first evidence to hint that there might be a controlled process specific to maintaining the chromosome end. She also, prophetically, hypothesized that the healing occurred "during

the reproductive cycle of the chromosome," now known as S phase, the phase of telomere elongation.

The Telomere: 1971-1978

Little else was revealed or speculated about the telomere until the early 1970's. In 1971 Dr. Alexey Olovinkov authored a theoretical paper entitled *Principles of marginotomy in template synthesis of polynucleotides*. Here he postulated that the end of the DNA molecule could not be completely replicated (the so called "end-replication [or end-underreplication] problem") because DNA polymerases cannot initiate *de novo* synthesis at the end of the chromosome. DNA synthesis requires a free 3'-hydroxyl (3'-OH) group, which for lagging strand polymerization is accomplished by the synthesis of a complementary RNA primer that primes DNA polymerization. When the terminal RNA primer is removed from the chromosome a 5'-gap or a 3'-overhang is established and DNA polymerase cannot fill-in this gap. (Figure 1.1) [4]. The predicted consequence is that the telomere would become shorter during every cell division eventually resulting in loss "of a critical portion of the telomeric DNA." Dr. James Watson independently suggested a similar hypothesis in 1972 through his work with linear phage DNA [5]. In addition, Dr. Olovinkov also speculated that the end-replication problem might cause the cellular senescence phenotype observed by Drs. Leonard Hayflick and Paul Moorhead in primary cell strains [4, 6, 7].

Since it had become plausible that telomeric shortening could be the cause of cellular senescence, hypotheses regarding how the telomere was maintained were

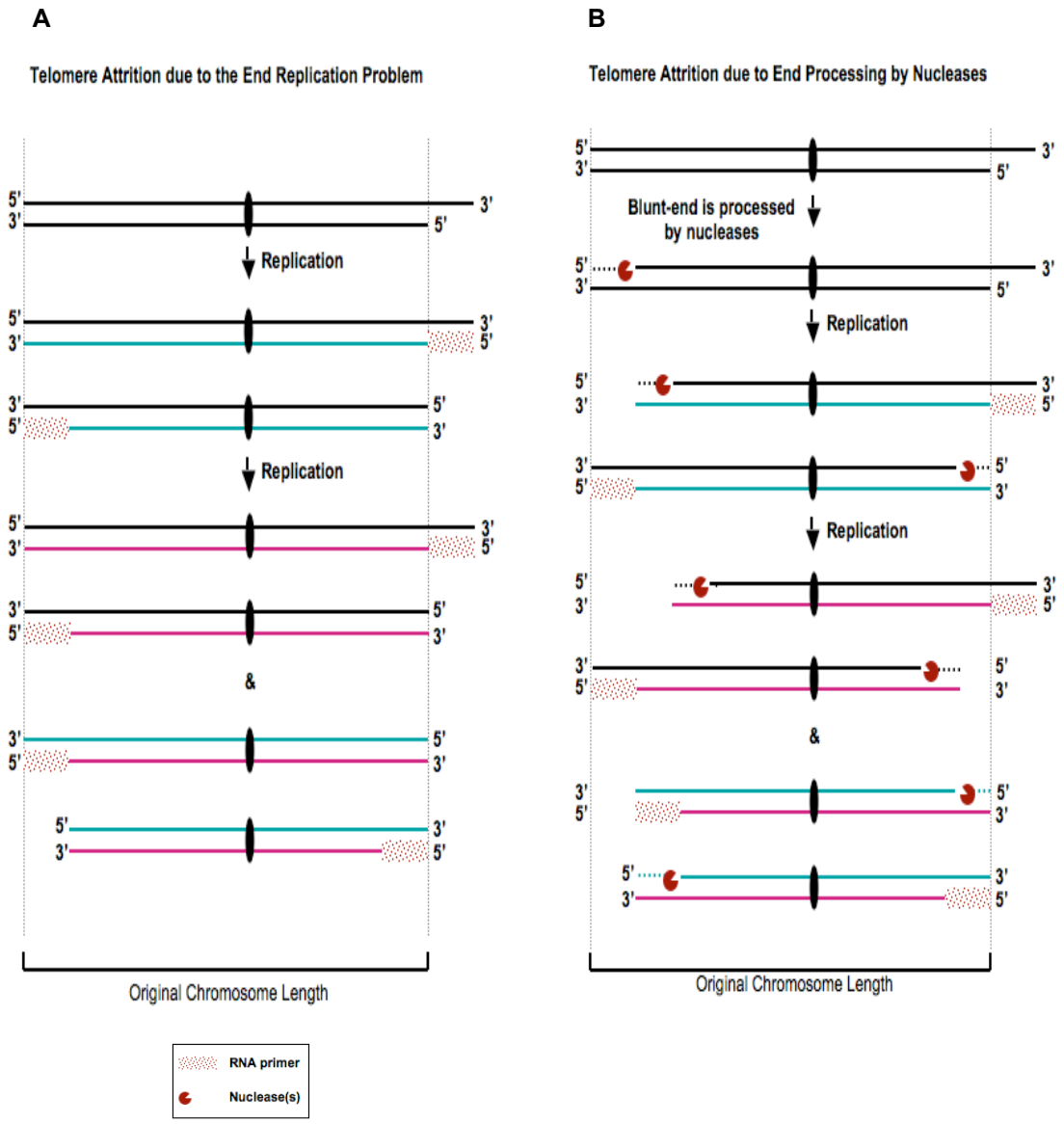

A
Telomere Attrition due to the End Replication Problem
Replication
Replication
&
Original Chromosome Length
RNA primer
Nuclease(s)
B
Telomere Attrition due to End Processing by Nucleases
Blunt-end is processed by nucleases
Replication
Replication
&
Original Chromosome Length

Figure 1.1 The End-(Under)Replication Problem. (A) Diagram of the original concept of the End-Replication problem hypothesis. Lagging strand synthesis results in an unreplicated 3'-overhang that through multiple rounds of replication becomes shorter and shorter as evidenced by comparing the original chromosome length (denoted by the bracket at the bottom) and the chromosome length after two rounds of replication. The teal and pink lines represent the first and second rounds of replication, respectively. The red-spotted rectangle represents the 5'-RNA primer that is removed at the end of replication. (B) Diagram of an updated understanding of the End-Replication problem that includes the role of exonucleases (represented by the red "pac-man"). Since telomeres require a 3'-overhang to establish appropriate capping, both the products of leading and lagging strand synthesis end in a 3'-overhang. This occurs at least in part by removal of the 5'-RNA primer (lagging strand) and by the exonuclease activity of Mre11 and Exo1 (leading strand). As a consequence, the parent strand involved in leading strand synthesis is continually attacked by exonucleases to re-establish a 3'-overhang in each cell cycle. This outcome is in stark contrast to the original hypothesis where the DNA strands involved in leading strand synthesis do not contribute to the End-Replication problem. The dashed line represents the length of the chromosome before 5'-resection by exonucleases.

proposed. Most common among them was that the telomere contained a palindromic or inverted repeat sequence that could fold on itself and thereby "self-prime" [8]. This would allow the conventional DNA polymerase to use the 3'-OH located at the 3'-end to initiate synthesis in the 5'-3' direction. This structure would be resolved by sequence specific endonuclease and/or recombination within the telomere. Because these models relied on palindromic or inverted repeat sequences, determining the sequence of chromosome ends became a high priority.

The Telomere and Telomerase: 1978-1985

The largest challenge that stood before the field at this point was isolating the relatively small amount of telomeric DNA from the rest of the chromosome. The macronucleus of the ciliated protozoan, *Tetrahymena thermophila*, provided a solution to this problem. Ciliated protozoans maintain two functionally distinct nuclei: a micronucleus and a macronucleus. The micronucleus is a transcriptionally inert nucleus important for meiotic exchange and maintenance of the germ line DNA. Alternatively, the macronucleus chromosomes are fragmented into minichromosomes ranging in size from ~21 kilobases (kb) to 1,500 kb (compare to an average chromosome length of ~44,000 kb in the micronucleus) (reviewed in [9]). The smaller minichromosomes (~21 kb) belong to the rDNA, which are maintained at ~10,000 copies/cell. The telomeres constitute ~ 0.0013% of the total DNA in the micronucleus, while they compose ~ 2.85% of the rDNA minichromosomes. Therefore isolation of rDNA minichromosomes more readily permitted purification of telomeres. In 1978, Drs. Elizabeth Blackburn and Joseph Gall reported the tandemly repeated hexanucleotide sequence (CCCCAA/TTGGGG) of

the rDNA minichromosome terminus [10]. The 3`-end of the chromosome bore the 5'-TTGGGG-3' sequence, while the 5'-end bore the 3'-CCCCAA-5' sequence. They observed that this sequence was repeated anywhere from 20-70 times at different chromosome ends. In the following few years, similar types of tandemly repeated sequences were discovered in other ciliates; each time the G-rich sequence being located at the 3'-end [11]. Collectively, these data began to argue against the prevailing hypotheses of a palindromic end or an inverted repeat that "self-primes," and the quest was on to determine if this sequence was specific to ciliates or if it was conserved in other eukaryotic organisms and to elucidate how this sequence was being maintained.

In 1982, Dr. Blackburn along with her colleague Dr. Jack Szostak discovered that the terminal sequence of *T. thermophila* chromosomes could function in a yeast (*Saccharomyces cerevisiae*) cell [12]. Typically when a linear piece of DNA is transformed into yeast, it is not stably maintained unless it integrates into the chromosome. They took advantage of this property to test whether a linear yeast plasmid (one that would normally be lost during cell division) could be maintained if the ends of this plasmid consisted of telomere sequence from *T. thermophila*. They discovered that in 14 out of 15 clones the plasmid was maintained as a linear molecule (the other clone had integrated into the chromosome, presumably by homologous recombination). Interestingly, the 14 linear clones that were maintained had become longer by ~100-300 basepairs (bp) within the terminal restriction fragments, suggesting that the *T. thermophila* telomere sequences were being maintained as telomeres in yeast. Then in 1984, Dr. Janis Shampay, a graduate student of Dr. Blackburn, cloned and sequenced yeast telomeres and found them to be a discontinuous sequence of $G_{1\text{-}3}T$ (~300 bp long)

[13]. Over the next few years, the sequences of telomeres from various species were determined and again, they contained a strand bias in which the 5`-strand was the G/T rich strand (reviewed in [14]). Together these data revealed that the maintenance of telomeres was (1) an evolutionarily conserved process and (2) began to argue for a specialized enzymatic process, specifically hypothesized by Blackburn and colleagues to be a terminal transferase [13].

Dr. Blackburn has written that in addition to the above evidence, a letter she received from Dr. McClintock solidified her belief that there was a unique enzyme that acted on telomeres [15]. In this letter Dr. McClintock revealed that during her work on maize she found one mutant (unpublished) that could not "heal" broken ends even in the early zygotic stages. This observation suggested that there was a gene responsible for the healing process [15]. In 1985, Dr. Blackburn and her graduate student Carol Greider published their discovery of the telomere field's "holy grail," an enzymatic activity specific to the telomeric sequence [16]. They found, using a primer extension assay and cell-free protein extract from *T. thermophila*, that the enzyme would processively add nucleotides, with a six-base periodicity, to a primer containing TTGGGG repeats. The enzyme required only dGTP and dTTP to extend the primer, was sensitive to proteinase K treatment and heat denaturation; it was not dependent upon endogenous double stranded (ds) DNA or alpha-polymerase, as pre-treatment of the extract with micrococcal nuclease or aphidicolin did not disrupt activity. In addition, it was noted that the enzymatic activity was present both in newly mated cells and those growing vegetatively, suggesting that telomere maintenance is an important function in both states. This enzyme was eventually termed telomerase (from Telomere Terminal Transferase).

Over the past 25 years a growing body of knowledge has been uncovered in the telomere and telomerase field. The telomeric sequence and the telomerase enzyme have been identified in many organisms (reviewed in [17]). As a result, many proteins that both positively and negatively regulate telomere maintenance have been discovered and unique roles for DNA replication and repair machinery, uncovered. The following section will provide a general overview of the function of both the telomere and telomerase and touch on some of the unique roles that telomeres play in the cell.

An Overview of the Function of the Telomere and Telomerase

The Telomere

The telomere is the protein/DNA complex found at the ends of linear eukaryotic chromosomes. Its primary functions are to cap and protect the chromosome from nucleolytic attack by nucleases and to distinguish the natural end from an internal DNA break, to regulate gene expression, to help facilitate organization of chromosomes in the nucleus and to limit the replicative capacity of the cell by serving as a "mitotic clock" (reviewed in [18-21]). Most telomeres have a unique repetitive sequence. For instance, in humans, an array of other metazoans and some plants the telomere consists of a TTAGGG repeat (reviewed in [17, 22]) while in budding yeast, a less well defined, $G_{1\text{-}3}T$ repeat is found [13]. Additionally, all telomeres discovered to date end with a single-stranded 3'-overhang important for telomere capping, suggesting it is an important, evolutionarily conserved structure. The 3'-overhang is established either by removal of

the 5'-primer left by lagging strand synthesis or by exonuclease activity to create an overhang on the blunt-ended product of leading strand synthesis (Figure 1.1b) [23-26].

The 3'-overhang is important for proper maintenance of the telomere, but these resulting sticky ends must be capped to provide end-protection from nucleases and to signal to the cell that the normal chromosome end is not a double-strand break in need of repair. The proteins involved in capping vary in number and complexity in different species, but are found in all eukaryotes (see Figure 1.2 for reference). For example, the primary capping proteins in the ciliated protozoans are TEBPα and TEBPβ, which cap the telomere by forming a heterodimeric clamp around the chromosome end [27]. In budding yeast **C**dc13, **S**tn1 and **T**en1 (CST complex) are the primary telomeric capping proteins [28]. Recent data have revealed that mammals and the evolutionarily distant fission yeast, *Schizosaccharomyces pombe*, have both a CST complex and a hexameric "shelterin" complex that functions as the telomeric cap by promoting the formation of large telomere-loops (t-loops) (reviewed in [29]). Even *Drosophila melanogaster*, an organism that maintains telomeres without telomerase, has capping proteins, collectively termed "terminin" (HOAP, HP1, Moi and Ver) [30, 31]. In addition to having **O**ligonucleotide/oligosaccharide-**B**inding (OB) folds, which are important for binding the ssDNA overhang and protein-protein interactions, some of these end-binding proteins may be evolutionarily related. For instance, the TEBPα and TEBPβ proteins in ciliates are thought to be homologous to the POT1 and TPP1 proteins in mammals, respectively [32, 33], while Stn1 in budding yeast and Ver in flies are hypothesized homologues [31].

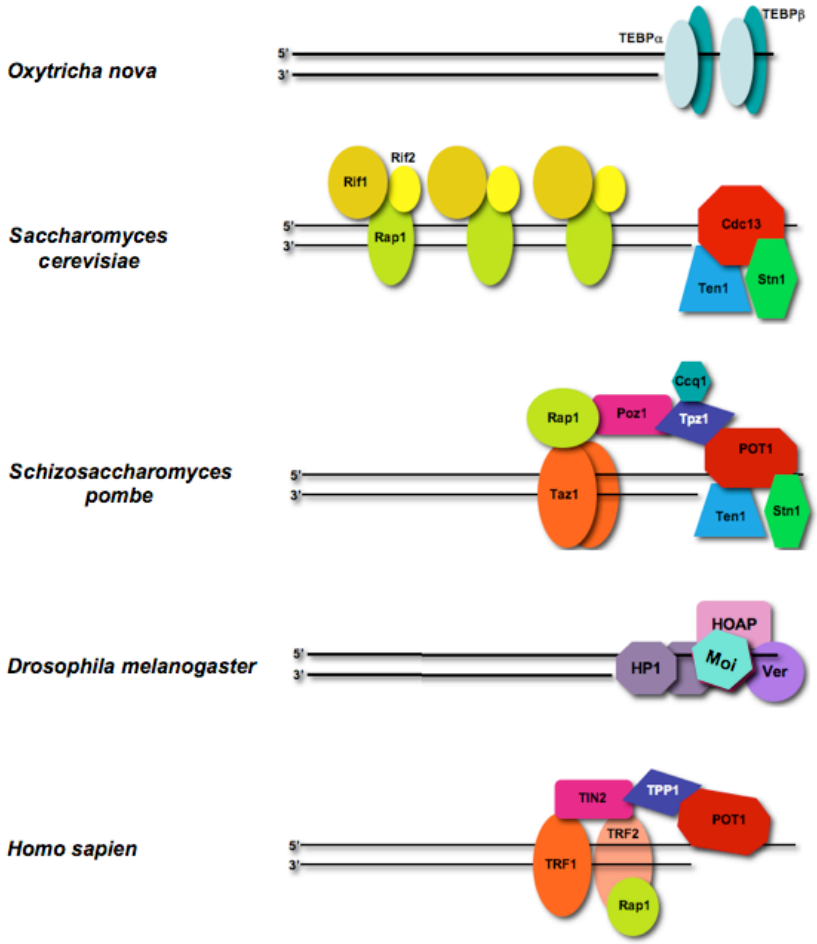


Figure 1.2 Telomere capping proteins in different eukaryotes. Analogous or homologous subunits between different species are the same color to denote either functional similarity or evolutionary relationship. Though the end structure for different species may consist of different proteins, the essential roles of protecting the telomere from nucleolytic degradation or recognition by DNA repair proteins are conserved.

The primary roles of the telomeric cap are to inhibit the telomere from being recognized as a broken DNA end and thereby, to inhibit DNA repair pathways from attempting to "heal" the telomere. The ATM kinase (**A**taxia **T**elangiectasia **M**utated) and ATR kinase (**A**taxia **T**elangiectasia and **R**ad3 related) pathways respond to dsDNA breaks and activate signaling pathways to recruit factors that repair the DNA (reviewed in [34]). Briefly, ATM kinase directly recognizes dsDNA breaks, whereas ATR kinase is activated when the 5'-end becomes resected and subsequently bound by RPA (**R**eplication **P**rotein **A**). After recognition of a dsDNA break, the chromosome can be repaired by two main DNA repair mechanisms: **H**omology **D**irected **R**epair (HDR) or **N**on-**H**omologous **E**nd **J**oining (NHEJ).

The mammalian model systems have contributed extensively to understanding how the cap inhibits both the detection and repair of dsDNA breaks. Figure 1.2 illustrates the mammalian end-protection complex, termed shelterin. TRF1 and TRF2 bind as homodimers to the double-stranded telomere sequence TTAGGG through their respective Myb-type domains [35]. TRF2 can generate higher order DNA structures called t-loops *in vitro* that are speculated to help "hide" the 3'-overhang *in vivo* [36]. Rap1 interacts with TRF2. TIN2 binds to both TRF1 and TRF2 and acts as a bridge to connect them to TPP1, which associates with POT1 [37]. POT1 binds the single-stranded telomeric overhang [32].

How do these proteins inhibit the telomere from recognition as dsDNA breaks? In mammals, TRF2 appears to inhibit activation of the ATM kinase, NHEJ and HDR as evidenced by an increase in Chk2 phosphorylation (specific to ATM activation) and telomere fusions upon TRF2 knocked-down [38-40]. Such inhibition is likely achieved

indirectly to its role in t-loop formation. Since TRF2 is important in "hiding" the 3'-overhang, ATM kinase cannot be activated if it cannot detect ssDNA, thereby reducing the ability to activate NHEJ or HDR. When RAP1 is depleted from cells or unable to bind TRF2, there is no phosphorylation of Chk2 or Chk1 (specific to ATR activation) and no telomere fusions occur. However there is a dramatic increase in telomere-sister chromatid exchanges (T-SCEs), a measure of HDR [41-43]. Because RAP1 has both a BRCT and Myb-type domain, it is speculated that it may interact with other proteins that signal the inhibition of telomere recombination [44]. Loss of POT1 in mammalian cells activates ATR kinase and HDR is upregulated as evidenced by phosphorylation of Chk1 and an increase in T-SCEs, respectively [45-47]. The POT1 binding partner, TPP1, inhibits ATR kinase activation, through its recruitment of POT1 to the telomere [46, 47]. Since POT1 binds the long single-stranded overhang, depletion of POT1 could cause an increase in RPA binding at the telomere and subsequently activate the ATR kinase pathway. Intriguingly, this is supported by two recent publications that show that there is a dynamic interplay between POT1 and RPA for telomere binding [48, 49]. In addition to preventing ATR and HDR signaling, POT1 also inhibits NHEJ in G_2 phase of the cell cycle [45, 50].

As well as its role in capping the chromosome end, the telomere is important for the proper localization of chromosomes in meiosis. In the latter part of the 19th Century, biologists were attempting to understand the biology of the chromosome in different phases of the cell cycle. One unique observation was that in some meiotic cells chromosome ends were clustered at their ends to one side of the nucleus (e.g. [51]). In 1900, Gustav Eisen, who was studying meiosis in salamanders, termed the structure

"chromosomal bouquet" due to its similarity to bundled flower stems [52]. The polarization of chromosomes to a restricted section of the **N**uclear **E**nvelope (NE) is conserved among most eukaryotes, suggesting that this is an important cellular mechanism (reviewed in [53]).

Telomeric clustering is important for proper progression through meiosis, as telomere dysfunction caused by reduced telomerase activity results in an inability for yeast and mice to complete meiosis [54-57]. Telomeres are anchored to the NE by telomere end-binding proteins. For example, if Taz1 or Rap1's (*S. pombe* shelterin proteins-Figure 1.2) ability to bind the telomere is disrupted, then telomere clustering and attachment at the NE are disrupted as well [58-61]. In budding yeast when the telomere sequence is altered so that Rap1 (a telomere-binding protein) can no longer bind efficiently, cells do not pass through meiosis [62]. In addition, a budding yeast meiotic-telomere specific protein, Ndj1/Tam1, interacts with the **S**pindle **P**ole **B**ody (SPB) component Msp3 and is important for crossing-over and telomere clustering [63-65]. Interestingly, when nuclear envelopes are purified from frog oocytes, TRF2 has also been purified, suggesting that telomere binding proteins may also be important for telomere anchoring in higher eukaryotes [66].

Why is it important for telomeres to be attached to the NE in meiosis? During meiosis, it is critical that homologous chromosomes locate one another (termed homology search) and undergo recombination to ensure proper chromosomal segregation. Homologous recombination cannot occur if the homologous chromosomes do not first locate each other. It is hypothesized that attachment of the telomere to the NE during meiosis simplifies the homology search by clustering chromosome ends in one place

(reviewed in [53]). This idea is supported by several observations: *S. pombe taz1*$^{-}$ or *rap1*$^{-}$ cells have significantly lower recombination rates than WT (wild type) and *ndj1Δ* cells in *S. cerevisiae* have delayed kinetics of homologue pairing [58-61, 63-65]. Additionally, telomeric clustering is generally limited to the leptotene to zygotene (sometimes pachytene) phases of meiosis but once the synaptonemal complex is formed, the bouquet does not persist, perhaps because the chromosome pairing is complete (reviewed in [53]). Interestingly, in species that do not form a synaptonemal complex, as in *S. pombe* or *T. thermophila*, the bouquet is present until the end of prophase I, when recombination is complete [67, 68]. This apparent correlation between the completion of homologous chromosome pairing and of the dissolution of chromosome bouquets also supports that telomeric clustering is important for homology search.

Telomeres also play an important role in gene silencing. The role of telomeres in gene silencing is best understood in *S. cerevisiae* where many of the proteins involved and some of the mechanisms have been elucidated. Silencing at the end of the chromosome involves both the telomere and subtelomeric regions, which consist of special DNA structures termed X-elements and Y`-elements. Proteins that bind within the telomeric and subtelomeric DNA nucleate the formation of a specific chromatin structure that can spread inward from the telomere and is thought to sterically hinder the transcription machinery's access to genes within this region (reviewed in [69]). This phenomenon is called the **T**elomere **P**osition **E**ffect (TPE).

The chief proteins involved in TPE in *S. cerevisiae* are Rap1 and the Ku70/80 heterodimer, which bind directly to the yeast telomeric repeat, and the Sir family proteins (Sir2, Sir3 and Sir4). Rap1 binds, in a sequence-specific manner, to the double-stranded

portion of telomeric DNA. Rap1 then recruits either the Rif1/2 proteins, which regulate telomere length (discussed below) or the Sir proteins (Sir2/3/4), which are involved in chromatin silencing. Sir4 binds to Rap1 and in turn recruits Sir2 and Sir3 (See Figure 1.3) [70-73]. Sir2, an NAD^+-dependent histone deacetylase, deacetylates lysines in the histone tails of neighboring histone 3 (H3) and histone 4 (H4). The resulting hypoacetylated histones can be bound by Sir3, independent of the Sir4-Rap1 interaction [74], which then, in turn, recruits more Sir complexes to the DNA to spread chromatin silencing. The extent of silencing is dependent upon the pool of Sir3 as overexpression of Sir3 increases the range of the TPE [75].

Interestingly, Sir4 and Ku70/80 are also important for tethering telomeres to the nuclear envelope (NE). Specifically, Sir4 interacts with Esc1, an NE integral membrane protein, to anchor telomeres in S phase of the cell cycle. The Ku70/80 interacts with both Esc1 and Mps3, another NE integral membrane protein, to tether telomeres to the NE in G1 phase [76, 77]. Mutations in both Sir4 and Ku80 that separate telomeric anchoring from silencing, demonstrate that these two functions are independent of each other [77]. However, there is likely and important functional interplay between telomeric silencing and telomeric tethering since the same proteins accomplish these functions. Indeed, it has been hypothesized that clustering telomeres at the NE helps to create a sub-nuclear compartment to facilitate concentration of silencing factors that are important for gene silencing (reviewed in [21]). For example, both mating-type loci are located near the terminal regions of the opposite arms of Chromosome III in budding yeast. Silencing of these loci is critical for maintaining the haploid mating type. Therefore by clustering the

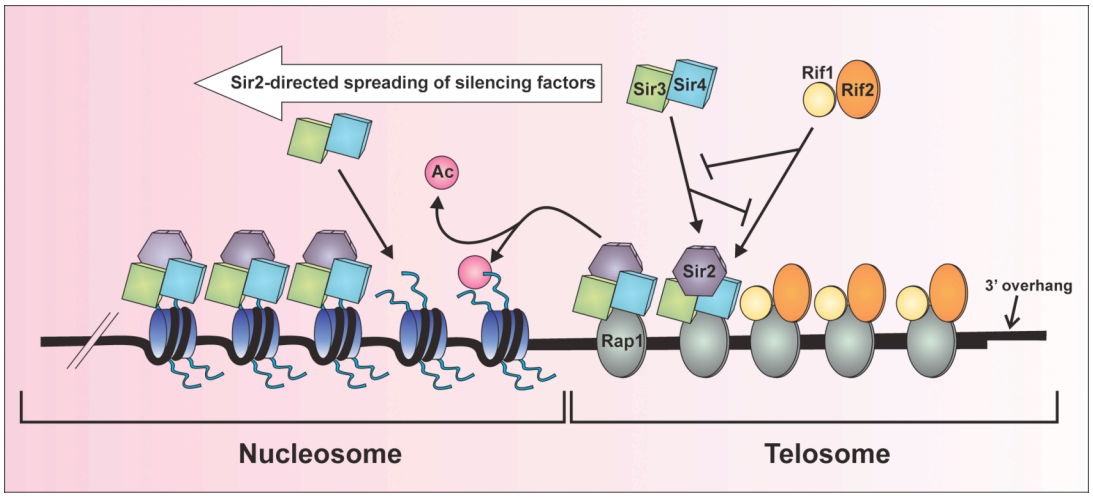


Figure 1.3 Telomere Position Effect (TPE) in *Saccharomyces cerevisiae*. TPE has been most extensively studied in budding yeast. Rap1 binds to the telomere in a sequence specific manner and recruits both Rif1 and Rif2, which bind to the C-terminus of Rap1. Sir4 competes with Rif1 and Rif2 for Rap1 binding at the telomere. Once Sir4 binds, it recruits Sir2 and Sir3. Sir2 then deacetylates histones which can recruit more Sir3 and Sir4 in a Rap1-independent manner. The telomeric region of the DNA that is bound by Rap1 is called the telosome while the region proximal to the centromere is comprised of nucleosomal chromatin. This figure was adapted from Ottaviani *et al*., 2008 [21].

telomeres and subsequently the silencing factors, these genes can be transcriptionally silenced even if they are not located directly in silenced chromatin [78].

TPE plays a role in DNA replication, recombination and repair. Origins of replication that are located in the heterochromatic sub-telomeric regions of DNA are fired later during S-phase than those more internal and their firing time is dependent upon Sir3. When Sir3 is inactivated, late origins are prematurely fired earlier in S-phase, and conversely, when Sir3 is tethered to an early-firing origin, it becomes a late-firing origin [79, 80]. TPE is responsible for decreasing rates of recombination within the telomere, which could result in non-homologous chromosome exchange and interestingly, anchoring of telomeres near the nuclear pore is important for proper repair of dsDNA breaks located in the sub-telomere [81, 82].

TPE is not a phenomenon restricted to yeast; when a luciferase reporter gene is integrated directly adjacent to telomeres in mammalian cells the expression of this gene is decreased 2 to 10-fold compared to integration more internal in the genome. [83]. Forced telomere elongation in this context caused further repression of the reporter, while addition of trichostatin A, a histone deacetylase inhibitor, restored expression [83]. Since the expression of a reporter gene can be modulated by changes at the telomere in a manner similar to that seen in yeast, these data support the existence of TPE in mammals. The telomere and subtelomere in humans vary significantly among each other in both size and arrangement (reviewed in [21]). This variability makes it difficult to understand what importance, if any, there maybe in differential expression of sub-telomeric genes in humans. One potential way that TPE may affect human disease is if a normally active gene(s) is rearranged, perhaps via translocation or terminal deletion, to be located

adjacent to the telomere. This situation might down-regulate gene expression resulting in a human disease phenotype (reviewed in [21]). Indeed, approximately 5-10% of patients with idiopathic mental retardation have small karyotypic changes in subtelomeric regions of their chromosomes, suggesting that deletions or rearrangements near the telomere can influence human disease (reviewed in [21]).

Telomerase

Eukaryotic species, excepting *Dipterans*, maintain telomeres using telomerase, a ribonucleoprotein complex, consisting minimally of the **Te**lomerase **R**everse **T**ranscriptase protein (TERT) and the **T**elomerase **R**NA (TR). TERT, as the name implies, is a specialized DNA polymerase that adds nucleotides to the telomere using **r**everse **t**ranscription (RT). The RT domain of TERT is functionally and structurally homologous to viral RNA polymerases and reverse transciptases and the B-type family of polymerases [84, 85]. TR is the RNA template for the telomerase complex as it contains a sequence that is complementary to the telomere DNA [86, 87]. In *Euplotes* the RNA template contains the sequence 5`-CAAAACCCCAAA-3`, which is complementary to the telomere sequence TTTTGGGG, found at the *Euplotes* telomere [87]. Though the existence of an RNA subunit and some aspects of tertiary structure have been conserved through evolution the size and sequence of the RNA has not. For example, in ciliates the RNA is ~150 nucleotides long, while in vertebrates it is ~500 nucleotides and in yeast it is much larger, ~1,300 nucleotides [86-90]. The divergence is attributed to the fact that in different species, different factors bind to the RNA and aid in its maturation and stability in the cell [91]. *In vitro* TERT and TR (along with the chaperones Hsp90 and p23) are the

only two telomerase components required for telomerase activity, as production of TERT and TR from humans in rabbit reticulocyte lysate can reconstitute telomerase activity [92]. *In vivo* telomerase activity is regulated by different, often species specific, accessory factors that are necessary for both proper association of telomerase with the telomere and elongation of the telomere.

Telomerase adds to G/T rich 3'-overhangs, specifically the 3'-overhang of the telomere [93]. Telomerase binds with the DNA (as assessed *in vitro*) in at least two independent mechanisms: TERT binds to DNA upstream of the terminal 3'-nucleotide using the "anchor site" and through base pairing between TR and the end of the telomeric DNA (see Figure 1.4) [94-96]. To ensure proper telomeric sequence replication, the RNA has both a 3' and 5'-template boundary. The 3'-boundary is defined by its complementarity to the telomeric sequence, whereas the 5'-boundary of the template RNA is often restricted by secondary structural elements within the RNA (reviewed in [97]). Once anchored and base paired to the DNA, telomerase elongates the telomere by adding nucleotides to the 3'-overhang one nucleotide at a time until it reaches the end of the RNA template. Then telomerase translocates to reposition the DNA and continues to add more nucleotides (Figure 1.4). Other protein interactions with the telomere and interactions between lagging strand DNA replication machinery and telomerase are important for regulating the extent of telomere elongation (discussed below).

TERT consists of three distinct domains: an **N**-**t**erminal **e**xtension (NTE), the RT domain and a **C**-**t**erminal **e**xtension (CTE) (Figure 1.5) [98]. The NTE is specific to TERT, as it is not found in other reverse transcriptases; it is loosely conserved in most

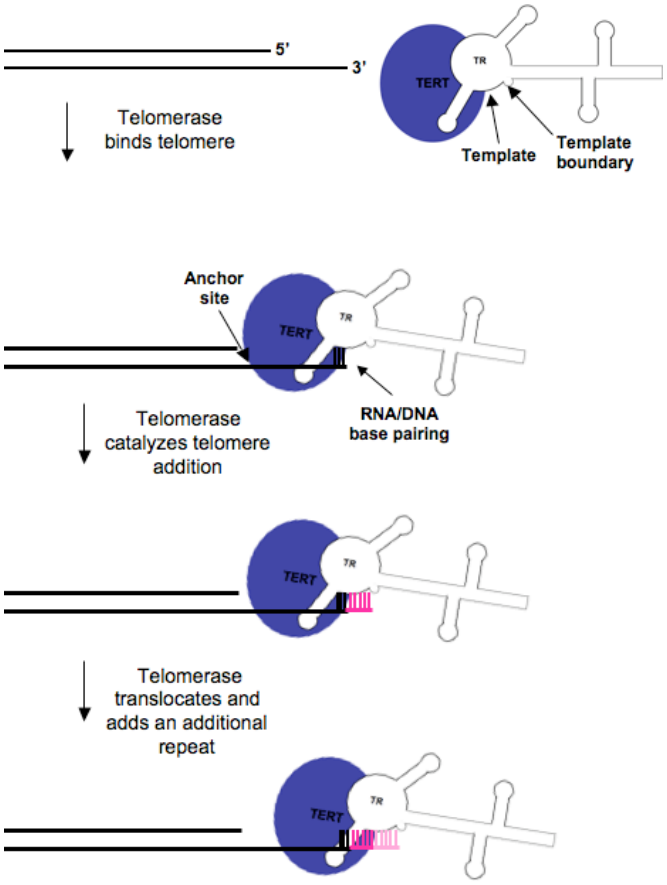


Figure 1.4 Telomerase catalyzes the addition of nucleotides to the end of the telomere. First telomerase must associate with the DNA template. This is accomplished minimally by two interactions within the telomerase complex: (1) the TERT protein binds to the template through its N-terminus in a region termed the anchor site and (2) the TR (telomerase RNA) base pairs with complementary residues at the extreme 3'-end of the telomere (the black lines denote base pairing). Next telomerase adds nucleotides to the telomere using the template region of the RNA until the template boundary is reached or telomerase dissociates from the telomere (the hot pink lines denote new addition). Finally, telomerase translocates and adds another repeat or dissociates completely and telomere elongation is complete (the light pink lines denote an additional round of telomere addition).

eukaryotes, but is absent in some insects [85, 97]. The NTE is comprised of four evolutionarily conserved regions, the most amino-terminal domain is called the **T**elomerase **E**ssential **N**-terminus (TEN) followed by Region II, Region III, and the T-motif [98, 99]. The NTE is important for interacting with TR, nucleolar localization, and multimerization in some species [100-107]. In addition, there is a small motif defined within the TEN domain of human TERT that can **d**issociate **a**ctivities of **t**elomerase (DAT). Mutations within this motif of both the human and budding yeast TERT disrupt *in vivo* function but not *in vitro* function, implying that TERT has a function in the cell other than catalysis [98, 102]. Interestingly, direct fusion of hPOT1 to $hTERT^{DAT}$ mutants rescues the *in vivo* phenotype in a manner dependent upon hPOT1 association with telomeric chromatin, suggesting that the DAT motif is important for assembly of TERT with the telomere [108].

The RT domain is the catalytic center of the protein and has the same conserved motifs as all other reverse transcriptases (1, 2, A, B`, C, D and E) (reviewed in [109]). Recently, the structure of full-length TERT from the flour beetle, *Tribolium castaneum*, was published and revealed that TERT retains important structural features also found in the HIV RTs [85]. The RT domain and the CTE form a ring configuration that has a groove hypothesized to bind an RNA/DNA duplex. The RT domain is organized into "finger" and "palm" subdomains. The "finger" subdomain is created by motifs 1 and 2, while the "palm" is created by motifs A-E [85]. There are three absolutely conserved aspartate residues in motifs A and C that function together in the tertiary structure to coordinate two magnesium ions important for positioning the incoming dNTP and the 3`-hydroxyl of the DNA strand in the active site [84, 85, 110]. Mutation of these aspartate

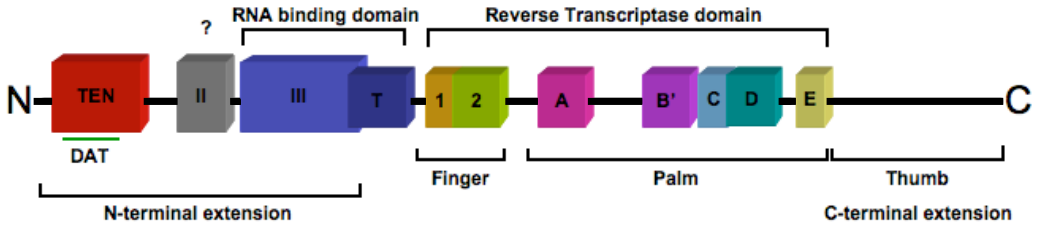


Figure 1.5 Schematic of the *TERT* domains. Most *TERT* genes have a highly conserved N-terminal extension that is important for interacting with TR and accessory proteins. The Telomerase Essential N-terminal (TEN) domain is important for associating with the DNA template via the anchor site. Additionally, mutations within a region of the TEN domain in higher eukaryotes specifically disrupts telomerase activity *in vivo* but not *in vitro* this is termed the DAT (dissociate activities of telomerase) domain. In budding yeast the TEN domain is important for assembling two accessory proteins (Est1 and Est3). The central portion of *TERT* consists of the reverse transcriptase domain, which contains both the finger and palm subdomains. *TERT* also has a C-terminal extension that is important in stabilizing DNA/RNA interactions, facilitating interactions with accessory proteins and it also influences telomerase activity (see text for a more detailed description).

residues results in a catalytically dead TERT [84, 110]. Mutations in both motifs C and E can either positively or negatively affect telomerase processivity [111].

The CTE is considered the "thumb" domain in TERT; this domain is mostly dispensable in yeast [98, 101, 103, 104, 111-113]. In the *T. castaneum* structure, the CTE appears to stabilize a modeled RNA/DNA duplex through interactions between lysines in the "thumb" domain and the DNA backbone [85]. Mutations within the CTE also affect telomerase processivity and hTERT multimerization [107, 111-115]. The CTE in hTERT also has binding sites for 14-3-3 and CRM1 proteins, which aid in proper intracellular localization and export of TERT, respectively [116].

Maintenance of Human Telomeres

Some of the main questions in the human telomere research area are: (1) What are the components of the active telomerase holoenzyme *in vivo*? (2) What mechanisms regulate telomerase activity? (3) How is the complex trafficked in the cell to become competent to extend telomeres? In the past several years there have been many advances in the understanding of each of these questions using a variety of techniques from bioinformatics and human genomics to cellular biology and biochemistry. These advances have identified homologous genes between yeasts and humans related to telomere maintenance and have aided in understanding the cellular localization of telomerase components and interaction partners [89, 117-123].

Components of Active Telomerase

The primary method used to determine if a protein is complexed with active telomerase is to determine if the proteins can immunoprecipitate telomerase activity. hEST1A and hEST1B both purify telomerase activity [117, 118]. These proteins were identified as homologues to the telomerase complex *EST1* gene from yeast. hEST1A is important in both telomere capping and telomere elongation as overexpression of this protein results in telomere uncapping and anaphase bridge formation [118]. The role of hEST1B at the telomere has yet to be uncovered, though binding to HDAC8 does protect hEST1B from being targeted to the proteosome by the ubiquitin E3 ligase, CHIP (**C**-terminal **h**eat **s**hock **p**rotein interacting factor). Depletion of HDAC8 affects telomerase activity *in vivo* presumably due to loss of hEST1B [124].

The RNA component in humans, hTR, is transcribed by RNA polymerase II and is subsequently bound at its 3'- H/ACA box by a complex of proteins composed of dyskerin, NHP2, NOP10 and GAR1. This dyskerin complex remains associated with the active telomerase complex and is important in processing, localizing and stabilizing the RNA [125-128]. hTR is also bound by TCAB1, a recently identified telomerase complex component that is also important for its localization in the cell [121].

Human telomerase also requires two chaperones, Hsp90 and Hsp23, for appropriate assembly both *in vivo* and *in vitro*. Inhibition of Hsp90 by geldanamycin inhibits the assembly of an active telomerase complex [129, 130]. hTERT complexes with nucleolin, SMN, and 14-3-3 to regulate its localization in the cell [116, 131, 132]. Only nucleolin has been shown to associate with the active telomerase complex. Additionally, other proteins associate with telomerase activity, though their function and

contribution to the complex remains enigmatic; these are: Ku, La and hnRNP D [133-136].

Regulation of hTERT

Telomerase is active in stem cells, germ cells, some highly proliferative cells and in ~85% of cancer cell lines, but is inactive in the vast majority of human somatic cells [137]. This inactivity is due primarily to the transcriptional repression of *hTERT* in these differentiated cells [123]. The *hTERT* promoter does not have a TATA or CAAT box, but instead is highly GC rich [138]. This sequence may form a CpG island near the ATG to regulate gene transcription. The promoter also contains an array of motifs that can bind different transcriptional repressors and/or activators. In addition, the methylation and acetylation status of nucleosomes in the promoter is regulated to alter the chromatin structure and subsequently modify the transcriptional state of *hTERT* (reviewed in [139]).

Transcription of *hTERT* can be activated by a number of transcription factors. The oncogene c-Myc heterodimerizes with its binding partner, Max, and binds to E-boxes located within the promoter of *hTERT* to activate transcription [140, 141]. There are also GC boxes within the promoter that can recruit SP1, a transcription factor that interacts with TATA-box binding protein-associated factors that are important in initiating transcription in TATA-less promoters [139]. Mutation of these GC boxes decreased transcription of telomerase *in vivo* [142]. The estrogen receptor and the human papilloma virus 16 E6 protein also activates *hTERT* expression. Estrogen binds to the estrogen receptor protein which then binds the estrogen responsive elements found in the *hTERT*

promoter, while the E6 protein interacts with Myc to bind the E-box within the promoter to increases transcription [139, 143, 144].

There are a number of factors that repress *hTERT* expression. As is true at other genes, the Mad1/Max heterodimer competes for binding at the E-boxes with the Myc/Max heterodimer to repress transcription [145]. Cells that are exiting the cell cycle or undergoing differentiation up-regulate Mad1 expression and down-regulate Myc expression resulting in a decrease in hTERT mRNA, explaining, at least in part, why most differentiated cells have silenced *hTERT* [139]. p53 inhibits expression of *hTERT* by binding SP1 and preventing it from binding to the promoter [146]. In 2003, Lin and Elledge screened for repressors of *hTERT* expression and discovered a number of factors: a tumor suppressor termed Menin; the transcriptional target of TGF-β, *SIP1*; Rak, a protein kinase that interacts with the tumor suppressor Rb; and a novel protein called Brit1 were all found to repress *hTERT* expression [147]. Another tumor suppressor, Wilms' tumor 1 (WT1), binds to the promoter in a sequence specific manner. Mutation of WT1 binding sites in the promoter results in increased hTERT (reviewed in [139]).

It is interesting to note that the vast majority of genes found to regulate *hTERT* are often categorized as either tumor suppressors or oncogenes, suggesting that these pathways are important in *hTERT* regulation. Additionally, this observation suggests that mutation within these genes may directly result in the dysregulation of *hTERT* in cancers.

Epigenetic changes to chromatin, such as methylation and acetylation of histones are important in regulating gene expression [148]. Acetylation does seem to play an important role in *hTERT* silencing. As observed elsewhere, an increase in acetylation results in an increase in *hTERT* expression. For example, addition of trichostatin A, an

inhibitor of histone deacetylases, to cell culture increases *hTERT* expression [149]. This result is consistent with the fact that Mad1, an *hTERT* silencer, associates with a histone deacetylase [150]. Methylation also modifies *hTERT* expression. Methylation of histone H3 on lysine 9 (H3K9) and histone H4 on lysine 20 (H4K20) are associated with *hTERT* repression, while methylation of histone H3 at lysine 4 (H3K4) is associated with *hTERT* expression [151]. Interestingly, depletion of SMYD3, a histone methyltransferase, decreases H3K4 methylation which reduces hTERT mRNA and telomerase activity [152]. Conversely, inhibition of LSD1, a lysine-specific demethylase, increases H3K4 methylation in human fibroblasts resulting in increased *hTERT* transcription [153]. The CpG islands of the *hTERT* promoter are also methylated (reviewed in [154]). To date there have been no recognizable differences between the *hTERT* promoter methylation profile of normal cells versus cancer cell lines or even within different cancer cell lines, suggesting that methylation of the DNA may not be a critical regulator of *hTERT* expression [154].

In addition to transcriptional regulation, hTERT is post-translationally modified to regulate its activity. Phosphorylation of hTERT both positively and negatively regulates its activity. Akt kinase and PKC phosphorylate hTERT to activate telomerase activity *in vitro*, while c-Abl, a tyrosine kinase, negatively regulates telomerase activity (reviewed in [139]). Overexpression of c-Abl decreases telomerase activity *in vivo* and cells that lack c-Abl not only have increased telomerase activity but also increased telomere length [155]. hTERT is ubiquitinated by MKRN1 and targeted to the proteasome for degradation when its chaperone, Hsp90, is inhibited from associating with hTERT, suggesting that ubiquitination is another level of post-translational control [156].

Trafficking of Telomerase

Exactly how hTR and hTERT are localized to the telomere during S phase, the phase of telomere extension, remains enigmatic, but recent discoveries have elucidated how these components are trafficked in the cell differentially during the cell cycle [157]. Both the nucleolus and Cajal bodies are important subnuclear compartments of telomerase assembly and maturation. The nucleolus is most notably the site of ribosome assembly and RNAs that traffic to nucleoli are termed small nucleolar RNAs (snoRNAs). Cajal bodies are important in maturation of spliceosomal components and RNAs that traffic here are termed small Cajal body RNAs (scaRNAs) (reviewed in [158, 159]).

hTERT is most likely trafficked into the nucleus once it is phosphorylated via the PI3K/Akt pathway [160]. Once in the nucleus during G1 and G2 phases of the cell cycle, hTERT is found in foci in the nucleoplasm, but not associated with the nucleolus or Cajal bodies [157]. As the cell progresses into early-S phase, hTERT localizes to the nucleolus and during mid-S phase it is found in foci adjacent to Cajal bodies and no longer co-localizes with the nucleolus or Cajal bodies [157]. hTR is primarily associated with Cajal bodies throughout the cell cycle, though it can also associate with the nucleolus [161]. hTR has two specific domains within its 3'-end that regulate its nuclear localization, the H/ACA box domain and the CAB domain. The dyskerin complex (dyskerin, GAR1 NOP10, and NHP2) binds the H/ACA box while TCAB1 binds the CAB domain. TCAB1 is a Cajal body specific protein and is thought to be responsible for shuttling hTR to the Cajal body [121]. Interestingly hTERT, hTR/Cajal bodies, and a subset of

telomeres co-localize during S-phase, suggesting that only a fraction of telomeres are elongated each cell cycle [157, 162].

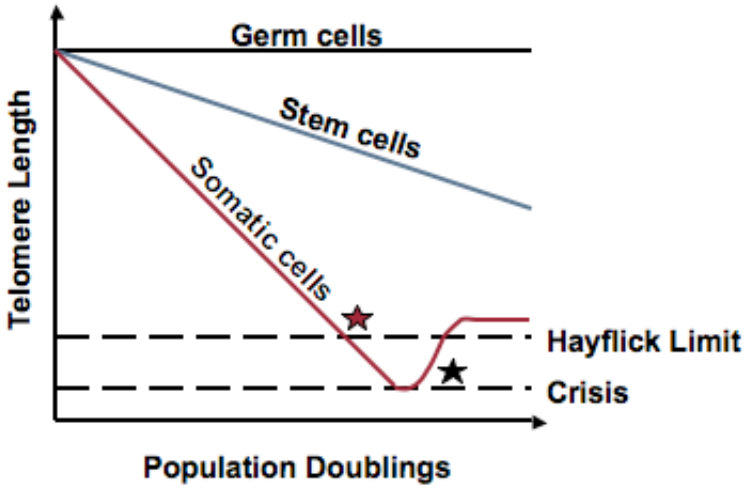
Telomeres, Telomerase and Human Disease

In 1961, Drs. Leonard Hayflick and Paul Moorehead elegantly showed that karyotypically normal primary cells, from 25 different cell strains, derived from fetal tissues, stopped dividing (or senesced and then died) in culture after ~40-50 generations [6]. When the cells were removed from culture and frozen at different generations and subsequently thawed and re-initiated to grow, the culture stopped dividing at what would have been a total of ~40-50 generations. Drs. Hayflick and Moorehead were never able to maintain these cells indefinitely in culture and this result caused a paradigm shift in the field of cellular biology. It had previously, albeit erroneously, shown that cells given the correct milieu of serum could grow indefinitely in culture. (It has since been hypothesized that in this earlier experiment cells were unknowingly contaminated with stem cells when harvested). It was Hayflick and Moorehead's observation that cells have a finite number of cell divisions that originally stirred the end-replication hypothesis proposed by Olovnikov in 1971. Three decades after Hayflick's finding, Harley *et al.* found that as mitotically differentiated cells divide, their telomeres become progressively shorter (acting as a "mitotic clock") [163]. The following year Allsopp *et al.* demonstrated that telomere length was a predictor of cellular senescence [164]. These three seminal pieces of work established a strong association between telomere length and cellular senescence. The following section is dedicated to examining how telomere length and telomerase activity may contribute to human disease.

Telomere Length of Cultured Cells

Telomeres of most differentiated cells shorten with each cell division because the transcriptional repression of *TERT* expression leaves no active mechanism to combat the end-replication problem (Figure 1.6) [165]. Consequently, these cells have a limited replicative capacity before they undergo cellular senescence. Alternatively, undifferentiated human stem cells express low levels of telomerase, which helps maintain the telomere, but this regulated expression of telomerase is insufficient to counteract telomere attrition, ultimately leading to a decline in tissue renewal (an aging phenotype) (reviewed in [166]). Interestingly, germ cells and $\geq$85% of cancer cells maintain high *TERT* expression and can maintain telomeres (reviewed in [139]). In germ cells, telomere maintenance ensures that progeny begin with sufficiently long telomeres. In cancer cells, telomere maintenance is required to induce cellular immortality, a hallmark of cancer. The telomere length in cancer cells is generally held at a short, stable length. In the other ~15% of cancer cells, telomeres are maintained by an alternative method that is dependent upon telomere recombination (reviewed in [139]). It is important to note that the maintenance or lack thereof, of the telomere, unlike any other DNA structure, is linked both to normal cellular aging and cancer formation. As a result, the telomere sits at the forefront of understanding some of the molecular mechanisms related to human aging and their ensuing disease-related states.

A


Germ cells
Stem cells
Somatic cells
Telomere Length
Hayflick Limit
Crisis
Population Doublings

B

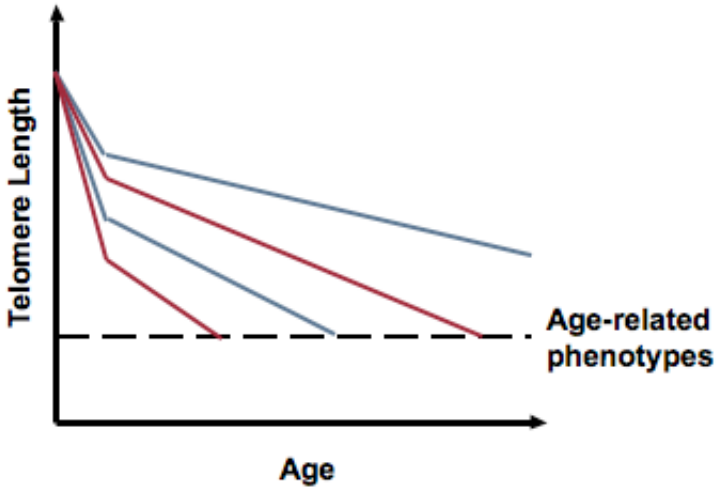

Telomere Length
Age-related
phenotypes
Age

Figure 1.6 The relationship of telomere length to cellular senescence and aging phenotypes. (A) Telomere length (y-axis) changes as cells divide (x-axis) depending on telomerase activity. Germ cells (black line) express sufficient levels of telomerase to maintain their telomere length through out population doublings. Stem cells (blue line) do not maintain sufficient levels of telomerase causing a modest decrease in telomere length over the lifetime of the cell. Most somatic cells (red line) are telomerase negative and lose telomere length as they divide. In culture, most somatic cells will eventually reach the Hayflick limit and trigger a cell-cycle checkpoint arrest, leading to cellular senescence. Cells with mutations in p53 or pRb can bypass the Hayflick limit allowing them to continue to divide until they reach crisis. Crisis is the point at which telomeres are so short that the capping function is lost. This loss ultimately results in chromosome fragmentation, vast genomic instability and cell death. A small number of these cells will gain the ability to maintain telomeres by either activating telomerase or up-regulating a recombination-based pathway of telomere maintenance. These cells are now immortal and have the propensity, given that they have undergone such dramatic mutagenesis, to become cancerous. Adapted from Shay and Wright, 2006 [167]. (B) Telomere length (y-axis) decreases as age (x-axis) increases. The telomere length of stem cells (blue lines) and somatic cells (red lines) are influenced by defects in telomerase function. Telomeres from stem cell populations will decrease more slowly than somatic cells due to greater telomerase expression. The lower colored-lines represents the lower telomere-length boundary of each cell type due to compromised telomerase expression. The large decrease in telomere length diagramed early in life is due to the rapid cellular division and turnover that occurs in childhood. As adulthood is reached, cell division reaches a more steady state and the slope of telomere attrition is less steep. Individuals who have defects in telomerase activity are more likely to reach age-related phenotypes earlier in life (the lower boundaries) compared to individuals with wild-type telomerase expression. Adapted from Kappei and Londono-Vallejo, 2008 [168].

The Link Between *in vivo* Telomere Length and Human Disease

Once the link between telomere length and cellular senescence (aging) was established in cell culture, there was great interest in elucidating if this correlation existed *in vivo*. Large population-based studies in the late 1990's and early 2000's established a negative (or inverse) correlation between age and telomere length, suggesting that the same phenomenon (telomeres shorten with cellular age) also exists within the organism (reviewed in [169]). This correlation alone does not mean that telomere shortening *causes* cellular aging or age-related phenotypes. To determine if it is causative, researchers have developed mouse models and used human genomics to determine genes involved in diseases that greatly perturb telomere maintenance.

Laboratory mice begin with extraordinarily long telomeres compared to humans or even wild mice, therefore when using them as a model it is important to note that aging phenotypes will be delayed a few generations. In TR knockout mice (TR-/-), mice grow normally for the first 3-4 generations, revealing that telomerase activity is not required for viability. As the mice reach the 5^{th} and 6^{th} generations they show age-related phenotypes such as graying fur, skin lesions, decreased tissue renewal in intestine, testes, and bone marrow (these are highly proliferating tissues), as well as a shorter life span and an increased propensity for precancerous lesions [170]. Reintroduction of TR into TR-/- mice rescues these aging phenotypes. Introduction of the TR-/- mutation into wild mouse strains with naturally shorter telomeres results in earlier expression of phenotypes, suggesting that short telomeres do influence aging phenotypes [170]. In this same study, telomere maintenance in mice heterozygous for TR (TR+/-) was also compromised and had phenotypic consequences, although those phenotypes were delayed by several

generations compared to the TR-/- mice. This result suggests that loss of one TR allele causes haploinsufficiency. The strongest evidence in this system that telomere shortening alone can cause disease is that genotypically wild-type progeny that are offspring from later generation heterozygotes (and thus start with short telomeres) also develop disease phenotypes. Since these mice have normal telomerase expression but very short telomeres, the authors convincingly argue that it is the short telomere and *not* the lack of telomerase activity *per se* that gives rise to the disease state [170].

Dyskeratosis **C**ongenita (DC), **A**quired **A**plastic **A**nemia (AAA) and **I**diopathic **P**ulmonary **F**ibrosis (IPF) are three telomerase spectrum diseases recently discovered to be caused, at least in some cases, by mutations in telomerase components or telomere capping genes (reviewed in [171]). The telomere lengths in these patients are generally (though not always-as in some AAA patients) much shorter than their age-matched-control subjects. DC is the more severe telomerase-deficiency related disease compared to AAA or IPF and patients tend to present symptoms earlier in life. Population studies of DC patients have identified mutations in dyskerin, NOP10, NHP2, TIN2, TR and TERT, all genes involved in telomere maintenance (reviewed in [171]). DC has a spectrum of phenotypes related to it that may include, but are not limited to: bone marrow failure, nail dystrophy, oral leukoplasia (a precursor for oral carcinomas) and predisposition to cancer [171]. Additionally, families with DC show genetic anticipation, an associated phenomenon in which disease symptoms appear earlier in each subsequent generation. This phenomenon is expected in DC as each generation begins with shorter telomeres.

AAA is a less severe telomerase spectrum disease that can be genetically inherited or induced through exposure to environmental agents or drugs such as benzene or

chloramphenicol that give rise to an immune-mediated bone marrow failure. Patients with AAA typically present symptoms later in life compared to DC patients. Mutations in TERT have been found to be associated with AAA in some patients [172]. Approximately 33% of patients with AAA have short telomeres and those with short telomeres do not respond well to the typical immunosuppressive treatment (reviewed in [171]).

IPF is also a less severe telomerase spectrum disease compared to DC that presents later in life. It can be inherited in families or occur sporadically by mutation in either TR or TERT. Pedigrees affected by IPF often show phenotypes similar to DC and patients have short telomeres. The development of IPF is influenced by environmental factors such as smoking, which might explain why the disease shows variable penetrance (reviewed in [171]).

Werner's **S**yndrome (WS) is a premature-aging disease that also affects telomere length, though the mechanism affecting telomeres is unknown. WS is caused by mutations in the *WRN* gene, which encodes a RecQ Family DNA helicase that plays a role in DNA replication, repair, and recombination and is particularly important in the restart of stalled replication forks (reviewed in [168]). The main phenotypes associated with WS are: short stature, early graying, formation of early cataracts and early death (40-50 years old) resulting from cancer or heart disease. Interestingly, studies have demonstrated a strong inverse correlation between telomere length and cardiovascular complications in this disease (reviewed in [168]).

These are the diseases in which changes in telomere length are most directly linked with aging or disease-phenotypes. Intriguingly, TERT mutations have been

reported for DC, IPF and AAA, but there does not appear to be any correlation between either the site of mutation within the protein or the extent to which telomerase activity is affected in these patients. Furthermore, different diagnoses can occur in the same family where the same mutation of TERT is segregating, suggesting that there must be other factors influencing the disease phenotype. Additionally, it is interesting that these diseases share some phenotypes but are also extremely varied, begging the question of why diseases that negatively affect telomere length give rise to such variation in disease presentation? The answer is likely due to multiple reasons: (1) Mutations within some proteins such as dyskerin, NOP10, NHP2, and WRN most likely disrupt multiple pathways in the cell in addition to telomere maintenance, which could instigate variable phenotypes. (2) The genetic environment may influence how a particular mutation affects the phenotype. (3) Environmental differences may explain a considerable amount of the variation, as smoking, obesity, stress and sex-hormones, exercise, diet and even marriage status have all been shown to influence telomere length [173-179]. Therefore, environmental pressures may negatively or positively impact telomere length, resulting in differential disease states.

It is and will be challenging to concretely establish that telomere shortening *causes* normal aging and its related diseases, as it is difficult to distinguish between cause and effect. However, given the above spectrum of premature aging diseases, the genetic anticipation in DC patients, and the data observed from mouse models, it does seem certain that telomere length is an important factor influencing health and longevity.

Telomere Maintenance in Yeast

The budding yeast, *Saccharomyces cerevisiae*, is an excellent model system for the study of telomeres and telomerase as yeast maintain their telomeres similarly to mammals and telomerase actively elongates telomeres during every S-phase of the cell cycle. Because telomerase is active in yeast, the yeast cell, at least at the level of telomerase assembly and function, is somewhat analogous to a cancer cell. These characteristics allow investigators to address fundamental questions regarding telomere structure and maintenance and telomerase assembly and function in a tractable system that can directly impact the research in the human cancer and telomerase fields.

The Cell Cycle Regulated Assembly of Telomerase Components

Telomerase activity is confined to late S phase and G2/M, implying that there is a mechanism(s) restricting activity to these phases [180, 181]. Interestingly the telomere structure in yeast changes during the cell cycle. In G1 and G2/M phases, the telomeric overhang is ~12-14 nucleotides long, whereas the overhang in S phase is $\geq$ 30 nucleotides [25, 182]. The length of the overhang is not determined by telomerase since deletion of Est2 does not alter the single-stranded telomere segment in the cell cycle [24]. It is instead regulated by the yin-yang relationship between capping proteins, Cdc13-Stn1-Ten1 (CST complex), Rap1 and Ku70/80 and exonucleases, Exo1 and Mre11 (reviewed in [183]). In G1, capping by both Ku70/80 and Rap1 are important in limiting nucleolytic cleavage of the 5'-strand, while the CST complex is essential in S phase for the same function [184, 185].

In the early 2000's, Dr. Virginia Zakian's group began detailing the temporal association of telomerase components with the telomere using chromatin immunoprecipitation (ChIP). Cdc13, though not an integral member of the complex, is an essential single-stranded telomeric binding protein and is important in recruiting telomerase to the telomere [186-189]. It associates with the telomere in S-phase, coincident with the increase in the single-stranded 3'-overhang [190]. In G1, both Est2 and TLC1 (the catalytic core of yeast telomerase) are bound to the telomere through an interaction with the double-stranded DNA binding protein, Ku70/80 [191]. As cells move into S phase, the association of Est2 and TLC1 with the telomere is decreased and peaks again in late-S phase, the time of telomere replication [190]. The Est1 protein (a telomerase accessory protein) levels are very low in G1 precluding it from telomere association, but as the cell moves into late-S phase, Est1 protein levels are higher and it can be detected at the telomere by ChIP, presumably due to its interaction with Cdc13p [190, 192]. Importantly, low Est1 protein levels alone do not restrict telomerase activity at the telomere in G1, since stabilization of Est1 in G1 using MG132 is not sufficient to promote telomere elongation, even though Est1 associates with Est2 under these conditions in G1 [192]. This finding supports the idea that there is a mechanism restricting telomere elongation to S phase. The association of Est3 (another telomerase accessory protein) with the telomere throughout the cell cycle has recently been elucidated and it generally mimics the Est1 profile [193]. In addition to having a peak of telomere association in late-S phase it also has a very small peak in G1, suggesting that Est3 can assemble with the telomere, at a low level, in the absence of Est1 [193]. This

Est3 assembly is presumably through Est2 as it is not thought that Est3 binds to telomeric DNA independent of the other telomerase components [194, 195].

Telomere Length Regulation

There are a number of positive and negative regulators of telomere length whose interactions are dynamically orchestrated to ensure that telomeres in yeast are maintained within a set average length. Telomere length homeostasis is ultimately a balance between activities that deplete telomeric DNA (incomplete replication and nucleolytic processing of the telomere) and addition of telomeric DNA by telomerase. Interestingly, the actual length of each individual telomere is important in regulating telomere addition *in cis*. A long telomere restricts telomere addition (unextendible), while telomerase generally acts upon a shorter telomere (extendible) [196]. This phenomenon is due to a telomere length "counting" mechanism regulated by Rap1 and its interacting partners Rif1 and Rif2 (discussed below) [197].

Maintenance of the telomere can be broken down into four general steps: (1) replication of the telomere, (2) nucleolytic processing the 3'-end, (3) telomerase-mediated elongation and (4) "fill-in" synthesis by the lagging strand replication machinery. DNA replication is important for proper telomere processing and telomere elongation [181, 198]. Deletion of the DNA replication origin on a linear yeast plasmid inhibited processing of the 3'-end of the telomere [198]. In 2000 Marcand *et al.* observed that removal of an origin on a yeast minichromosome disrupted telomere elongation, while strains that retained the origin maintained and elongated their telomeres, suggesting that DNA replication and telomere elongation are linked [181]. A recent publication from

the Shore lab elegantly showed that the timing of origin firing is strongly correlated with the extent of telomere elongation [199]. When a late firing origin located near (~1 kilobase upstream) the telomere is replaced with an early firing origin, telomere length is increased over wild type (on that chromosome only). Inversely, if an origin many kilobases upstream (~20) of the telomere is used to replicate the telomere, telomere length is significantly shorter than wild type. These data strongly support the idea that replication of the telomere is important for telomere elongation. Why might DNA replication and telomere elongation be strongly linked? One possibility is that the heterochromatic state of the telomere needs to be disrupted to allow telomere elongation. It is also conceivable that as the replication fork passes through the telomeric DNA, Cdc13p binds to the single-stranded DNA (similar to RPA in other regions of DNA), increasing the pool of Cdc13p near or at the telomere thereby stimulating telomerase recruitment [200].

Telomerase can only extend a 3'-single-stranded telomeric DNA substrate [93]. Therefore the telomere product of leading-strand synthesis (a blunt end) must be resected or processed before telomerase can act. Not all of the nucleases involved in telomere processing are known, but Exo1 and Mre11 (a member of the MRX complex: Mre11-Rad50-Xrs2; analogous to MRN in higher eukaryotes) are key players. Deletion of Exo1 suppresses the temperature sensitive phenotype in yeast strains with elongated ssDNA overhangs resulting from defective telomere capping, suggesting that in the absence of Exo1 the telomere is not degraded as readily [201]. Deletion of Mre11 in yeast cells decreases the telomeric overhang throughout the cell cycle and inhibits telomerase activity *in vivo*, revealing an important role for Mre11 in telomere processing [25, 202].

While nucleolytic processing of the telomere is required, it also could be detrimental since a large amount of single-stranded DNA triggers a cell-cycle checkpoint arrest. As a result, telomere resection must be highly regulated. Proteins involved in telomere capping are important in regulating the extent of resection, although the mechanism of this inhibition is poorly understood (reviewed in [183]).

As mentioned above, passage of the replication fork and telomere processing are important for telomere elongation, but once these processes are completed, how is telomerase recruited to the telomere? Once the telomere is processed, Cdc13 and its associated proteins, Stn1 and Ten1, most likely bind the long G/T rich 3'-overhang to protect the telomere from extensive nucleolytic degradation (Figure 1.7). The Cdc13 protein may be post-translationally modified (perhaps phosphorylation by the cyclin dependent kinase) to recruit the telomerase complex (reviewed in [183]). Interestingly, the initiation of telomere elongation appears to be dependent, at least in part, on the Rif1 and Rif2 proteins (Figure 1.7). Deletion of either protein increases the *frequency* but not the *extent* of telomere elongation events, suggesting that their normal role is to restrict telomerase association with the telomere [196]. *In vivo*, yeast telomerase appears to add repeats in a non-processive manner [203]. As a result, after telomerase replicates the RNA template region, it dissociates from the telomere and either the same or a different complex may re-associate. The lagging-strand polymerase machinery may regulate the extent of telomere elongation. The catalytic subunit of polymerase alpha, Pol1, and an accessory protein Pol12 interact with Cdc13 and Stn1, respectively [204, 205]. These interactions have been hypothesized to limit telomerase access to the telomere since Stn1 and the telomerase component Est1 compete for Cdc13 binding (Figure 1.7) [183]. The

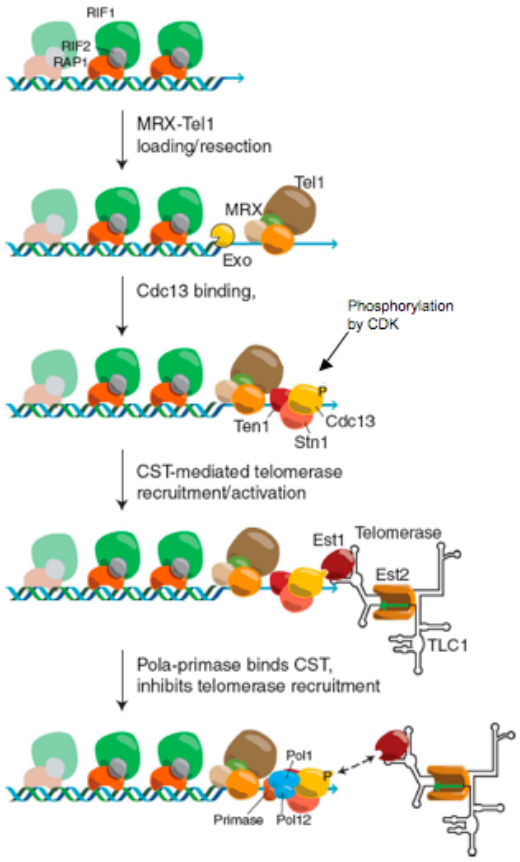


Figure 1.7 The telomere is processed to recruit telomerase. Briefly, the Mre11/Rad50/Xrs2 (MRX) complex along with Tel1p processes short telomeres. Cdc13p binds to the overhang and recruits the capping proteins Stn1 and Ten1 (together the CST complex). Cdc13p is phosphorylated by the Cyclin Dependent Kinase (CDK) to enhance recruitment of telomerase to the telomere through its interaction with Est1. Telomerase extends the 3'-overhang by catalyzing the addition of nucleotides. Polymerase alpha-primase competes with telomerase for binding to Cdc13p and inhibits telomerase action at the 3'-end. This figure was modified slightly from Shore and Bianchi, 2009 [183].

exact mechanism for how the CST complex and telomerase complex compete for telomere binding is unclear, but may be regulated by post-translational modifications to the Cdc13 protein (reviewed in [183]).

Telomere length in yeast is maintained between ~250-300 base pairs and interestingly, only ~6-8% of wild-type telomeres each cell cycle are elongated, most being short telomeres [196]. This begs the question; how does telomerase recognize short telomeres? There are a number of different protein complexes at the telomere, one such complex is the Rap1/Rif1/Rif2 complex. Rap1 binds the double-stranded portion of the telomere and dictates the extendibility of the telomere [197]. When the number of Rap1 molecules is artificially increased at the telomere there is a concomitant and dose-dependent decrease in the length of that telomere, revealing that Rap1 acts *in cis* to negatively regulate telomere length [197]. A C-terminal region of Rap1 interacts with Rif1 and Rif2, to negatively regulate telomere elongation, in part, through the Tel1 protein (reviewed in [183]). It is thought that the amount of Rif2 (low at a short telomere and high at a long telomere) dictates whether Tel1 can bind to the telomere. Indeed, Tel1 preferentially binds short telomeres, as assessed using chromatin immunoprecipitation (ChIP) and this preference is eliminated upon deletion of *RIF2* [206]. This result suggests that Tel1 can discriminate between the levels of Rif2 at the telomere. Tel1 may sense Rif2 levels through its interaction with Xrs2, a member of the MRX complex [207]. The Xrs2 protein can also interact with Rif2 but how this interaction may regulate the association of Tel1p with the telomere is not understood. Additionally, the Rif1 and Rap1 proteins can negatively regulate Tel1 and the MRX complex, respectively, but again the exact inhibitory relationships have not yet been well described [207]. Overall, it appears

that low levels of Rif2 at the telomere may bias Tel1 binding to short telomeres. Interestingly, the telomerase proteins Est1 and Est2 also preferentially bind short telomeres and this preference is dependent upon Tel1 [208]. Therefore, though the exact mechanism of telomere length control is not completely understood, it does appear that the Tel1 protein along with Rap1, Rif1, Rif2 and the MRX complex are playing very important roles.

Yeast Telomerase Components

Drs. Greider and Blackburn first measured the enzymatic function of telomerase in 1985 in the ciliate *Tetrahymena thermophila* [16]. Then in 1989 the first gene involved in telomerase function was identified; it was termed *EST1* for the **E**ver **S**horter **T**elomere phenotype it elicited when deleted or mutated [209]. Three other *EST* genes, *EST2-EST4*, were identified in 1996 and the RNA template (**T**e**l**omerase **C**omponent 1 or *TLC1*) was characterized in 1994 in budding yeast, establishing *S. cerevisiae* as a leading genetic model system for telomere and telomerase research [90, 210].

As with humans, the TERT subunit, *EST2*, and the RNA template subunit, *TLC1*, are sufficient for *in vitro* telomerase activity in yeast [211, 212]. Both Est1 and Est3 co-purify with telomerase from yeast extract but deletion of either gene from yeast strains does not prevent telomerase activity *in vitro*, therefore, *EST1* and *EST3* are thought to play accessory or regulatory roles *in vivo* [211, 213]. *EST4* was later determined to be a separation-of-function allele of *CDC13* [214]. The Cdc13 protein is not considered a member of the telomerase complex as it does not co-purify with telomerase activity from yeast extract and Cdc13p cannot co-purify subunits of the telomerase complex from yeast

[213]. The essential function of Cdc13p is to cap the 3'-overhang of the telomere and protect it from nucleolytic degradation [189, 215]. A mutation of *CDC13* was isolated in the EST genetic screen due to its other role in telomerase recruitment via an interaction with Est1 [186, 200].

Est2 is the evolutionarily conserved telomerase reverse transcriptase subunit. In yeast, Est2 has a conserved N-terminus that consists of four regions. The most N-terminal is termed the TEN (**T**elomerase **E**ssential **N**-terminus) domain and is thought to contribute to the assembly of the accessory components Est1 and Est3. Alanine-block mutations within this domain phenocopy deletions of either *EST1* or *EST3* [98]. The TEN domain is also important for anchoring telomerase to the DNA substrate by the so-called anchor site and regulating telomere length, as some mutations within this domain result in longer-than-normal telomeres [95, 216-218]. The other three regions are termed Region II, Region III and the T-motif; the latter two are critical for proper TLC1 binding, but the function of Region II is unknown (See Figure 1.5 for graphical representation) [98, 100]. The canonical reverse transcriptase domain is located centrally in Est2 (immediately C-terminal to the T-motif) and there is a **C**-**T**erminal **E**xtension (CTE) that is thought to be the "thumb" domain of the reverse transcriptase. In yeast, this region is non-essential since the cells can survive, albeit with short telomeres, without most of the CTE [85, 98].

TLC1 contains the RNA template for the telomerase complex [90]. It is transcribed in yeast by RNA polymerase II and it has both a 5'-2,2,7 trimethyl-guanosine (TMG) cap and polyadenosine tail. At least two forms of the RNA exist in cells: a precursor, telomerase-unbound, polyadenylated form and a mature, telomerase-bound, depolyadenylated form [219]. The RNA has non-overlapping binding sites for Est1 and

Est2 in the central region of the RNA. As with components of the splicing machinery, TLC1 is considered a small nuclear RNA (snRNA) because the heptameric Sm protein complex binds to the 3'-end of TLC1 and is important for its maturation [219, 220]. The Sm proteins are retained in the active telomerase holoenzyme since immunoprecipitation of Sm proteins co-purifies telomerase activity. Because the heptameric Sm protein complex binds TLC1 it is considered an snRNA. TLC1 also has a binding site for the Ku70/80 heterodimer that is required for localization of telomerase to the telomere in G1 phase [191, 221]. The RNA in yeast is much longer than the template RNA in human (~1,300 bp versus ~500 bp, respectively) (reviewed in [97]). The variation in size between different organisms may be due, in part, to variability in the number and identity of protein interaction partners. Interestingly, a mini-TLC1 (500 bp) that has been modified to remove RNA regions that are not involved in protein interactions retains enzymatic function. However, cells expressing mini-TLC1 have reduced fitness compared to cells with wild-type TLC1. This result suggests that the intervening regions of TLC1, though not essential, do contribute to function, perhaps by providing structural support or appropriate spacing and/or flexibility between protein-binding molecules [212].

Est1 is an accessory protein that recruits telomerase to the telomere through an interaction with the ssDNA binding protein Cdc13p. When Cdc13p is fused to Est2 (the catalytic subunit) yeast cells can survive without Est1, but die in the absence of Est3. This genetic result strongly suggests that the normal function of Est1 is to recruit telomerase to the telomere [188]. Interestingly, in wild-type yeast cells when Cdc13 and Est2 are fused, telomeres become hyper-elongated due to increased telomerase

recruitment at the telomere. If the only function of Est1 is to recruit telomerase to the telomere then deletion of *EST1* should not appreciably affect telomere maintenance. Strikingly, deletion of *EST1* from these cells results in a shorter than wild-type telomere phenotype, suggesting that Est1 has, at minimum, two functions at the telomere and that the second function is only partially rescued by the Cdc13-Est2 fusion. Our lab suggests that this function is stimulating the recruitment of Est3 to the complex. The observation that over-production of Est1 results in a concomitant increase in the association of Est3 with telomerase supports this hypothesis [192]. Not only is Est1 sufficient for Est3 recruitment, it is also necessary because deletion of *EST1* precludes Est3 assembly with the complex [192]. Est1 assembles with TLC1 independent of Est2 via a specific interaction with a bulge stem structure within the central region of TLC1 [220, 222]. It can also bind single-stranded RNA or DNA telomeric substrates *in vitro* and was recently shown to stimulate telomerase activity *in vitro* [223, 224]. Since the telomere is rich in guanosine, higher-order DNA structures, such as G-quadraplexes are hypothesized to form *in vivo*. The Est1 protein has been shown to stimulate G-quadraplex formation *in vitro* and mutations that disrupt this activity result in cellular senescence *in vivo* [225]. This result suggests that Est1 may function to stimulate telomere addition by modifying the structure of telomeric DNA.

Est3 is a 21kDa protein that has structural similarity to the mammalian shelterin protein TPP1, which is important in capping the telomere and is a processivity factor for telomerase activity [33, 226, 227]. Indeed, it has very recently been demonstrated that the Est3 protein in a closely related yeast species, *Saccharomyces castelli*, influences the processivity of telomerase *in vitro*, suggesting that it might be functionally related to

TPP1 [194]. Est3 has been suggested to have non-sequence specific helicase activity *in vitro*, although the possibility of a contaminating function remains since no mutation tested disrupted the unwinding activity [228]. More recent data using **N**uclear **M**agnetic **R**esonance (NMR) uncovered no Est3 peak shifts upon titration of DNA oligos, raising doubt about the ability of the *S. cerevisiae* protein to bind DNA [194, 228]. Est3 can multimerize *in vitro* and addition of magnesium appears to stimulate multimerization [229-231]. However, the significance of potential Est3 multimerization for its function *in vivo* is unknown. Indeed, immunoprecipitation using differential tagged alleles of *EST3* was unable to detect Est3 multimerization in cell extract (Robin Bairley, unpublished data).

Two forms of Est3 are translated *in vivo*: a small 92 amino acid protein resulting from an in-frame stop codon and the telomerase-bound, full-length Est3 protein (181 amino acids) that results from a programmed +1 translational frameshift at a rare serine codon [232]. At steady-state, the smaller form constitutes ~75% of the total Est3 protein in the cell [232]. To date, there is no known function for the truncated Est3 protein. Deletion of the rare codon, to ensure only the full-length protein is made *in vivo*, yields no measurable mutant phenotype associated with telomere maintenance or cellular viability [232].

Significance of This Study

Striving to gain greater knowledge of natural processes is a method of seeking truth, while working toward understanding the fundamental mechanisms underlying cellular processes is important for appreciating and respecting the environment and life.

Often these discoveries can improve the length and quality of life for individuals. Specifically the study of telomeres and telomerase is proving to be critical in understanding the finite nature of tissue renewal (aging) and the establishment and persistence of cancer and other age-related diseases.

Studying fundamental processes is often more simply accomplished and less expensive in lower eukaryotes. Due, in part, to evolutionary constraint, many of the cellular mechanisms discovered in more simple organisms are frequently conserved in humans. Consequently, our lab studies telomere biology in the genetic model system, *Saccharomyces cerevisiae*. In Chapter II, I demonstrate that the telomerase accessory protein Est1 is degraded in G1 phase of the cell cycle in a manner dependent upon the proteasome. This finding has contributed significantly to our understanding of telomerase assembly and activity in the cell cycle. Chapter III focuses on elucidating an interaction that is important for the assembly of Est3 with telomerase and finally in Chapter IV, I uncover a function for Est3 in telomerase activity. Both of these findings regarding the Est3 protein are the first to establish an *in vitro* assay for determining how Est3 assembles with components of telomerase and show that Est3 does contribute to telomerase activity in *S. cerevisiae*.

CHAPTER II

THE EST1 PROTEIN IS DEGRADED IN G1 PHASE OF THE CELL CYCLE IN A PROTEASOME-DEPENDENT MANNER[1]

Introduction

The action of telomerase in *Saccharomyces cerevisiae* is regulated in the cell cycle. The enzyme does not extend telomeres in G1 phase, but acquires that capability in late S phase and G2 phase of the cell cycle [180, 181]. The catalytic core of the complex (Est2 and TLC1 RNA) is bound to the telomere in G1 through an interaction of the TLC1 RNA with the yKu70/80 heterodimer [191]. Its association with the telomere dips in early S phase and then peaks again in late S/G2 phase [190]. The accessory protein Est1 is not detected at the telomere in G1, but does peak in late S/G2 phase, concurrent with both the presence of the catalytic core and telomere elongation [190]. The association of Est3 with the telomere was recently shown to also be regulated in the cell cycle, with a very minor peak in G1 and a strong peak of association in late S/G2 phase, concomitant with both the Est1 and Est2 peaks [193].

The assembly of the telomerase complex is also cell cycle regulated. Both Est1 and Est3 co-purify with Est2 in G2/M-blocked cells, but neither protein co-purifies with Est2 in G1 [192]. Interestingly, the modest association of Est3 with the telomere during G1 phase suggests that there may be some residual interaction between the catalytic

[1] This chapter represents my contribution to the published work: [192] Osterhage, J.L., J.M. Talley, and K.L. Friedman, *Proteasome-dependent degradation of Est1 regulates the cell cycle-restricted assembly of telomerase in Saccharomyces cerevisiae.* Nat Struct Mol Biol, 2006. **13**(8): p. 720-8.

subunit and Est3 [193]. Overall, these data suggest that the Est1 and Est3 proteins are primarily precluded from telomerase assembly in G1, but are competent for assembly in S phase, begging the question: what is precluding these components from assembly in G1? The EST3 RNA does not appear to vary during the cell cycle and Est3 protein levels are constant throughout [192, 233]. In contrast, the levels of EST1 RNA are modestly reduced in G1 phase when compared to G2 phase (~ three-fold reduction), but the protein levels are reduced by greater than four-fold, suggesting that in addition to modest transcriptional regulation, the Est1 protein may also be degraded more rapidly during G1 phase [192, 234]. Interestingly, over-expression of Est1 stimulates the association of Est1 with Est2 in G1 phase, implying that Est1 protein levels account, at least in part, for regulated assembly [192]. In addition, Est3 assembly with telomerase is dependent upon Est1 as deletion of *EST1* reduces Est3 association with the catalytic core while over-production of Est1 stimulates Est3 binding to the complex [192]. These results suggest that Est1 is a critical determinant of telomerase assembly in G1 phase and further suggest that Est1 stability during G1 phase may play an important role in the regulation of telomerase assembly and/or activity.

There are two major pathways of protein degradation in yeast cells: the lysosome and its associated proteases and the ubiquitin-proteasome system. The proteasome is an important regulator of protein stability and is often involved in cell cycle regulation of protein abundance (reviewed in [235, 236]). Ubiquitin is a 76 amino acid protein moiety that is covalently attached to one or more lysine residues of proteins targeted for protein trafficking, cell signaling, or proteolysis [236]. There are a number of steps involved in marking a protein with ubiquitin. First, ubiquitin is activated by the E1 activating

enzyme. Second, the ubiquitin moiety is transferred to the E2 ubiquitin-conjugating enzyme and finally, an E3 ligase covalently attaches ubiquitin to the targeted lysine residue on the protein of interest. There are two types of ubiquitination: mono-ubiquitylation and poly-ubiquitination. Mono-ubiquitylation is typically involved in cell signaling and protein trafficking, while poly-ubiquitination usually results in the proteasome-dependent degradation of proteins (reviewed in [237]).

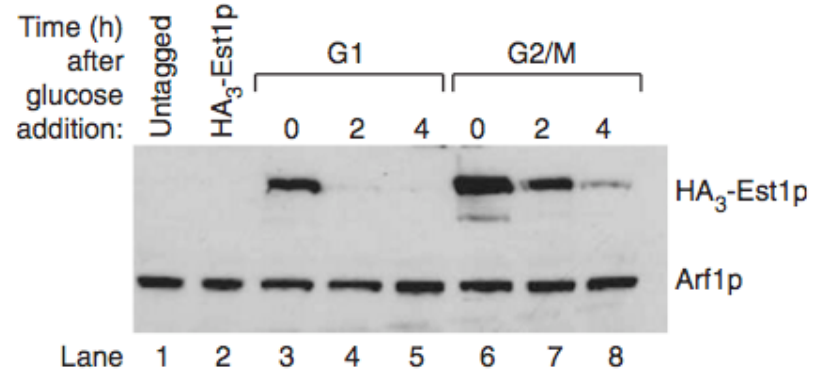
Because Est1 is not at the telomere or associated with the catalytic core in G1 phase and because its protein abundance is extremely low in G1 phase relative to other phases of the cell cycle, I hypothesized that the Est1 protein is specifically degraded in G1 phase of the cell cycle. To test this hypothesis, I monitored the half-life of the Est1 protein in G1-blocked cells. Consistent with a role for protein degradation, the half-life of Est1 during G1 phase is markedly shorter than during G2/M. Finally, I determined that enhanced degradation of the Est1 protein during G1 phase is dependent upon the ubiquitin-proteasome system.

Results

The half-life of Est1 is lower in G1-blocked cells compared to G2/M-blocked cells

To analyze Est1 stability in the cell cycle, I used cells deleted for *EST1* at the endogenous locus and complemented by a plasmid-borne HA_3-*EST1* allele under control of the galactose-inducible *GAL1* promoter. Use of an inducible-promoter system facilitates the determination of protein half-life by allowing gene expression to be turned off at the transcriptional level. Cells were grown to mid-log phase in 0.2% galactose and

A


Time (h) after glucose addition:
Untagged
HA3-Est1p
G1
G2/M
0 2 4 0 2 4
HA3-Est1p
Arf1p
Lane 1 2 3 4 5 6 7 8

B

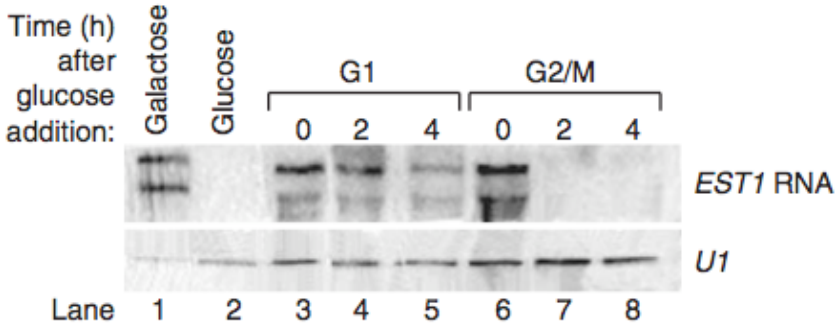

Time (h) after glucose addition:
Galactose
Glucose
G1
G2/M
0 2 4 0 2 4
EST1 RNA
U1
Lane 1 2 3 4 5 6 7 8

Figure 2.1 HA_3-Est1 stability is decreased during G1 phase. (A) The half-life of Est1 is decreased in G1-arrested cells. Cultures of strain YKF332 (*Myc_9-EST2 EST3-HA_3 bar1::URA3*) containing plasmid pVL242 (*GAL1-HA_3-EST1 LEU2*) were arrested with alpha factor (G1; lanes 3-5) or nocodazole (G2/M; lanes 6-8) in medium containing galactose. Glucose was added at time 0 to repress *EST1* transcription. Protein extracts were prepared from cells harvested at the indicated times after glucose addition and analyzed by Western blotting with anti-HA and anti-Arf1p antibodies. Extracts prepared from asynchronous cultures of AVL78 (untagged; lane 1) and TVL288 (*HA_3-EST1*; lane 2) are included as negative controls. Note that expression of endogenous HA_3-Est1 (lane 2) is below the detection limit of this experiment. (B) *EST1* mRNA shows increased stability in G1-arrested cells compared to G2/M-arrested cells. RNA was isolated from the identical extracts shown in (A) (lanes 3-8) and analyzed by Northern blotting with *EST1* and U1 radiolabeled probes (shown in lanes 3-8). RNAs isolated from strain YKF332 (*Myc_9-EST2 EST3-HA_3 bar1::URA3*) containing plasmid pVL242 (*GAL1-HA_3-EST1 LEU2*) grown with (lane 1) or without (lane 2) induction are included as controls. The upper band is of the size expected for *HA_3-EST1* expression. At this exposure, endogenous *EST1* transcript is not detected (lane 2). Results shown in (A) and (B) are representative of two independent experiments.

arrested with alpha factor (G1-block) or nocodazole (G2/M-block). Glucose was added (time zero) to repress *HA$_3$-EST1* expression once greater than 95% of the cells were arrested in the appropriate cell-cycle phase. Markedly, HA$_3$-Est1 is virtually undetectable two hours after the addition of glucose in G1-arrested cells (Figure 2.1a, lanes 3-5), while the Est1 protein persists for at least four hours (the length of the experiment) in G2/M-arrested cells (Figure 2.1a, lanes 6-8). Interestingly, as expected if the protein is rapidly degraded in G1 phase, the steady-state levels of HA$_3$-Est1 were lower at time zero in cells arrested in G1 phase with alpha factor than in those arrested in G2/M with nocodazole (Figure 2.1a, lanes 3 and 6).

Since it had previously been demonstrated that EST1 RNA levels are modestly reduced (~ 3 fold) in G1 phase compared to G2/M phase, I wanted to ensure that the differences in protein levels were not due to differences in the persistence of the EST1 mRNA following inhibition of transcription by glucose addition [192, 234]. Therefore, the identical samples used in Figure 2.1a were monitored for levels of EST1 mRNA by Northern blot. Intriguingly, the EST1 mRNA is more stable in G1-blocked cells than in cells arrested in G2/M, opposite to the trend observed for the HA$_3$-Est1 protein (Figure 2.1b). Together, these data support the hypothesis that the Est1 protein is turned over more rapidly during G1 phase compared to G2/M.

Degradation of Est1 in G1 phase of the cell cycle is dependent upon the proteasome

During the course of analysis, it was discovered that a consensus destruction box sequence (D-box: RxxLxxxxN) was created when the HA-epitope tag and was cloned at the N-terminus of Est1 [238]. D-boxes are known to target proteins to the proteasome in a

cell-cycle dependent manner via recognition by the anaphase promoting complex (APC), an E3 ubiquitin ligase [239]. To eliminate concerns that this sequence was contributing to Est1 protein degradation, I mutated the highly conserved arginine residue to alanine and found no obvious change in Est1 stability using an assay similar to that shown in Figure 2.1 (data not shown). This version of HA_3-Est1 was used in future experiments.

To obtain an accurate estimate the half-life of the Est1 protein and to eliminate any confounding effects of mRNA stability, I modified the above promoter shut-off assay. Cells were initially grown to mid-log phase in media containing 2% raffinose to ensure that the *GAL1* promoter was neither stimulated (galactose) nor repressed (glucose). Cultures were arrested in G1 or G2/M phases as described previously. Once the cells were arrested, 2% galactose was added to induce transcription of *HA_3-EST1* for one hour. After the one-hour incubation, glucose and cycloheximide were added (time zero) to prevent both transcription of EST1 mRNA and new protein synthesis. In G1-arrested cells, HA_3-Est1 has a half-life of 15-30 minutes and is not detected by 60 minutes (Figure 2.2a, lanes 2-7). In contrast, cells blocked in G2/M phase maintain a detectable level of HA_3-Est1 for at least 120 minutes (Figure 2.2a, lanes 9-14). I conclude that Est1 degradation is accelerated during G1 phase when compared to G2/M.

The yeast strain used in these experiments is deficient in vacuolar proteases. Therefore, I hypothesized that the ubiquitin-proteasome system could be responsible for Est1 protein degradation in G1 phase. MG132 is a drug that reversibly binds to and inhibits the active sites in the proteasome, thereby restricting this pathway of protein

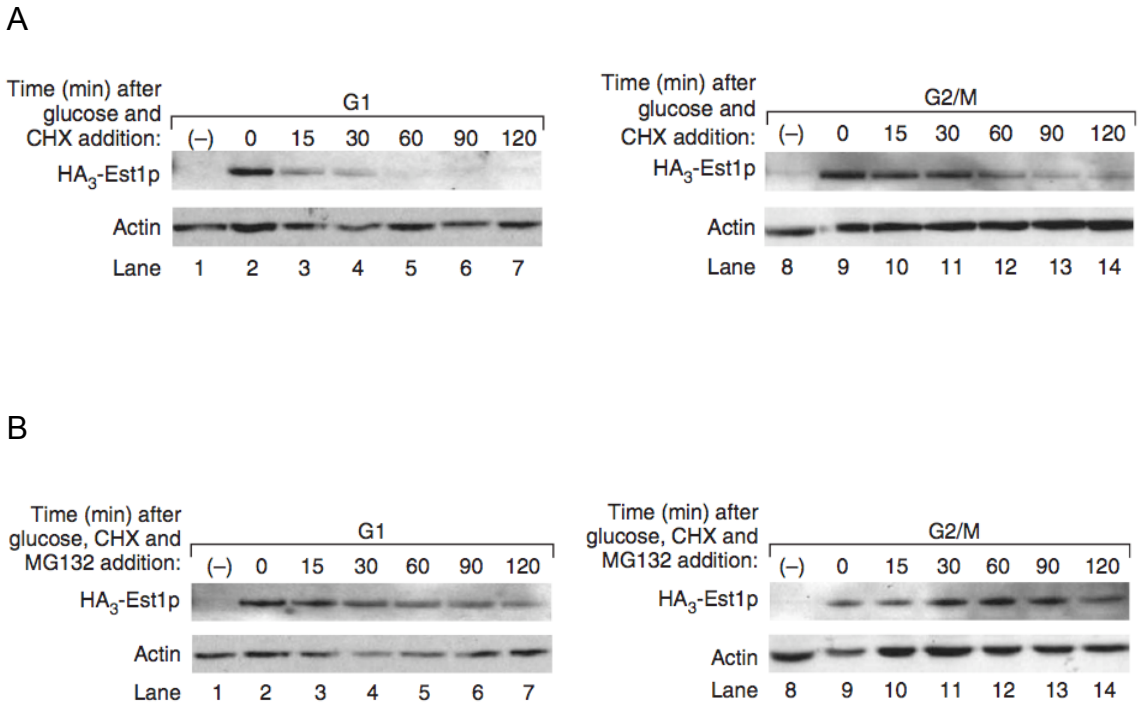


Figure 2.2 Inhibition of the proteasome by MG132 stabilizes HA_3-Est1 in G1.
(A) Half-life of HA_3-Est1 monitored in the presence of cycloheximide. Cultures of strain YKF333 (*Myc$_9$-EST2 EST3-HA$_3$ bar1::URA3 erg6::kanMX6*) containing plasmid pVL242RtoA (*GAL-HA$_3$-EST1RtoA LEU2*) were arrested with alpha factor (G1; lanes 2-7) or nocodazole (G2/M; lanes 9-14). Following galactose induction, glucose and cycloheximide were added (time 0), and cells were harvested at the indicated times. Samples were analyzed by Western blotting with antibodies against the HA epitope tag and actin. Lanes 1 and 8 contain extract from an uninduced control strain. (B) Stabilization of HA-Est1 in G1 phase after the addition of MG132. Identical to (A), except that MG132 was added at time 0 in addition to glucose and cycloheximide. All results shown in this figure are representative of at least three independent experiments.

degradation [240]. To assess the contribution of the proteasome to Est1 protein degradation, the promoter shut-off strategy described above was used. MG132 was added at time zero in addition to glucose and cycloheximide to inhibit the proteasome. Because yeast cell walls are not normally permeable to MG132 (a cation), *ERG6* was deleted to increase cell permeability [240]. Strikingly, addition of MG132 to G1-arrested cells stabilizes HA_3-Est1 abundance out to 120 minutes (Figure 2.2b, lanes 2-7), while treatment of G2/M-arrested cells with MG132 had little effect (Figure 2.2b, lanes 9-14). These results demonstrate that proteasome function is required for HA_3-Est1 degradation during G1 phase of the cell cycle.

Discussion

These data support the hypothesis that Est1 protein levels are reduced during G1 phase due to regulated degradation. How and why the Est1 protein is degraded is unclear. It could be targeted to the proteasome directly by ubiquitination, or alternatively, it might be targeted indirectly. If Est1 is a direct target of the proteasome, it is most likely ubiquitinated. To date our lab has not been able to demonstrate that Est1 is itself ubiquitinated, thus it is possible that the effect on Est1 stability is indirect. For example, a protein that is itself a direct proteasome target during G1 phase may normally be required to stabilize Est1 throughout the cell cycle. I favor the hypothesis that Est1 is targeted to the proteasome by ubiquitination, potentially by the Anaphase Promoting Complex (APC). The APC is a large, cell cycle regulated, E3 ubiquitin ligase that targets specific proteins such as S/M phase cyclins for degradation during a period from anaphase to the following G1 phase [239]. The APC is activated by two separate proteins, Cdc20, an

early APC activator (anaphase to the completion of mitosis), and Cdh1, the G1 activator (reviewed in [241]). Jenifer Ferguson, a graduate student in our lab, has demonstrated that the steady-state levels of endogenous Est1 protein mimic the profile of known APC-target proteins. Furthermore, Est1 is stabilized in G1 phase by a temperature-sensitive mutation of Cdc16, an essential APC component (J. Ferguson, unpublished data). Finally, overexpressed HA_3-Est1 protein becomes stabilized in G1 phase in cells disrupted for APC function (J. Ferguson, unpublished data). Taken together, these data argue strongly that the APC influences Est1 stability. However, Jenifer has been unable to demonstrate that recombinant Est1, made by *in vitro* transcription/translation, is degraded in an *in vitro* APC assay utilizing *Xenopus* egg extract. One interpretation is that the degradation of Est1 is more complex than previously thought (J. Ferguson, unpublished data). Alternatively, since the APC components used in this assay are from the evolutionarily distant *Xenopus lavis* (APC core complex) and human (Cdh1) and not budding yeast, the specificity of the APC/Est1 interaction could be weakened or completely disrupted.

What is the physiological importance of Est1 degradation during G1 phase? One hypothesis is that low levels of Est1 ensure that telomerase remains inactive in G1. Jennifer Osterhage, a previous graduate student in the Friedman lab, demonstrated that stabilization of the endogenous Est1 protein in G1 phase using MG132 resulted in association of both Est1 and Est3 with Est2, an interaction that does not normally occur in G1 phase [192]. This result suggested that Est1 could be the limiting factor for active telomere elongation in G1 phase. However, even though the complex was able to assemble and this "G1-telomerase" was active *in vitro*, it was not competent to extend telomeres *in vivo* [192]. It is not known if the G1-telomerase complex is present at the

telomere, the site of elongation. If it is localized but unable to extend the telomere, then there must be an additional mechanism that restricts telomerase action in G1 phase. Alternatively, if it is not localized to the telomere, then the telomere structure (perhaps an open vs. closed conformation) may be critical for the regulation of telomerase action. No matter what the answer may be regarding the location of the fully complexed telomerase in G1 phase, the fact that Est1 stabilization in G1 is insufficient for telomere elongation suggests that there are other factors or mechanisms restricting telomerase activity to late S/G2 phase.

CHAPTER III

THE EST3 PROTEIN BINDS DIRECTLY TO AN ESSENTIAL N-TERMINAL DOMAIN OF THE CATALYTIC SUBUNIT OF TELOMERASE (EST2)[2]

Introduction

Telomeres are protein-DNA complexes that protect chromosome termini from nucleolytic digestion and distinguish natural chromosome ends from internal DNA breaks. While the majority of telomeric DNA is double stranded, the G/T-rich strand forms a protruding 3'-overhang. In the absence of a counteracting mechanism, telomeres shorten during each cell division, ultimately activating cell-cycle checkpoints and cellular senescence [165]. If these checkpoints are disrupted or bypassed, end-to-end fusions and bridge-breakage cycles can ensue. Telomerase, a ribonucleoprotein complex, promotes telomere maintenance and genomic stability by elongating the 3'-overhang by reverse transcription [84].

In budding yeast, telomerase consists minimally of four dedicated subunits: TLC1 RNA, the template RNA [90]; Est1, an accessory protein important for recruiting and activating telomerase at the telomere [188, 224]; Est2, the reverse transcriptase [84]; and Est3, an additional accessory protein necessary for proper activity *in vivo* [213]. Deletion of any of these components eliminates telomerase function *in vivo*, yielding the EST (**E**ver **S**horter **T**elomere) phenotype [90, 209, 210].

[2] The work presented in this chapter are published in: [242] Talley, J.M., et al., *Stimulation of yeast telomerase activity by the Ever Shorter Telomeres 3 (Est3) subunit is dependent on direct interaction with the catalytic protein Est2.* J Biol Chem, 2011. **In Press**.

Although these and other telomerase components have been known for well over a decade, few details of their assembly into the ribonucleoprotein complex are understood. Described interactions are largely confined to protein associations with the RNA template. Est1 and Est2 independently bind distinct regions within the central portion of TLC1 RNA [220, 222, 243]. Sm proteins facilitate RNA stability through interaction with a site near the 3' end of the RNA and association of the catalytic core of telomerase with the telomere in G1 phase is mediated by interaction of the yKu heterodimer with TLC1 RNA [191, 219]. Protein-protein interactions are less well understood. Est1 and Est2 may interact in an RNA-independent manner. When Est1 is tethered to an internal chromosomal site via fusion with the Gal4 DNA binding domain, Est2 is recruited to that site in the absence of TLC1 RNA [244]. Furthermore, recombinant Est1 and Est2 can bind directly, although TLC1 RNA enhances this interaction [224].

Both the function of Est3 and the mechanism of its assembly into the telomerase complex are unknown. Est1 stimulates the association of Est3 with telomerase in a cell-cycle dependent manner that requires Est2 [192]. Very recently, Est1 protein purified from yeast was shown to bind recombinant Est3 protein, yielding strong evidence for a direct interaction between these two proteins [193]. Genetic evidence suggests that Est3 interacts with an N-terminal (Telomerase Essential N-terminus: TEN) domain of Est2, but no direct protein/protein or protein/RNA interactions have been reported [213, 245, 246]. It has been suggested that Est3 binds nucleic acid and possesses helicase activity [228]. However, sensitive analysis by NMR spectroscopy of Est3 in the presence and absence of DNA failed to detect an interaction [246].

Here I show the first biochemical evidence that Est3 binds directly to the TEN domain of Est2 in *Saccharomyces cerevisiae* and additionally reveal amino acids within Est3 that are important for this interaction both *in vitro* and *in vivo*.

Results

Est2^{TEN} and Est3 interact directly *in vitro*

The previously reported allele-specific suppression of temperature-sensitive (ts) mutations within the Est2 TEN domain by over-production of Est3 [245] may reflect a direct interaction between the two proteins. To test this hypothesis, *EST3* was fused with an N-terminal *His$_6$* tag and the TEN domain of *EST2* (*EST2^{TEN}*; residues 1-162) was fused to the C-terminus of maltose binding protein (Mbp). Tagged proteins were individually expressed in *Escherichia coli* and purified to greater than 95% apparent homogeneity (Figure 3.1). An ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) experiment was used to assess His$_6$-Est3 tertiary structure. If the protein were unfolded, there would be a collapse of crosspeaks in the region near 8 ppm. Instead, the HSQC of Est3 revealed excellent spectral dispersion with backbone amide peaks that range from ~6.1 to 9.9 ppm in the proton dimension (Figure 3.2).

The individually purified proteins were mixed and their co-purification was monitored. His$_6$-Est3 did not detectably associate with amylose resin alone (Figure 3.3c, lane 6) or when co-incubated with Mbp (Figure 3.3a, lane 7). In contrast, His$_6$-Est3 robustly co-purified with Mbp-Est2^{TEN} (Figure 3.3a, lane 10), indicating that His$_6$-Est3 and Mbp-Est2^{TEN} bind directly *in vitro*.

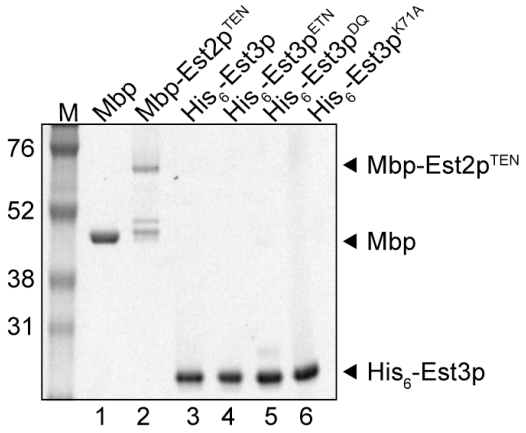


Figure 3.1 Coomassie-stained protein gel of recombinant proteins. Mbp-Est2^{TEN} and His$_6$-Est3 were individually expressed and purified from *E.coli*. Marker sizes (M) are shown in Kilo Daltons (KDa). Mbp, Mbp-Est2^{TEN} and His$_6$-Est3 are 42KDa, 62KDa, and 21KDa, respectively. The proteins were estimated to be >95% pure. Mbp was purchased from New England Biolabs.

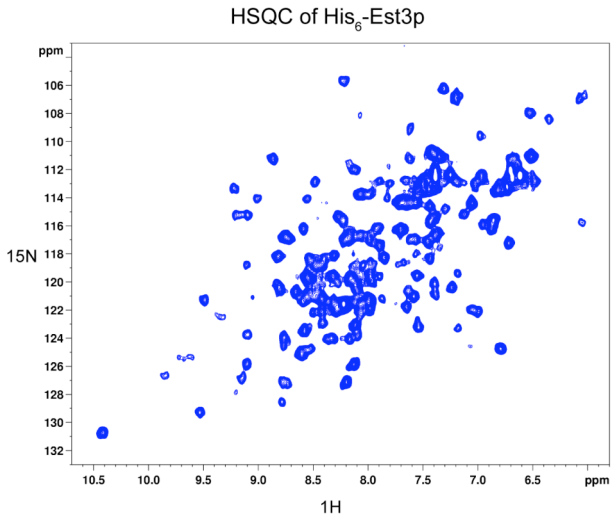


Figure 3.2 ^{1}H-^{15}N Heteronuclear Single Quantum Correlation (HSQC) of His$_6$-Est3 purified from *E. coli.* NMR spectra were recorded at 15°C on a 500 MHz Bruker Avance spectrometer equipped with cryoprobe. Data were processed and visualized using Topspin 2.1 software. The intensity of the crosspeaks and relatively robust spectral dispersion suggest that Est3 retains tertiary structure.

To examine the specificity of the interaction between Est3 and Est2^{TEN}, I created and characterized *EST3* mutants known or suspected to influence telomerase assembly and/or function. Based on structural modeling, these mutations are predicted to affect surface-exposed residues and to have minor effects on protein stability [227]. Est3^{K71A} (lysine 71 mutated to alanine) has been shown to disrupt telomere maintenance but retain assembly with telomerase *in vivo*, as assessed by co-purification of the mutant protein with TLC1 RNA [227]. In contrast, individual mutations in residues glutamate 114, threonine 115 or asparagine 117 were shown to shorten telomeres and reduce Est3 association with TLC1 RNA [227]. To completely disrupt this charged region, I simultaneously mutated all three residues to create Est3^{ETN114,115,117AAK} (Est3^{ETN}). Aspartate 166 also contributes to the association of Est3 with TLC1 RNA [227]. Because glutamine 167 is conserved in related fungal species, I mutated both residues to create Est3^{DQ166,167AA} (Est3^{DQ}).

These mutant proteins were purified as His_6 fusion proteins from *E. coli* and their secondary structure characterized by circular dichroism. Both the wild-type Est3 and each of the altered Est3 proteins displayed strong evidence of secondary structure, suggesting that the amino acid changes did not strongly perturb protein folding (Figure 3.4). Compared with the interaction observed for wild-type His_6-Est3, co-purification of His_6-Est3^{ETN} with Mbp-Est2^{TEN} was greatly decreased (Figure 3.3a and b; 17 fold, p=0.0007). In contrast, the interaction between recombinant Mbp-Est2^{TEN} and His_6-Est3^{DQ} was only slightly decreased compared to wild type (Figure 3.3a and b; 1.5 fold, p=0.0002). The identification of point mutations that disrupt the Est3-Est2^{TEN} interaction *in vitro* suggests that the association between these two proteins is both direct

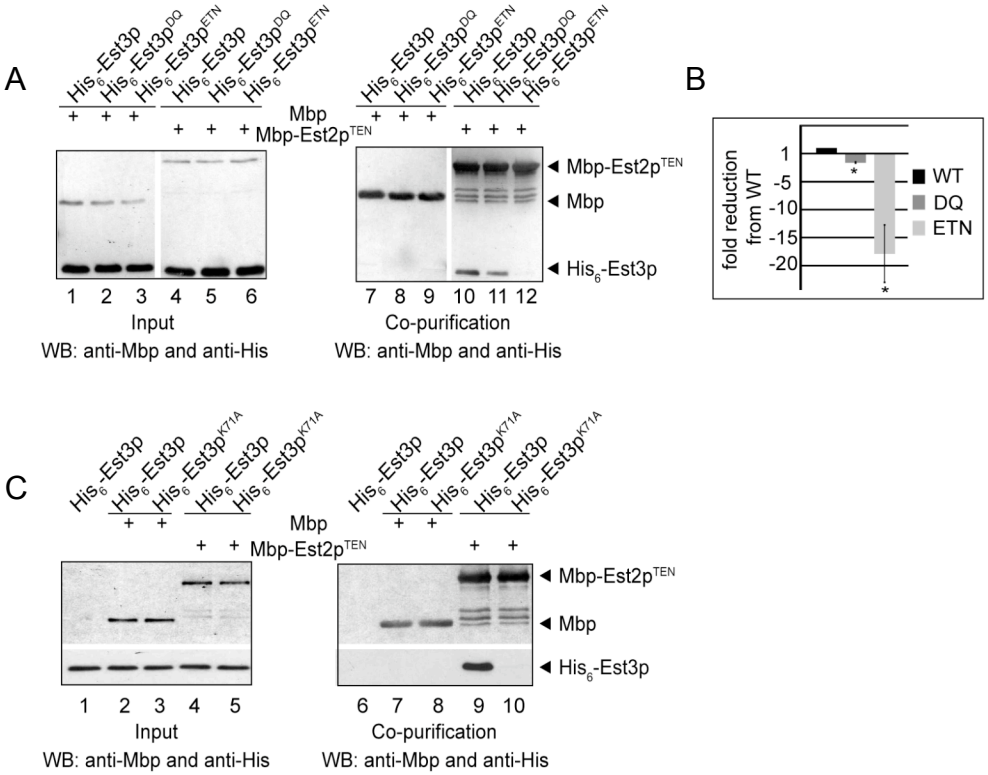


Figure 3.3 His_6-Est3 interacts directly with Mbp-$Est2^{TEN}$. (A) 200 pmol Mbp or Mbp-$Est2^{TEN}$ were incubated as indicated (+) with 1 nmol His_6-Est3, His_6-$Est3^{DQ}$ or His_6-$Est3^{ETN}$ and captured on amylose resin. Input (1% of total; left panel) and amylose-bound proteins (right panel) were analyzed by Western blotting using anti-Mbp and anti-His antibodies. Data shown are representative of four independent experiments. (B) Quantification of data shown in (A). Fold reduction in recovery of His_6-$Est3^{DQ}$ and His_6-$Est3^{ETN}$ compared to WT was averaged over four independent experiments. Bars are standard error. Both His_6-$Est3^{DQ}$ and His_6-$Est3^{ETN}$ binding are statistically different from His_6-Est3 by one-tailed paired t-test (p-values 0.0002 and 0.0007, respectively) as denoted by *. (C) 200 pmol Mbp or Mbp-$Est2^{TEN}$ were incubated as indicated (+) with 1 nmol His_6-Est3 or His_6-$Est3^{K71A}$ and captured on amylose resin. Input (1% of total; left panel) and amylose-bound proteins (right panel) were analyzed by Western blotting using anti-Mbp and anti-His antibodies. Data shown are representative of three independent experiments.

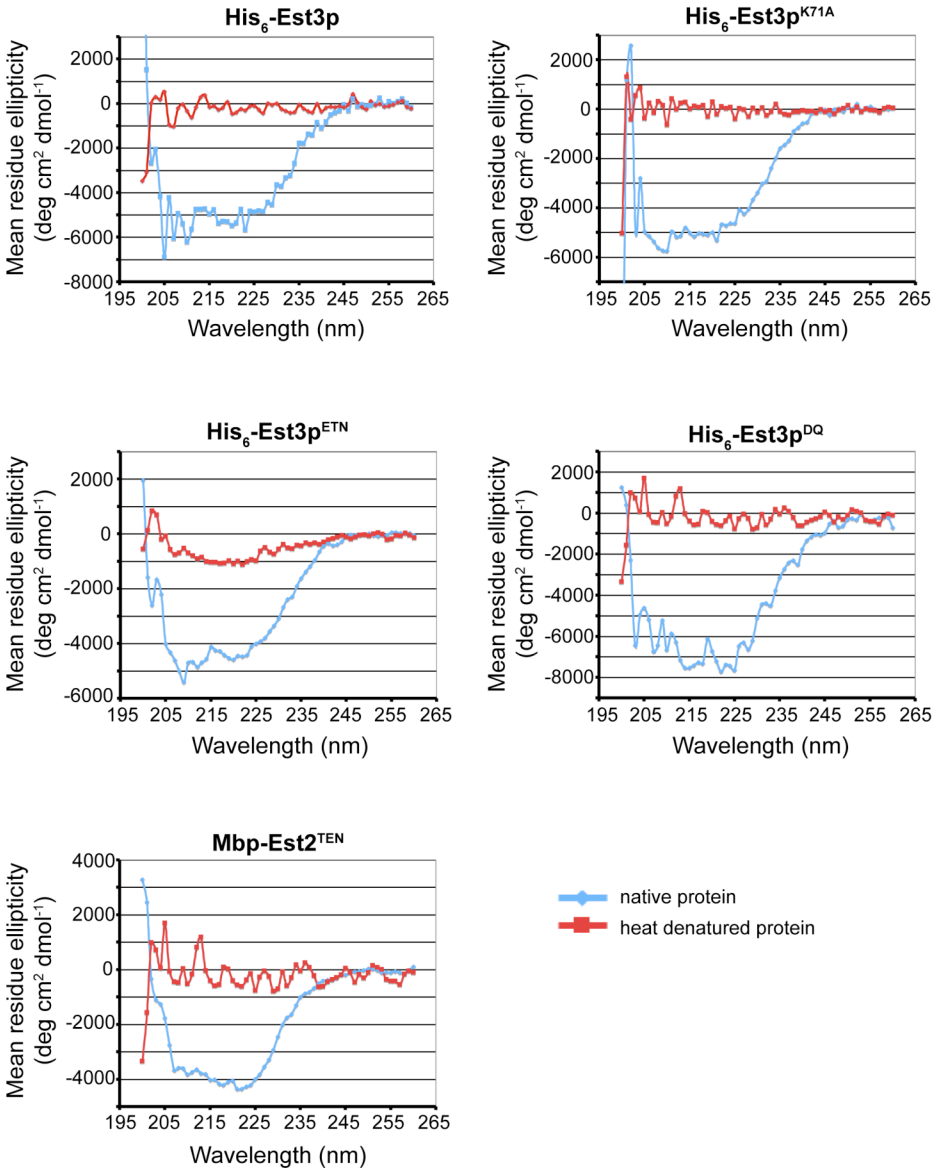


Figure 3.4 Circular Dichroism (CD) of His_6-Est3 proteins and Mbp-$Est2^{TEN}$. Circular dichroism (CD) was used to determine if the recombinant proteins used in this study retained substantial secondary structure characteristics. Secondary structural elements such as α-helix, β-sheet, and random coil exhibit distinctive CD signals in the far-UV region (180nm-260nm), while unfolded proteins do not. Circular dichroism spectrum of each native protein was collected at 20°C, shown in blue. The temperature of the identical protein sample was gradually increased from 20°C to 80°C over the course of an hour. The sample was returned to 20°C after heat denaturation and an additional CD spectrum was collected, shown in red. The difference between the spectra is consistent with a folded protein.

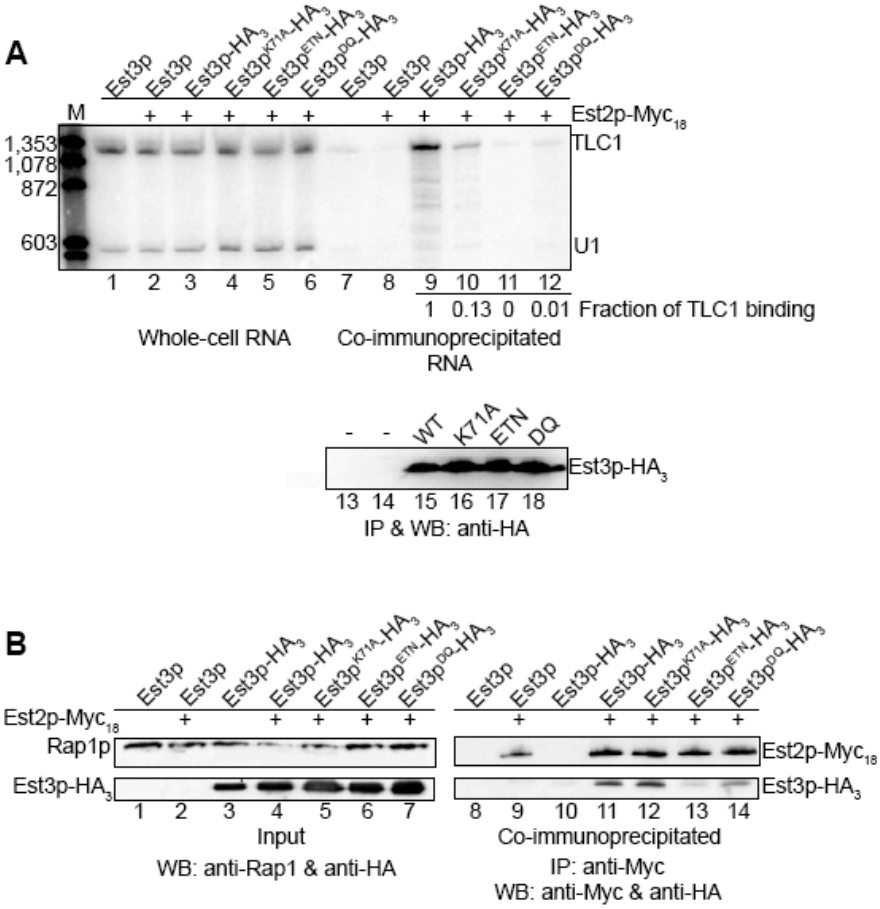
and specific. Furthermore, residues near E114 contribute to the association of Est3 with the TEN domain of Est2.

Surprisingly, no association between His_6-$Est3^{K71A}$ and Mbp-$Est2^{TEN}$ could be detected in the pull-down assay (Figure 3.3c, lane 10), even though this mutant can assemble with the telomerase complex *in vivo* ([227] and Figure 3.5). This result may suggest that multiple interactions are required for the association of Est3 with $Est2^{TEN}$ *in vitro*, but that additional redundancy exists *in vivo*. However, I cannot dismiss the possibility that mutation of lysine 71 to alanine disrupts the integrity of the recombinant protein in a manner not detected by circular dichroism.

EST3 mutant alleles alter telomerase assembly and function *in vivo*

Since the $est3^{ETN}$ and $est3^{DQ}$ alleles evaluated in this study are different from those previously investigated, I characterized their *in vivo* phenotype. Telomerase assembly was monitored by co-immunoprecipitation of HA-tagged Est3 with TLC1 RNA and with Myc_{18}-tagged Est2 (Figure 3.5a and b). *EST2* was epitope-tagged at its N-terminus by two-step integration of eighteen copies of the Myc epitope at the endogenous locus. To avoid complications that might arise from measuring telomerase assembly in senescent strains, the epitope-tagged *EST3* variants were expressed from a plasmid in the presence of the endogenous, untagged *EST3* gene. Previous work has shown that the untagged Est3 protein does not appreciably interfere with the ability of epitope-tagged Est3 to co-immunoprecipitate with its binding partners [227].

I first verified that TLC1 RNA levels are not affected by the *EST3* mutations. As shown in Figure 3.5a (lanes 1-6), TLC1 RNA levels detected by Northern blot from total


A
Est3p
Est3p
Est3p-HA3
Est3pK71A-HA3
Est3pETN-HA3
Est3pDQ-HA3
Est3p
Est3p
Est3p-HA3
Est3pK71A-HA3
Est3pETN-HA3
Est3pDQ-HA3
M
Est2p-Myc18
1,353
1,078
872
603
TLC1
U1
1 2 3 4 5 6 7 8 9 10 11 12
1 0.13 0 0.01 Fraction of TLC1 binding
Whole-cell RNA
Co-immunoprecipitated RNA
- - WT K71A ETN DQ
Est3p-HA3
13 14 15 16 17 18
IP & WB: anti-HA

B
Est3p
Est3p
Est3p-HA3
Est3p-HA3
Est3pK71A-HA3
Est3pETN-HA3
Est3pDQ-HA3
Est2p-Myc18
Rap1p
Est3p-HA3
1 2 3 4 5 6 7
Input
WB: anti-Rap1 & anti-HA
Est2p-Myc18
Est3p-HA3
8 9 10 11 12 13 14
Co-immunoprecipitated
IP: anti-Myc
WB: anti-Myc & anti-HA

Figure 3.5 *EST3^{ETN}* reduces association with telomerase *in vivo*. (A) Whole-cell RNA (lanes 1-6) or anti-HA immunoprecipitations (lanes 7-12) were generated from yeast strains AVL78 (untagged; lanes 1 and 7) or YKF126 (*EST2-MYC$_{18}$ est3::KAN^{R}*) with pKF441 (*CEN EST3 URA3*) and pKF448^{HA} (*CEN EST3-HA$_3$ LEU2*), or pKF449^{HA} (2 µm *est3$^{K71A, ETN}$* or *^{DQ}-HA$_3$ LEU2*) as indicated. TLC1 and U1 RNA were detected by Northern blot (top panel); (M) is marker in base pairs (bp). The amount of TLC1 retained in each co-immunoprecipitation (normalized to the wild-type value) is notated under lanes 9-12. This value was determined by first subtracting the amount of TLC1 bound non-specifically (untagged Est3; lane 8) from the amount of TLC1 observed in each lane and then dividing by the wild-type value (lane 9). Est3-HA$_3$ recovery was measured by Western blot (bottom panel; lanes 13-18) in the identical immunoprecipitation. Results are representative of two independent biological replicates. (B) Protein extracts were isolated from strains AVL78 (lanes 1 & 8), YKF126 containing plasmid pKF441 (lanes 2 & 9), YKF122 (*est3::KAN^{R}*) containing pKF442^{HA} (*CEN EST3-HA$_3$ URA3*; lanes 3 & 10), or YKF126 containing plasmids pKF441 and pKF448^{HA} or pKF449^{HA} mutants, as indicated. Anti-Myc immunoprecipitations were analyzed by Western blot with monoclonal anti-Myc and anti-HA antibodies (lanes 8-14). Input (1% of total) was probed with anti-HA and anti-Rap1p antibodies as a loading control (lanes 1-7). Results are representative of two biologically independent experiments.

cellular RNA are equivalent across strains. Est3 was immunoprecipitated from whole-cell extract and an aliquot was analyzed by Western blot (Figure 3.5a, bottom panel). Each of the mutant proteins was purified at a level equivalent to or slightly higher than wild-type Est3 (mutants are expressed from a high copy number 2 um plasmid). TLC1 RNA was detected by Northern blot in the identical immunoprecipitates (Figure 3.5a, lanes 7-12). After correcting for minor nonspecific recovery of TLC1 RNA from control extract lacking the HA tag on Est3 (Figure 3.5a, lane 8), the amount of TLC1 RNA immunoprecipitated with each mutant protein was expressed as a fraction of the association observed for wild-type Est3. Highly congruent results were obtained in two independent biological replicates (Figure 3.5a and data not shown). $Est3^{ETN}$-HA_3 and $Est3^{DQ}$-HA_3 showed very little if any residual association with TLC1, in agreement with the effects previously observed for the corresponding single amino acid mutations in *EST3*. $Est3^{K71A}$-HA_3 consistently retained higher levels of association with TLC1 than the other mutants, but was reduced to approximately 10% of the wild-type level (Figure 3.5a).

As an alternate measure of telomerase complex assembly, I monitored the co-immunoprecipitation of each Est3 variant with Myc_{18}-Est2. In agreement with the effects on TLC1 association, $Est3^{ETN}$-HA_3 and $Est3^{DQ}$-HA_3 were reduced in their ability to co-immunoprecipitate with Myc_{18}-Est2 (Fig. 2B). Interestingly, $Est3^{K71A}$-HA_3 immunoprecipitated with Myc_{18}-Est2 at a similar level to wild type. The discordance between the ability of $Est3^{K71A}$-HA_3 to immunoprecipitate with TLC1 and Myc_{18}-Est2 suggests that the interaction between TLC1 RNA and Est3 (likely indirect) is more easily disrupted by the wash conditions than the interaction between Est2 and Est3. In

agreement with previous results [227], I conclude that the residues near glutamate 114 and aspartate 166 are important for Est3 assembly with telomerase, whereas residue lysine 71 plays a less critical role *in vivo*.

est3^{ETN} does not complement an *EST3* delete strain

To address the ability of the *EST3* variants to support telomere replication, each allele was expressed from a low-copy number centromere plasmid in yeast lacking endogenous *EST3*. The resulting strains were monitored for the ability to support growth over three consecutive restreaks on solid medium. As expected, the yeast expressing wild-type *EST3* maintained robust cellular growth (Figure 3.6a), while the empty vector control senesced after three restreaks. Interestingly, the strain expressing *est3*^{ETN} resembled the empty vector control for growth (Figure 3.6a), while *est3*^{K71A} and *est3*^{DQ} looked similar to wild type. As a more sensitive measure of telomerase function, the telomere length of cells taken from the final restreak was measured by Southern blot. *Xho*I releases a terminal fragment from chromosomes containing one or more subtelomeric Y' elements, generating a heterogeneous DNA band of 1.2 to 1.3 kilobases. In comparison with the telomere length of cells expressing wild-type *EST3*, cells containing either the empty vector control or expressing *est3*^{ETN} displayed severe telomere shortening (Figure 3.6b, compare lanes 1, 6 and 7). In the absence of telomerase function, these cells were unable to maintain telomeres and instead utilized a *RAD52*-dependent recombination pathway to maintain telomeres, manifested on the Southern blot as stochastic lengthening of the TG-rich telomeric repeats or amplification of Y` elements located internal to some telomeres (brackets, Figure 3.6b) [247].

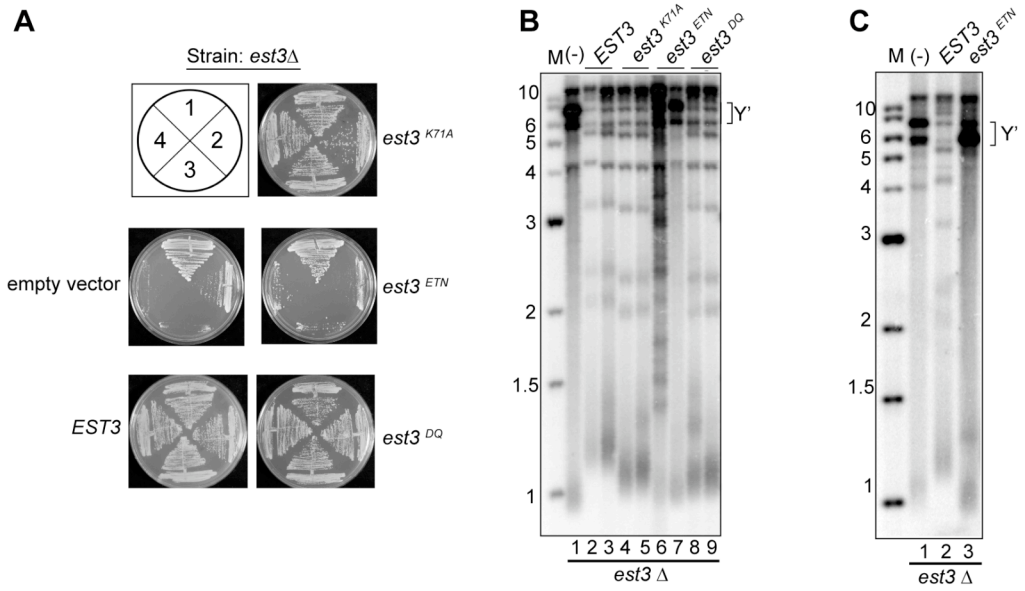


Figure 3.6 *est3*^{ETN} does not complement an *est3*Δ strain even when overexpressed. (A) Yeast strain YKF122 complemented with pKF441 was transformed with pRS315 (*CEN LEU2* empty vector), pKF448 (*CEN EST3 LEU2*) or pKF448 mutants (*CEN est3*$^{K71A,\ ETN\ \text{or}\ DQ}$ *LEU2*) and loss of the *URA3* complementing plasmid was selected on plates containing 5-FOA. Cells were restreaked four times; numbers represent restreaks following loss of the complementing plasmid. (B) DNA was extracted from cells grown in liquid culture from the 3rd restreak of strains shown in (A). DNA was digested with *XhoI*, Southern blotted, and probed with a randomly labeled telomeric probe. (-) indicates the empty vector control. Y'-elements are bracketed. Two independent transformants are shown. (M) is marker in kilobases. (C) YKF122 (*est3::KAN*^{R}) complemented with pKF441 (*CEN EST3 URA3*) was transformed with pRS425 alone (2 μm *LEU2*), lane 1; pKF449 (2 μm *EST3 LEU2*), lane 2 or pKF449^{ETN}, lane 3. After selection for loss of the complementing plasmid on plates containing 5-FOA, cells were restreaked three times on plates lacking leucine (–leu). Telomere blots were performed as described in (B). Y'-elements are bracketed.

Expression of *est3*^{ETN} from a high-copy number 2 um plasmid allowed the protein to accumulate at wild-type levels (Figure 3.5b compare lanes 4 and 6) but did not improve complementation for either growth (data not shown) or telomere length (Figure 3.6c), indicating that low protein levels alone do not account for the lack of telomere maintenance.

Although both *est3*^{DQ} and *est3*^{K71A} supported normal cellular growth (Figure 3.6a), the telomeres in cells expressing these *EST3* alleles were maintained at a length shorter than wild type (Figure 3.6b), similar to a previous report [227]. All of the *est3* alleles were dominant-negative when overexpressed in the presence of wild-type Est3, suggesting that they are expressed and retain some function (Figure 3.7). Together, these data reveal that residues E114, T115, and N117 are critically important for telomerase assembly both *in vivo* and *in vitro*, while D166 and Q167 appear to have a lesser role *in vitro*. As previously described [227], K71 has a modest influence on telomerase assembly *in vivo*, though mutation of this residue to alanine disrupts the interaction between Est3 and the TEN domain of Est2 *in vitro*.

Discussion

Although the major components of yeast telomerase have been known for more than a decade, it has been difficult to determine the details of subunit interactions within the complex. Here I provide the first evidence of a direct Est2/Est3 interaction. Recombinant Est3 binds the purified N-terminal (TEN) domain of Est2 *in vitro* and this interaction is largely dependent upon several predicted surface residues of Est3 including residues glutamate 114, threonine 115 and asparagine 117. Our lab previously

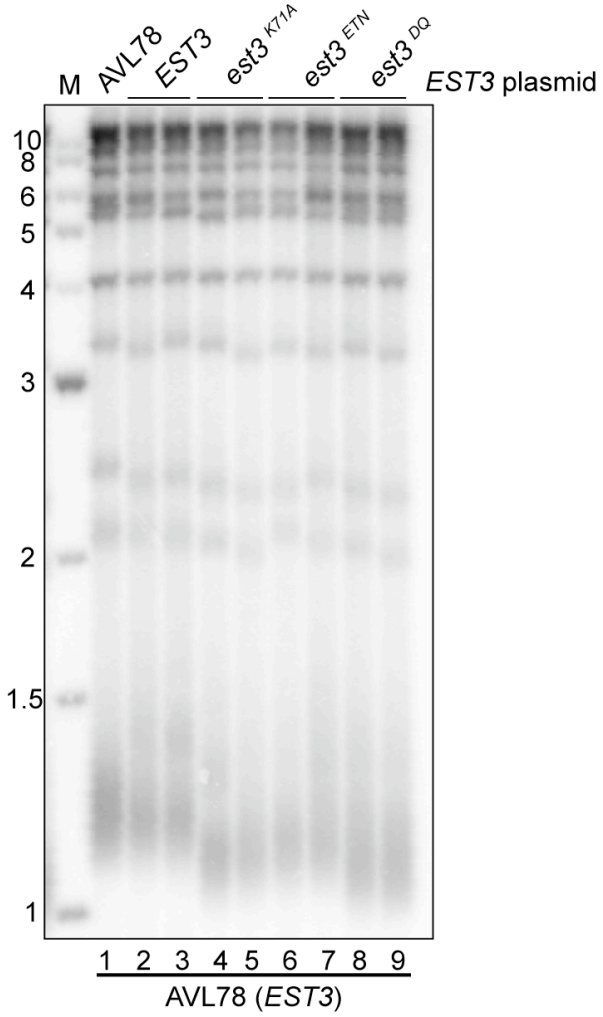


Figure 3.7 All of the *EST3* alleles are dominant negative. AVL78 was transformed with pKF449 (2 μm *EST3 LEU2*) expressing the indicated *EST3* alleles. After three successive restreaks –leu, genomic DNA was digested with *XhoI,* blotted, and probed with a randomly labeled telomeric probe. (M) is marker in kilobases. Two biological replicates are shown. The parental wild-type strain (AVL78) is shown for reference.

showed that Est1 stimulates the assembly of Est3 with telomerase [192]. However, our failure to detect an Est1/Est3 interaction in the absence of Est2 suggested that Est1 might not be the primary binding site for Est3. Additionally, *EST1* function is bypassed in a strain expressing a Cdc13-Est2 fusion protein, while *EST3* remains essential [188]. This result implies that Est3 can, in some circumstances, assemble and contribute to telomerase function in the absence of Est1. Prior to work presented here, genetic results revealed that overexpression of Est3 suppresses mutations located in the Est2 TEN domain, but evidence for a direct interaction between these components was lacking [245].

Here I have combined an *in vitro* co-purification assay using recombinant proteins with experiments that assess both RNA and protein interactions in the native telomerase complex to gain a more clear understanding of telomerase complex assembly. Using these assays I have demonstrated that the Est2 TEN domain (residues 1 to 161) interacts directly with Est3 *in vitro* (Figure 3.3). Mutation of three predicted surface-exposed residues (E114, T115, and N117) of Est3 significantly perturbs this interaction *in vitro*, while also disrupting the assembly of Est3 with telomerase *in vivo* (Figures 3.3 and 3.5).

Interestingly, the *est3*^{DQ} mutation decreases the co-immunoprecipitation of Est3 with both TLC1 RNA and Myc$_{18}$-Est2 *in vivo* (Figure 3.5), but has a fairly minor (less than two-fold) effect on Est2 TEN binding *in vitro* (Figure 3.3a and b). One interpretation of these data is that the interaction between Est3 and Est2^{TEN} is insufficient for the assembly of Est3 with the telomerase complex *in vivo*, consistent with a model in which Est3 makes multiple contacts with telomerase components. This proposal is consistent with the recent report that Est3 interacts directly with Est1 [193]. However, because

telomere length is only moderately affected by the DQ mutation, it is also possible that the co-immunoprecipitation experiment overemphasizes the defect of Est3 association *in vivo*. Indeed, even though the general trends are the same for $Est3^{ETN}$-HA_3 and $Est3^{DQ}$-HA_3, each mutation has an apparently more severe effect on TLC1 RNA association than on the ability to co-immunoprecipitate with Myc_{18}-Est2 (compare Figure 3.5a, lanes 11 and 12 to Figure 3.5b, lanes 13 and 14), suggesting that TLC1 RNA might be more sensitive to co-immunoprecipitation conditions than Est2. This effect is even more pronounced with the $Est3^{K71A}$-HA_3 mutation. While the co-immunoprecipitation of TLC1 is reduced to approximately 10% of wild-type levels, the association of $Est3^{K71A}$-HA_3 with Myc_{18}-Est2 is equivalent to that of wild-type Est3. This difference may arise because Est3 and Est2 interact directly, while Est3 and TLC1 do not.

Although $Est3^{K71A}$-HA_3 and Myc_{18}-Est2 co-immunoprecipitate from cellular extract, the *est3*^{K71A} mutation abolishes the *in vitro* interaction between Mbp-$Est2^{TEN}$ and His_6-Est3 (Figure 3.3c). This result may suggest that a second site of interaction between Est3 and telomerase, such as the interaction with Est1 [193], can mediate Est3 assembly when the TEN domain interaction is compromised. If true, the *est3*^{ETN} mutation must disrupt both contacts. However, I cannot eliminate the possibility that the $Est3^{K71A}$ protein has defects *in vitro* (such as a minor disruption in tertiary structure) that are not reflected *in vivo*.

CHAPTER IV

THE EST3 PROTEIN INFLUENCES TELOMERASE ACTIVITY *IN VITRO* IN *SACCHAROMYCES CEREVISIAE*[3]

Introduction

The function of Est3 in *Saccharomyces cerevisiae* is unknown, even though it was identified in a genetic screen fifteen years ago. Until recently there were no clues to any structural or functional homologues in higher eukaryotes. Based on modeling algorithms, both the Lundblad and Lue groups published in 2008 that the Est3 protein from *S. cerevisiae* and *Candida albicans*, respectively, may be structurally similar to the characterized oligosaccharide/oligonucleotide-binding fold domain (OB fold) of TPP1, a telomere binding protein in mammals [37, 226, 227]. Interestingly, mutations in Est3 that were predicted to disrupt tertiary folding resulted in dramatic decreases in Est3 protein abundance while mutations in predicted surface residues disrupted telomerase function and or assembly *in vivo*, suggesting that their structural models were functionally relevant [226, 227].

One role of TPP1 at the telomere is to positively affect telomerase processivity through an interaction with POT1 [33]. Additionally, although Est3 has been reported to be dispensable for the catalytic activity of *S. cerevisiae* telomerase [211], recent work in a related species, *Saccharomyces castelli*, implicates the Est3 homologue in the stimulation

[3] Work from this chapter (Figure 4.1) has been published in: [242] Talley, J.M., et al., *Stimulation of yeast telomerase activity by the Ever Shorter Telomeres 3 (Est3) subunit is dependent on direct interaction with the catalytic protein Est2.* J Biol Chem, 2011. **In Press**.

of nucleotide addition processivity [246]. Moreover, Est3 from *C. albicans* is required for robust telomerase activity *in vitro* with specific primers [248]. These data raise the possibility that Est3 affects the enzymatic activity in *S. cerevisiae*, but that technical limitations have, to date, precluded the detection of such effects.

I show that recombinant Est3 stimulates telomerase activity during *in vitro* primer extension and that telomerase immunopurified from yeast extracts lacking *EST3* or containing an *est3* mutant allele ($est3^{ETN}$) are modestly reduced in overall telomerase activity. These data suggest that Est3 from *S. cerevisiae*, like its closely related fungal homologues, can stimulate telomerase function. This observation further strengthens the similarities between Est3 and its hypothesized TPP1 homologue. Interestingly, the interaction of Est3 with the Est2 TEN domain appears to be required for this function both in the *in vitro* and *in vivo*.

Results

Est3 stimulates telomerase activity *in vitro*

The function of Est3 in *S. cerevisiae* has remained elusive since its discovery as a telomerase complex component [213]. While early studies did not detect an obvious effect on the catalytic activity of telomerase upon *EST3* deletion in *S. cerevisiae* [211], more recent studies of *S. castelli* and *C. albicans* have demonstrated roles for these Est3 homologues during primer extension *in vitro* [246, 248]. These observations raise the possibility that *S. cerevisiae* Est3 may have a similar function, but that the particular assay conditions utilized either minimize or mask that effect.

To address this issue, I collaborated with Brian Freeman's lab at the University of Illinois to measure the ability of recombinant Est3 to stimulate the primer extension activity of partially purified yeast telomerase. It is thought that proteins that stimulate telomerase activity above basal levels (like Est1; ref. 10) may induce a more functional confirmation for the telomerase complex in the primer extension assay, thereby increasing the overall activity on the enzyme (B. Freeman, personal communication). The Freeman lab has extensive knowledge in assaying the effects of exogenous protein factors in an *in vitro* telomerase activity assay [224, 249-251]. Diane DeZwaan performed the experiments in Figure 4.1 with recombinant protein that I generated at Vanderbilt University. Briefly, a biotin-labeled yeast telomeric primer was bound to streptavidin beads and incubated in the presence of radio-labeled nucleotides and wild-type telomerase extract that was partially purified over DEAE and Mono Q resins [224, 250]. As previously demonstrated (ref. 10), yeast telomerase adds seven nucleotides (5'-GGTGTGG) to the end of the primer and then terminates elongation. Strikingly, titration of His_6-Est3 into this reaction increased overall telomerase activity in a dose-dependent manner, whereas a bovine serum albumin (BSA) control did not (Figure 4.1a). Importantly, Est3 does not appear to affect the processivity of telomerase in this assay since neither the maximal length of the extended product nor the relative intensity of each band is altered. I conclude that Est3 (like Est1; ref. 9) stimulates telomerase activity by increasing the fraction of extended primers.

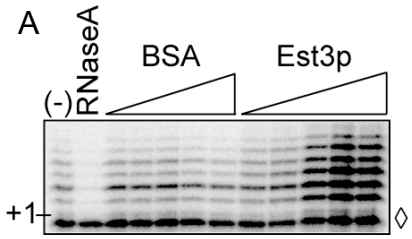
Having established that recombinant Est3 stimulates primer extension by telomerase, I next determined the effect of each previously characterized (Chapter III) mutant Est3 protein in this assay. Both $Est3^{K71A}$ and $Est3^{ETN}$ were significantly reduced

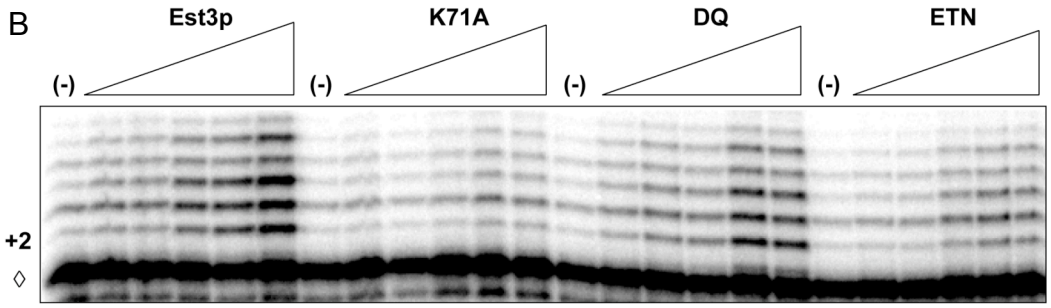
in their ability to stimulate telomerase activity compared with wild-type Est3 (Figure 4.1b and c; p = 0.0018 and 0.03, respectively). Because the association of $Est3^{K71A}$ and $Est3^{ETN}$ with the Est2 TEN domain was dramatically reduced *in vitro* (Chapter III), this result suggests that the interaction between Est3 and $Est2^{TEN}$ is required for telomerase stimulation. Consistent with its ability to bind $Est2^{TEN}$ *in vitro*, $Est3^{DQ}$ retained the capacity to stimulate telomerase (Figure 4.1b and c; p=0.87). Given the reduced assembly and activity of this mutant *in vivo*, this result suggests that $Est3^{DQ}$ affects a different, yet uncharacterized function of Est3. Overall, these data show that *S. cerevisiae* Est3 can stimulate telomerase activity in a manner dependent on direct interaction with $Est2^{TEN}$.

Telomerase activity is reduced in the absence of *EST3*

The observation that recombinant Est3 stimulates primer extension activity by telomerase suggests that telomerase isolated from strains lacking Est3 should have reduced activity in comparison to telomerase from *EST3* strains. While previous work demonstrated that neither *EST1* nor *EST3* is required for *S. cerevisiae* telomerase activity in a primer extension assay [211], in both cases a modest reduction in nucleotide incorporation was noted, although this reduction was not quantified. Given my results and the observations in related yeast species that Est3 is required for optimal telomerase activity [246, 248], I revisited the question of whether loss of *S. cerevisiae* Est3 has consequences for *in vitro* telomerase catalytic activity.

I addressed this question by using yeast strains containing tagged Est2 (Myc_{18}-*EST2*) that were deleted for *EST3* and either complemented or not with *EST3* on a low-


A
RNaseA
(-)
BSA
Est3p
+1
◊


B
Est3p
K71A
DQ
ETN
(-)
(-)
(-)
(-)
+2
◊

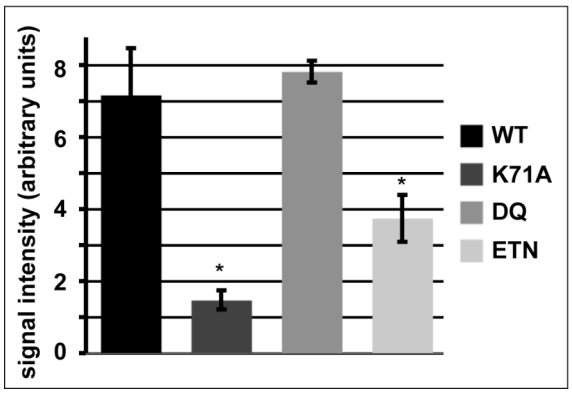

C
signal intensity (arbitrary units)
0
2
4
6
8
*
*
WT
K71A
DQ
ETN

Figure 4.1 Recombinant Est3 stimulates telomerase activity *in vitro* in a manner dependent on the Est3/Est2^{TEN} interaction. A. Partially purified telomerase extracts were prepared from YPH499 cells and incubated with a 7-base 3'-overhang immobilized DNA primer in the presence of dTTP and [α-^{32}P] dGTP. In lane (R) RNase A was added to telomerase prior to addition of the primer BSA or recombinant His_6-Est3 (0.5, 1, 2.5, 5 or 10 μM) was titrated into DNA extension reactions as indicated. (-) indicates basal telomerase activity. (◊) indicates a 27-mer loading control added prior to DNA precipitation. B. Recombinant His_6-Est3 (wild type or indicated mutant; 0.25, 0.5, 1, 2.5, and 5 μM) was titrated into DNA extension reactions as described above. (-) indicates basal telomerase activity. (◊) indicates a 27-mer loading control added prior to DNA precipitation. C. Recombinant His_6-Est3 (wild type or mutant) was added at 2.5uM to DNA extension reactions. Signal intensity of three replicates was determined using ImageQuant software and its significance was assessed using an analysis of variance (ANOVA) post hoc Dunnett's (Control= His_6-Est3). The stimulatory activities of His_6-Est3^{K71A} and His_6-Est3^{ETN} were lower than wild-type His_6-Est3 (p=0.0018 and 0.03, respectively), while His_6-Est3^{DQ} activity was similar to wild type (p=0.87). Bars represent standard error and * denotes a p-value below 0.05.

copy vector. I partially purified telomerase from these yeast cells by immunopurifying Myc_{18}-Est2 and assessing telomerase activity using primer extension. Telomerase purified from either the *Myc_{18}-EST2 est3*Δ or *Myc_{18}-EST2 EST3-HA_3* strains showed no statistical difference in overall telomerase activity (Figure 4.2c; p=0.224).

Since it is difficult to know what percentage of the active telomerase that I immunopurified has Est3 associated with it, I became concerned the association of Est3 with telomerase might be sensitive to the wash conditions of the immunoprecipitation. If Est3 is present at substoichiometric levels in telomerase isolated from the *EST3* strain, any reduction in activity upon deletion of *EST3* would be underestimated. To circumvent this concern, I used Est3 as the "handle" for immunoprecipitation of telomerase (anti-HA immunoprecipitation from an *Myc_{18}-EST2 EST3-HA_3* extract), to ensure that the catalytic core (Est2/TLC1) was associated with Est3 in the primer extension assay. Telomerase lacking Est3 was obtained as before, by immunoprecipitation from a *Myc_{18}-EST2 est3*Δ extract using antibodies against the Myc epitope. Isolation of telomerase via Est3-HA_3 gives consistently lower yield than when Myc_{18}-Est2 is used (in the *est3Δ* background), as measured by co-immunoprecipitation of the TLC1 RNA (data not shown). I cannot distinguish whether this discrepancy reflects differences in the efficiency of immunoprecipitation, differences in enzyme stoichiometry, or both. To account for this difference, standard primer extension assays were adjusted to contain approximately equal amounts of telomerase RNA and the telomerase activity measurements were normalized to the amount of TLC1 RNA present in each sample. Using this approach in three independent experiments (each representing the average of three independent assays), the *est3Δ* strain showed a decrease in overall telomerase activity

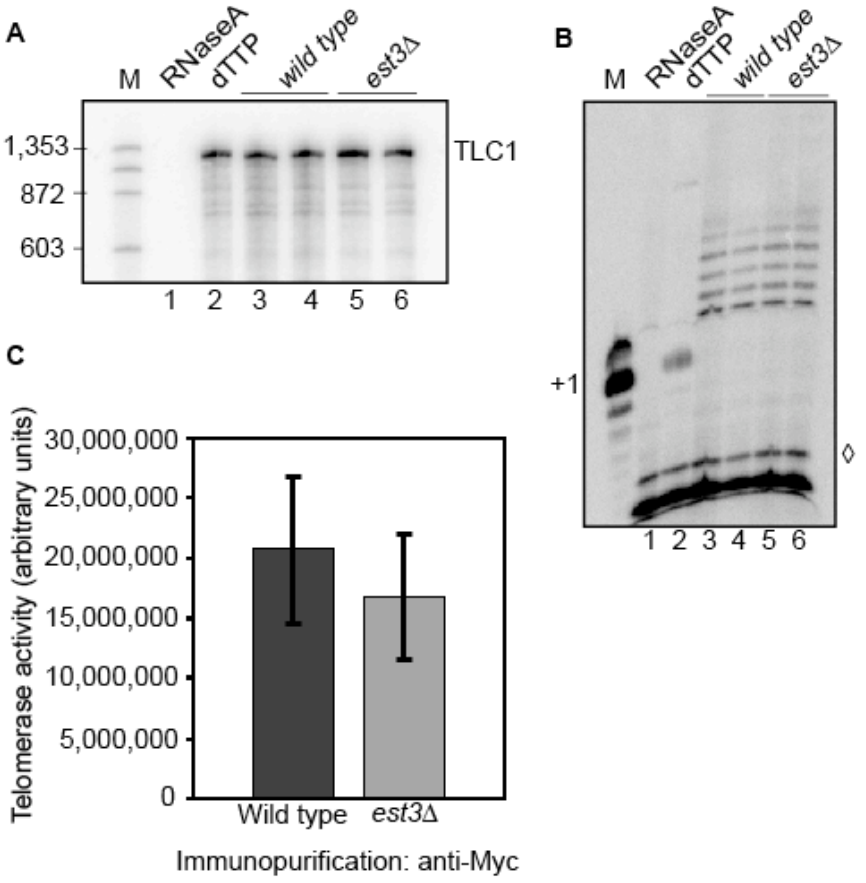

A
M
RNaseA
dTTP
wild type
est3Δ
1,353
872
603
TLC1
1 2 3 4 5 6
B
M
RNaseA
dTTP
wild type
est3Δ
+1
◊
1 2 3 4 5 6
C
Telomerase activity (arbitrary units)
30,000,000
25,000,000
20,000,000
15,000,000
10,000,000
5,000,000
0
Wild type
est3Δ
Immunopurification: anti-Myc

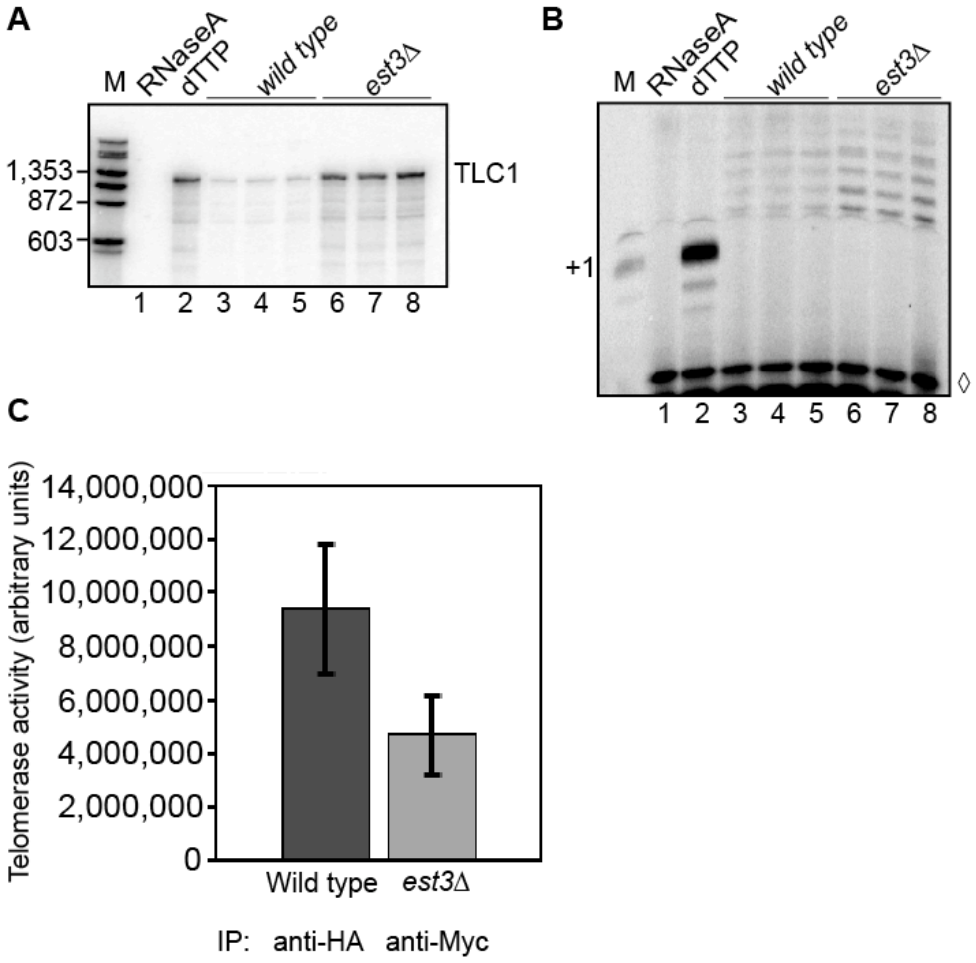
Figure 4.2 The activity of telomerase isolated by immunoprecipitation of Myc_{18}-Est2 is similar in the presence and absence of Est3. A. Co-immunoprecipitation of TLC1 RNA from strains YKF126 (*MYC_{18}-EST2 est3*Δ) complemented with pKF442 (*CEN EST3-HA_3 URA3*) or YKF126 alone was monitored by Northern blot. 10μL of immunoprecipitation beads were analyzed for both strains. B. Telomerase activity assay. Telomerase was immunoprecipitated using antibodies against the Myc epitope from extracts derived from strains YKF126 complemented with pKF442 or from YKF126 alone. Extension of a 14-nt telomeric primer was assessed by addition of dTTP and [α32-P] dGTP to the identical immunoprecipitations shown in (A). All the numbered lanes in (A) correspond directly to the numbered lanes in (B). A labeled oligonucleotide was added to each sample prior to DNA precipitation (◊). Lane (M) contains the 14-nt primer used in the telomerase assay extended by terminal transferase in the presence of [α-32P] dTTP to mark the +1 position. Lane 1 is addition of RNaseA prior to addition of the telomeric primer. In Lane 2 (dTTP), only [α-32P] dTTP was added to the extension reaction to allow addition of a single nucleotide (+1). Both lane 1 (RNaseA) and lane 2 (dTTP) show that activity is specific to telomerase. Each lane represents an independent biological replicate. C. Bar graph representation of quantification of three independent telomerase assay experiments. For each experiment telomerase activity was measured in two anti-Myc immunoprecipitations from either *MYC_{18}-EST2 est3*Δ extract or from *MYC_{18}-EST2 EST3-HA_3* extract. After correction for precipitation and loading, telomerase activity values were adjusted for the amount of TLC1 RNA in each sample as measured by Northern blot (A). Significance was assessed using a randomized block design with gel number as the block. There is no statistical difference in telomerase activity between the two yeast strains; $p=0.224$, but there was a significant contribution for gel number; $p=0.0001$, showing that there is significant variation between gels (or experiments). This is most likely due to variation in the specific activity of radionucleotides between experiments.

(Figure 4.3c; p=0.0046). These results are consistent with my observation that addition of recombinant Est3 stimulates telomerase activity. These data also support the idea that Est3 may be at substoichiometric levels when purified from wild-type extract using Est2.

Given that Est3^{ETN} was both unable to assemble with Est2 *in vivo* (Chapter III) and has reduced capacity for stimulation *in vitro* (Figure 4.1b and c), I predicted that telomerase activity should be reduced in the presence of this allele. I have previously shown (Chapter III-Figure 3.5) that Est3^{ETN}-HA$_3$ was unable to co-immunoprecipitate either TLC1 or Est2. Therefore the only way to isolate telomerase from *Myc$_{18}$-EST2 est3^{ETN}* yeast extract is by immunoprecipitation of Myc$_{18}$-Est2. Since immunopurification of Myc$_{18}$-Est2 from an *est3*Δ did not show a significant change in telomerase activity from wild-type extracts (Figure 4.2), I did not expect to see any differences, in this assay, between the wild-type and *est3^{ETN}* extracts. Unexpectedly, extracts made from yeast expressing only Est3^{ETN}-HA$_3$ displayed a significant reduction in telomerase activity (Figure 4.4c; p=0.03). Since *est3^{ETN}* has a dominant negative phenotype the reduction in telomerase activity detected in this assay may be due to this phenomenon (perhaps competitively binding other telomerase factors) instead of the lack of Est3.

Discussion

Two recent papers demonstrated that the Est3 homologues in *C. albicans* and *S. castelli* influence telomerase activity *in vitro* [211, 246, 248]. I report here that *S. cerevisiae* Est3 also stimulates basal telomerase activity (Figure 4.1). Interestingly, His$_6$-Est3^{DQ}, the mutant that retains assembly with the Est2 TEN domain, stimulates telomerase activity, while two mutants that compromise the Est3/Est2^{TEN} interaction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TLC1
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B
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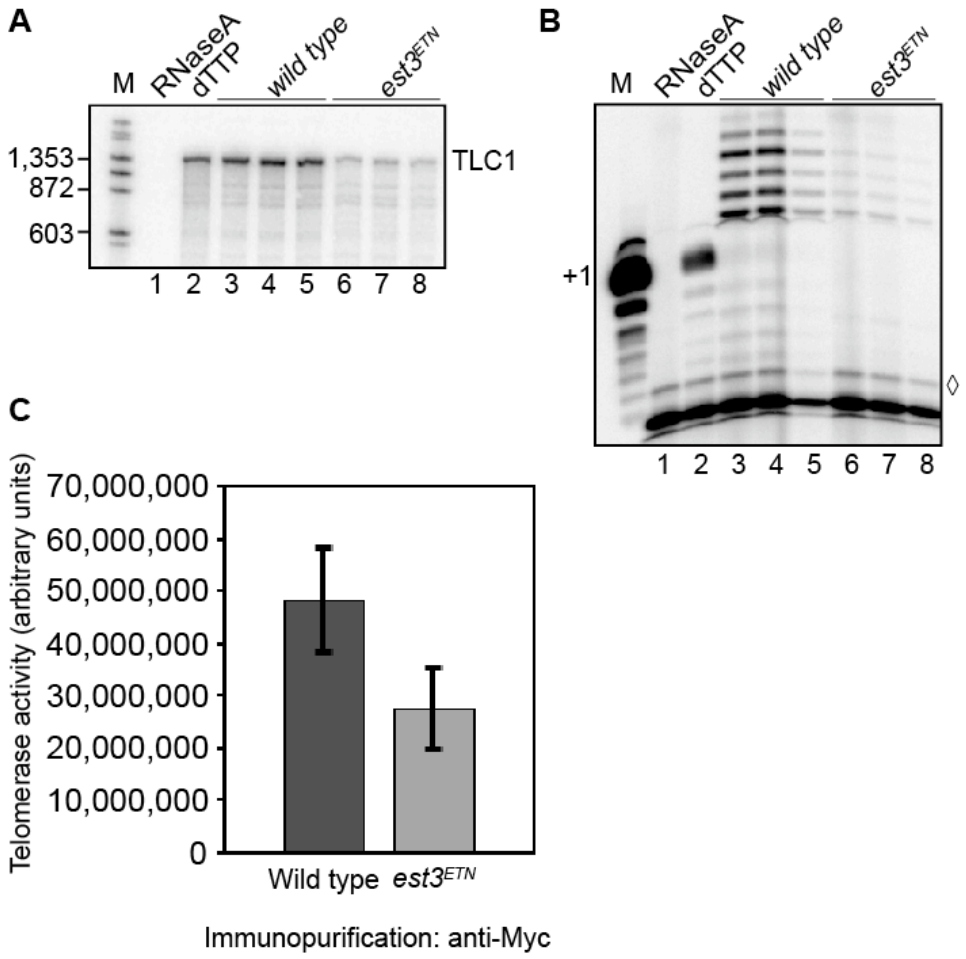
RNaseA
dTTP
wild type
est3Δ
+1
◊
1 2 3 4 5 6 7 8
C
Telomerase activity (arbitrary units)
14,000,000
12,000,000
10,000,000
8,000,000
6,000,000
4,000,000
2,000,000
0
Wild type
est3Δ
IP: anti-HA anti-Myc

Figure 4.3 Telomerase activity is decreased in *est3Δ* strains compared to wild-type extract enriched for Est3. A. Representative co-immunoprecipitation of TLC1 RNA from strains YKF126 (*MYC_{18}-EST2 est3*Δ) with or without pKF442 (*CEN EST3-HA_3 URA3*) was monitored by Northern blot. 20μL or 2.5μL of immunoprecipitation beads were added for the *MYC_{18}-EST2 EST3-HA_3* and *MYC_{18}-EST2 est3*Δ strains, respectively. B. Representative data used to measure telomerase activity. Extension of a 14-nt telomeric primer was assessed by addition of dTTP and [α32-P] dGTP to the identical immunoprecipitations shown in (A). All the numbered lanes in (A) correspond directly to the numbered lanes in (B). A labeled oligonucleotide was added to each sample prior to DNA precipitation (◊). (M) contains the 14-nt primer extended by terminal transferase in the presence of [α-32P] dTTP to mark the +1 position. In lane 2 (dTTP), only [α-32P] dTTP was added to the extension reaction to allow addition of a single nucleotide (+1). Both lane 1 [IP beads treated with RNaseA (R)] and lane 2 (dTTP) show that activity is specific to telomerase. Extracts were derived from three independent extracts of each strain. C. Bar graph representation of quantification of three independent telomerase assay experiments. For each experiment telomerase activity was measured in three anti-Myc immunoprecipitations from *MYC_{18}-EST2 est3*Δ extract or in three anti-HA immunoprecipitations from *MYC_{18}-EST2 EST3-HA_3* extract. After correction for precipitation and loading, telomerase activity values were adjusted for the amount of TLC1 RNA in each sample as measured by Northern blot (A). Significance was assessed using a randomized block design with gel number as the block. There is a statistical difference in telomerase activity between the two yeast strains; $p=0.0046$ and a significant contribution for gel number; $p < 0.0001$, showing that there is significant variation between gels (or experiments). This is most likely due to variation in the specific activity of radionucleotides between experiments.

(*est3*^{ETN} and *est3*^{K71A}) do not (Figure 4.1). These results suggest that Est3 requires interaction with the Est2 TEN domain to mediate its stimulatory effect. The requirement for this interaction in the stimulation assay may simply reflect a defect in the recruitment of recombinant Est3 to the telomerase complex *in vitro*. However, it is also plausible that the stimulatory effect of Est3 requires a specific interaction with the TEN domain that is separable from complex assembly.

Although previous work demonstrated that Est3 is not required for *in vitro S. cerevisiae* catalytic activity, these initial experiments hinted at a modest reduction in activity when telomerase was isolated from *est3*Δ or *est1*Δ strains [211]. I have revisited this issue by measuring telomerase activity in a manner that ensures the presence of Est3 in telomerase samples purified from wild-type yeast and that corrects for the efficiency with which TLC1 RNA is co-immunoprecipitated. Using this method, I detect a reproducible decrease in primer extension activity when telomerase is immunopurified from an *est3Δ* strain under conditions that ensure the presence of Est3 in the wild-type sample (Figure 4.3). Notably, there is not a significant difference between telomerase activity from *est3*Δ strains immunopurified using Myc_{18}-Est2 (Figure 4.2; p=0.224). This could be due to the small sample size (n=6), inherent variability between experiments (p=0.0001), and/or depletion of Est3 from the telomerase complex during immunoprecipitation washes. Though it is difficult to increase the concentration of Est3 co-immunoprecipitated from the wild-type yeast extract, repeating this experiment using a larger sample size and analyzing as many data points as possible on one gel could help in detecting a difference, if it exists, between wild-type and *est3*Δ strains.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RNaseA
dTTP
wild type
est3ETN
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TLC1
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B
M
RNaseA
dTTP
wild type
est3ETN
+1
◊
1 2 3 4 5 6 7 8
C
Telomerase activity (arbitrary units)
70,000,000
60,000,000
50,000,000
40,000,000
30,000,000
20,000,000
10,000,000
0
Wild type
est3ETN
Immunopurification: anti-Myc

Figure 4.4. Telomerase activity from yeast expressing Est3^{ETN} is reduced compared to wild type. A. Representative co-immunoprecipitation of TLC1 RNA from strains YKF126 (*MYC$_{18}$-EST2 est3*Δ) with or pKF442 (*CEN EST3-HA$_3$ URA3*) or pKF449^{ETN}(2micron *est3^{ETN}-HA$_3$ LEU2*) was monitored by Northern blot. 10μL of immunoprecipitation beads were processed. B. Representative data used to measure telomerase activity. Extension of a 14-nt telomeric primer was assessed by addition of dTTP and [α32-P] dGTP to the identical immunoprecipitations shown in (A). All the numbered lanes in (A) correspond directly to the numbered lanes in (B). A labeled oligonucleotide was added to each sample prior to DNA precipitation (◊). (M) contains the 14-nt primer extended by terminal transferase in the presence of [α-32P] dTTP to mark the +1 position. In lane 2 (dTTP), only [α-32P] dTTP was added to the extension reaction to allow addition of a single nucleotide (+1). Both lane 1 [IP beads treated with RNaseA (R)] and lane 2 (dTTP) show that activity is specific to telomerase. Extracts were derived from three independent extracts of each strain. C. Bar graph representation of quantification of three independent telomerase assay experiments. For each experiment telomerase activity was measured in two to three anti-Myc immunoprecipitations either extract. After correction for precipitation and loading, telomerase activity values were adjusted for the amount of TLC1 RNA in each sample as measured by Northern blot (A). Significance was assessed using a randomized block design with gel number as the block. There is a statistical difference in telomerase activity between the two yeast strains; $p=0.03$ and a significant contribution for gel number; $p=0.009$, showing that there is significant variation between gels (or experiments). This is most likely due to variation in the specific activity of radionucleotides between experiments.

Telomerase isolated from a strain expressing *est3*^{ETN} shows reduced catalytic activity compared to wild type (Figure 4.4). This result was not entirely expected because to assess telomerase activity in the *est3*^{ETN} strain I had to immunopurify telomerase using Myc$_{18}$-Est2 (caveats discussed above). Therefore, the observed decrease in the *est3*^{ETN} strain may reflect the dominant negative phenotype of Est3, rather than its lack of assembly into the complex. For example, it is possible that Est3^{ETN} disrupts formation of the telomerase complex in a manner that greatly perturbs the conformation and/or function of telomerase *in vivo*. If the Est3^{ETN} protein retains the ability to bind some telomerase components, both known (Est1) and/or unknown (other unidentified subunits), it may alter the telomerase complex stoichiometry through competition for association with those subunits. In support of this idea, it has recently been shown that Est1 and Est3 interact directly *in vitro*, though the site of interaction remains enigmatic [193].

Why is the effect of Est3 on *in vitro* telomerase activity greater in the "stimulation" assay compared to the "conventional" primer extension assay? I suggest that technical differences between the protocols may influence the effect of Est3. Specifically, our conventional assay immobilizes telomerase on a bead (possibly restricting important conformational changes) while the primer is free in solution. In contrast, the stimulation assay anchors the primer to a streptavidin bead, perhaps better mimicking the situation at a chromosome terminus and telomerase is free in solution. Therefore, the stimulation assay may allow a more dynamic examination of telomerase activity than the conventional assay. Exactly how Est3 aids in telomerase activity is unknown, but it is plausible that it could affect the position of telomerase at the 3`-

overhang, allowing it to initiate synthesis more efficiently. Alternatively, Est3 may enhance the ability of telomerase to recognize the substrate. Both of these possibilities are consistent with an increase in overall telomerase activity and no increase in telomerase processivity.

TPP1 is a member of the telomere-binding shelterin complex in mammals and influences the processivity of telomerase *in vitro* [33, 37]. Based on prediction algorithms, fungal Est3 has a similar structure to the oligonucleotide/oligosaccharide-binding (OB) fold domain of TPP1, although this observation alone does not imply an evolutionary relationship between the two proteins [226, 227]. Intriguingly, deletion of the TPP1 OB fold disrupts telomerase localization to the telomere in human cells [252]. Additionally, a sequence-specific interaction between TPP1 and the human TEN domain of TERT is important for telomerase processivity [253]. My observation that the Est2 TEN domain mediates a critical interaction with Est3 that influences both telomerase assembly *in vivo* and stimulation of telomerase activity *in vitro* provides an intriguing parallel between the two proteins. The glutamate 114, threonine 115, and asparagine, 117 of the Est3 primary structure align near some surface-exposed residues within a small alpha helix of the OB fold of TPP1: arginine 159, glutamate 160, aspartate 163 and threonine 164. Determining if these residues influence telomerase function in higher eukaryotes could give insight into a possible an evolutionary relationship between Est3 and TPP1.

CHAPTER V

MATERIALS AND METHODS

Plasmids for protein expression

The plasmid pET Duet EST3 (for expression of *His$_6$-EST3*) was made by moving *EST3* from YCPlac33 EST3 using primers M090 and M091 (Table 5.1) into pET Duet-1 (Novagen) using restriction sites *Bam*HI and *Sal*I. *EST3* mutants *K71A* (lysine 71 to alanine), *ETN* (glutamate 114, threonine 115, and asparagine 117) and *DQ* (aspartate 166 and glutamine 167) were introduced into pET Duet EST3 by Quikchange™ (Stratagene) using primer pairs: K71A For and K71A Rev; ETN114AAK F and ETN114AAK R; DQ For and DQ Rev, respectively. A vector for expression of *Mbp-EST2^{TEN}* was made by cloning *EST2^{TEN}* (residues 1-161) from pKF404 [98] using primer pair LM204.1 For and LM204.1 Rev into pLM204a (gift of L. Mizoue) using restriction sites *Eco*RI and *Pst*I. The *Mbp-EST2^{TEN}* fusion gene was then moved into the *Eco*RV and *Kpn*I sites of pET Duet-1 using primer pair EcoRV Mbp F and KpnI Est2RI R to create pET Duet Mbp-EST2^{TEN}. *Mbp* alone was cloned into the *Eco*RV and *Kpn*I restriction sites of pET Duet-1 from pLM204a using primer pair EcoRV Mbp F and KpnI Mbp R to create pET Duet Mbp.

Plasmids for *in vivo* characterization

A consensus destruction box (D-box) motif in plasmid pVL242 (*GAL-HA$_3$-EST1*; gift of V. Lundblad) was mutated by PCR using primers M086 and M087 [254]. The

resulting PCR product was cloned into the *Bam*HI and *Hin*dIII sites of pVL242 to create pVL242RtoA and verified by sequencing.

pKF441 (*CEN EST3 URA3*) was created by PCR amplification of the *EST3* promoter and frameshift-corrected gene from pVL901 (gift of V. Lundblad) using primer pair UEprimer1 and UEprimer4 to create an *Eco*RI site upstream of the promoter and a *Kpn*I site immediately before the *EST3* stop codon. The endogenous *EST3* termination sequence was amplified using primer pairs UEprimer5 and UEprimer6 to create a *Hin*dIII site downstream of the terminator. UEprimer1 and UEprimer6 were then used to amplify the full-length insert using these two PCR products as template. The resulting fragment was cloned into YCplac33 (*CEN URA3*) using *Eco*RI and *Hin*dIII. pKF442 (*CEN EST3HA URA3*) was created by PCR amplification of the HA_3 tag from pVL901 using primer pair KpnI For and XbaI Rev and ligation into pKF441 using *Kpn*I and *Xba*I. *EST3* and *EST3HA* were subsequently moved into pRS315 or pRS425 (*CEN LEU2* and 2µm *LEU2*, respectively; [255, 256]) using the *Pvu*II sites of pKF441 or pKF442. The $est3^{ETN}$ and $est3^{K71A}$ alleles were subcloned from pET Duet-1 (see above) using restriction sites *Msc*I and *Xho*I. $est3^{DQ}$ was created by Quikchange™ using the same primers as above and subcloned using *Spe*I and *Xma*I. All point mutations were verified by sequencing.

Yeast strains

YKF122 (AVL78 $est3::KAN^R$) was created by standard one-step gene replacement by PCR amplifying the kanamycin resistance gene from pFA6a-kanMX6 using primers Est3KanFor and Est3KanRev. The PCR product was cloned into the *Sac*I and *Kpn*I sites of pKF441 to create pKF441 *est3::KAN*. The deletion construct was

transformed into AVL78 using a standard lithium acetate protocol. YKF126 [AVL78 *est3::KAN*^{R} *EST2-G*$_{8}$*-Myc*$_{18}$ (G: glycine)] was created by linearizing pRS304-Est2-G8-Myc18 (Gift of V. Zakian) with *Swa*I and transforming it into YKF122 + pKF441. A list of yeast strains and plasmids can be found in Table 5.2.

Complementation and growth assay

Functional complementation of the *est3* mutant alleles was tested in YKF122 (AVL78 *est3::KAN*^{R}) using mutant constructs created in pRS315 (*CEN LEU2*). YKF122 was complemented with pKF442 (*CEN EST3-HA URA3*); loss of the complementing plasmid was selected on 5-fluoroorotic acid (5-FOA) plates and the WT or mutant pRS315 plasmids were subsequently transformed using the standard lithium acetate method. Resulting single colonies were restreaked three times on plates lacking leucine. Cell viability was assessed visually and telomere length was determined by Southern blot using *Xho*I as previously described [245].

Protein Purification

BL21 cells containing pET Duet *EST3* were grown in six liters of standard Luria broth (LB) with 50ug/mL ampicillin at 37°C to an OD_{600} 0.3-0.4. After shifting the culture to 16°C for 1h, protein expression was induced with 500μM IPTG overnight with moderate shaking (110 rpm). Cells were harvested by centrifugation at 4°C and resuspended in 10 mL TG buffer (+ 100 mM NaCl) per liter of original culture (TG: 50mM Tris pH 7.5, 10% glycerol and 3mM beta-mercaptoethanol [β-ME]). Cells were lysed using an EmulsiFlex (Avestin) by passing cells 3-4 times through the machine at

20,000 psi. The resulting extract (60mL) was incubated with 7.5mL Talon® resin (Clontech) for 1h at 4°C with gentle agitation and gravity packed into an empty glass EconColumn™ (BioRad). Resin was washed with 10 column volumes of TG + 300mM NaCl and 10mM imidazole. Protein was eluted with 5 column volumes TG + 100mM NaCl and 100mM imidazole. The elution was dialyzed (Spectra/Por® #7; 10,000KDa [Spectrum Laboratories]) at 4°C overnight into TG buffer, bound to a Source™ 15Q HR16/10 column (GE Healthcare) and eluted using a linear salt gradient (TG + 0mM NaCl to TG + 1M NaCl). The purest fractions were pooled and applied to a Superdex™ 200 26/60 gel filtration column (GE Healthcare) using TG + 100mM NaCl buffer. Again, His_6-Est3 fractions were pooled and concentrated to 3mL using a 15mL, 10KDa cutoff Amicon® Ultra concentrator (Millipore) and dialyzed into TG + 100 mM NaCl + 50% glycerol. The protein was stored at -20°C. Mutant proteins were purified in the same manner except that His_6-$Est3^{ETN}$ and His_6-$Est3^{DQ}$ were purified at pH 8.0. The Est3 protein sequence was verified by mass spectrometry.

Three-liter cultures of BL21 cells containing pET Duet *MBP-EST2^{TEN}* were grown and harvested as described above. Cells were lysed as above in 10mL TEG-200 per liter of original culture (TEG-200: 20mM Tris-HCl pH 7.4, 200mM NaCl, 1mM EDTA, 10% glycerol). Extract was incubated with 10mL amylose resin for 2 hours at 4°C and was packed similarly as above. Resin was washed with 15 column volumes TEG + 500mM NaCl. Mbp-$Est2^{TEN}$ was eluted with TEG-200 + 5mM maltose. Fractions containing Mbp-$Est2^{TEN}$ were further purified over an S200 gel filtration column using TG + 100mM NaCl buffer. Mbp-$Est2^{TEN}$ was concentrated as described for His_6-Est3 and

dialyzed into TG + 100mM NaCl, 50% glycerol. Protein was stored at -20°C. The Est2^{TEN} protein sequence was verified by mass spectrometry.

All purified proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine purity. All protein preparations used were judged by Bio-Safe™ Coomassie (BioRad) staining to be at least 95% pure. Protein concentration was determined either by spectrophotometry or by comparison of a serial dilution to a known concentration of protein standard.

Maltose Binding Protein (Mbp) was purchased from New England Biolabs and diluted to 20μM in TG + 100mM NaCl + 50% glycerol.

In vitro pulldown assay, immunoprecipitation and Western analysis

Mbp or Mbp-Est2^{TEN} (200 picomol) were incubated with 25μL amylose resin in buffer I (20mM Tris-HCl pH 7.4, 200mM NaCl, 1mM EDTA, 0.05% Tween-20 and 10% glycerol) for 2h at 4°C with gentle agitation. 1 nmol His_6-Est3 or mutant Est3 proteins were added and incubated 3h at 4°C. The resin was washed 3 times for 5 min. with 1mL buffer I, resuspended in 100μL buffer I, loaded into a 700μL spin cup (Pierce), and washed with 1.5mL buffer I. Retained resin was resuspended in 40μL buffer I and 20μL were separated by 12% SDS-PAGE. For detection of Mbp and Mbp-Est2^{TEN}, proteins were blotted onto nitrocellulose (GE Healthcare) and blocked with 5% Milk/phosphate buffered saline pH 7.4 with 0.05% Tween (PBS-T). For detection of His_6-Est3, proteins were blotted onto Hybond P membrane (GE Healthcare) and blocked with 5% BSA/PBS-T. HRP-conjugated anti-Mbp (NEB) was used at a dilution of 1:50,000. Primary antibody for detection of His_6 (Santa Cruz Biotechnology) was diluted 1:1,000. Secondary

antibody was peroxidase-conjugated mouse anti-rabbit at 1:10,000 (Chemicon). ECL plus Western Blotting Detection system (GE Healthcare) was used for detection.

Yeast protein extract was prepared as described [98]. Extracts were normalized to 20mg/mL and incubated with antibodies as previously described [98, 192]. Either 20μL of anti-Myc immunoprecipitated material (1/3 of total) or 2.5 μL of anti-HA immunoprecipitated material (1/24 of total) was separated by 10%-12% step-gradient SDS-PAGE. Proteins were transferred to Hybond P membrane (GE Healthcare). The membrane was blocked with 5% Milk/PBS-T followed by incubation with primary antibodies (HA: 1:500 dilution murine monoclonal HA.11 [Covance] or Myc: 1:250 dilution of murine monoclonal Myc Ab-1 [OP10L, EMD Biosciences]) in 5% Milk/PBS-T. Secondary antibody was peroxidase-conjugated goat anti-mouse (Chemicon) used at a 1:10,000 dilution in 5% Milk/PBS-T. Detection was done as described above.

Est1 protein stability assays

100 mL cultures containing plasmid pVL242RtoA were grown overnight in 2% raffinose medium lacking leucine to an OD_{600} > 0.3 but < 0.45. Cells were arrested by incubation with 1 mM alpha factor or 10 mg/ml nocodazole for 4h. When >95% of the cells were of the expected morphology, galactose was added at a final concentration of 2% for 60 minutes. At time zero, glucose (2% final concentration) and cycloheximide (10 mg/ml) were added. For proteasome inhibition, MG132 (Peptides International) was added to a final concentration of 50 mM. 10 mL samples were collected at 0, 15, 30, 60, 90 and 120 minutes. Protein was isolated by trichloroacetic acid (TCA) precipitation as described [257] and pellets were resuspended in 150 ml of 0.05 N NaOH. 10 μL of each

sample was immunoblotted as described above except anti-HA antibody was used at 1:500 at room temperature for 2h or at 4°C overnight.

Telomerase assays and Northern analysis

Telomerase was partially purified by immunoprecipitation from extracts of strains YKF126 [*est3Δ::kanMX6 EST2-G$_8$-MYC$_{18}$*], YKF126 + pKF442 (*EST3-HA*) or YKF126 + pKF449^{ETN} (*EST3^{ETN}*) using antibodies against Myc (A14, Santa Cruz Biotechnology) or HA (Y11, Santa Cruz Biotechnology), respectively, as previously described [98]. Telomerase activity was assessed using the Tel14 primer (Table 5.1) with either 2.5µL or 20µL of immunoprecipitation beads (YKF126 or YKF126 + pKF442, respectively) as previously described [98]. For each immunoprecipitation, an identical aliquot was analyzed by Northern blot for TLC1 RNA, as described below. Total radioactive signal corresponding to extension of the Tel14 primer was quantified for each sample using ImageQuant and divided by the signal from an end-labeled 12-base oligonucleotide used as a precipitation and loading control. This corrected telomerase activity value was divided by the TLC1 RNA signal obtained by Northern blot from the corresponding sample and values of two to four independent reactions were averaged. To facilitate statistical analysis of five experiments, these average values were normalized to the activity obtained in the presence of *EST3* (YKF126 + pKF442).

For Northern analysis, RNA was isolated from immunoprecipitation beads and detected by Northern blotting as previously described [98, 192]. Whole-cell RNA was prepared from 10mL mid-log phase cultures and hybridization was performed simultaneously with probes specific for *TLC1* RNA ([^{32}P]dCTP random-primed fragment

of *TLC1*) and *U1* snRNA (^{32}P-5'-end-labeled oligonucleotide 5'CTACTATTGGAAGCGCATGTTTG) [258]. For analysis of *EST1* mRNA stability, whole cell RNA was isolated from 50 ml cultures grown to mid-log phase and Northern blotting was performed as described [259]. Hybridization was performed with probes specific for *EST1* RNA ([^{32}P]dCTP random-primed fragment of *EST1*) and *U1* snRNA as described above.

Telomerase was partially purified from wild-type yeast (YPH499) by chromatography over DEAE sepharose fast flow resin (GE Healthcare) and MonoQ resin (GE Healthcare) as previously described [224, 251] and used in telomerase DNA extension assays. Briefly, partially purified telomerase extract was incubated with 2 pmol of a 7-base 3'-overhang template (GTGTGTG) immobilized on streptavidin paramagnetic beads (Promega) and extension buffer (50 mM Tris [pH 8.0], 1 mM $MgCl_2$, 1 mM spermidine, 1 mM DTT, 0.5% glycerol, 50 μM dTTP, 10 μCi of [α- ^{32}P]dGTP [3,000 ci mmol^{-1}; Amersham]). The 7-base 3'-overhang template was generated by annealing biotin-conjugated Backbone1 primer (Table 1) to GTG7 base consensus primer. Reactions were incubated for 30 min at 30°C followed by magnetic collection of DNA-bound beads. The beads were washed twice with 1x *Eco*RI buffer (New England Biolabs). Beads were resuspended in 50 μL of 1x *Eco*RI buffer, 100 μg/mL bovine serum albumin (BSA) and 10U of *Eco*RI and incubated for 1 hour at 37°C. The beads and cleaved DNA fragments were separated magnetically. A PNK (T4 Polynucleotide Kinase [NEB]) end-labeled, 27-base oligonucleotide was added and the DNAs were ethanol precipitated. DNA was reconstituted in formamide-NaOH loading buffer and run in a 14% acrylamide denaturing gel and subsequently visualized using a PhosphorImager. For

the experiments in Chapter IV, Figure 4.1, BSA or recombinant His_6-Est3 was titrated into the extension buffer at varying concentrations before addition of telomerase extract [224].

Table 5.1. Oligonucleotide primers

Primer Name	Primer Sequence 5'-3'
M086	CCCCGGATCCATGTACCCGTATGATG
M087	CATATTTTTAAGCTTGGATGCTGGACCACCCAAGC
M090	CGGGATCCGATGCCGAAAGTAATTC
M091	ACGCGTCGACTCATAAATATTTATATACAAATG
K71A For	CGCCAAAATTACAGCGTTTTATAACGTTTGCGAC
K71A Rev	GTCGCAAACGTTATAAAACGCTGTAATTTTGGCG
ETN114AAK F	GCAGGATCACATCTGCGGCGACGAAATGCTTAATGATCATTGGCG
ETN114AAK R	CGCCAATGATCATTAAGCATTTCGTCGCCGCAGATGTGATCCTGC
DQ For	CCAGGCCACGATATTTGATATTGCAGCTGTCGGATCGTTAAGTACTTTC
DQ Rev	GAAAGTACTTAACGATCCGACAGCTGCAATATCAAATATCGTGGCCTGG
LM204.1 For	CGAATTCGATGAAAATCTTATTCGAG
LM204.1 Rev	AACTGCAGTTAGACCCCATTTGGGC
EcoRV Mbp F	CGGATATCATGGGTATGAAAATCGAAG
KpnI Est2RI R	CGGTACCTTAGACCCATTTGGGCGGCAG
KpnI Mbp R	CGGTACCTTAATTCGGATCCCCGGGCCCC
UEprimer1	GATTACGCCAAGCTTGCATG
UEprimer4	GAAGGTCAGGTACCTAAATATTTATATACAAATGGG
UEprimer5	TATTTAGGTACCTGACCTTCTGCAGGCTCG
UEprimer6	CCAGTGAATTCGAGCTGCGTACCCGGGGATC
KpnI For	GTATATAAATATTTAGGTACCATGTACCCGTATG
XbaI Rev	CCTTTTGTTTCTCTAGAGGAGTAC
Est3::Kan^R For	GCTGTAAATCCTGGGTACAAATCGGGATAACAAGTAAACACGGATCCCCG GGTTAATTAA
Est3::Kan^R Rev	GTTTAAACGAGCTCGAATTCCTAACTCTCTCACACTTATAAAATATCGAGC CTGCAGAAGG
Backbone 1	ACACACCACACCACACGAATTCCCCACACACACCACAC[BIOTIN-TEG]
GTG7base consensus	GTGTGGTGTGTGTGGGGAATTCGTGTGGTGTGGTGTGTGTGTGTG
27mer (loading control)	GTGGGTGTGGGTGTGGGTGTGGGTGTG
Tel14	GTGTGGTGTGTGGG
12mer	GTATCCTGGCAT

Table 5.2. Strains and plasmids

Strains or Plasmids	Genotype	Chapter	Source
AVL78	*MATa leu2 trp1 ura3-52 prb1 prc1 pep4-3*	II-IV	V. Lundblad
YPH499	*MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2Δ1*	IV	B. Freeman
TVL288	*HA_3-EST1*	II	V. Lundblad
YKF122	AVL78 *est3Δ::kanMX6*	III	This study
YKF126	AVL78 *Myc_{18}-EST2 [TRP] est3Δ::kanMX6*	III & IV	This study
YKF332	AVL78 *EST3-HA_3 Myc_9-EST2 [TRP] bar1:URA3*	II	J. Osterhage
YKF333	AVL78 *Myc_9-EST2 EST3-HA_3 bar1::URA3 erg6::kanMX6*	II	J. Osterhage
pVL242	*GAL1-HA_3-EST1 LEU2*	II	V. Lundblad
pVL242RtoA	pVL242 RxxLxxxN to AxxLxxxN in linker between HA and *EST1*	II	This study
pLM204a	*MBP KAN*	III	L. Mizoue
pKF441	*CEN EST3 URA3*	III	This study
pKF442^{HA}	*CEN EST3-HA_3 URA3*	III & IV	This study
pKF448	*CEN EST3 LEU2*	III	This study
pKF448^{K71A}	*CEN $EST3^{K71A}$ LEU2*	III	This study
pKF448^{ETN}	*CEN $EST3^{ETN114,115,117AAK}$ LEU2*	III	This study
pKF448^{DQ}	*CEN $EST3^{DQ166,167AA}$ LEU2*	III	This study
pKF448^{HA}	*CEN EST3-HA_3 LEU2*	III	This study
pKF449	2 μm *EST3 LEU2*	III	This study
pKF449^{ETN}	2 μm *$EST3^{ETN114,115,117AAK}$ LEU2*	III	This study
pKF449^{HA}	2 μm *EST3-HA_3 LEU2*	III	This study
pKF449$^{K71A-HA}$	2 μm *$EST3^{K71A}$-HA_3 LEU2*	III	This study
pKF449^{ETN-HA}	2 μm *$EST3^{ETN114,115,117AAK}$ -HA_3 LEU*	III & IV	This study
pKF449^{DQ-HA}	2 μm *$EST3^{DQ166,167AA}$ -HA_3 LEU2*	III	This study
pKF1201	pET Duet-1 *MBP-$EST2^{TEN}$ AMP*	III	This study
pKF1301	pET Duet-1 *EST3 AMP*	III	This study
pKF1302	pET Duet-1 *$EST3^{K71A}$ AMP*	III	This study
pKF1303	pET Duet-1 *$EST3^{ETN114,115,117AAK}$ AMP*	III	This study
pKF1304	pET Duet-1 *$EST3^{DQ166,167AA}$ AMP*	III	This study

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

The potential roles for Est1 degradation in G1 phase of the cell cycle

The maintenance of the telomere is critical for faithful replication of DNA and proper cell division [165]. This thesis has focused on the regulation of telomerase complex assembly, defining interactions within the complex and elucidating an enzymatic contribution for the telomerase accessory protein, Est3. Understanding how members of the telomerase complex assemble is critical since the assembly of the complex is a prerequisite for activity *in vivo* [192, 226, 227, 248]. Telomerase is only active in S/G2 phases of the cell cycle, even though Est2 and TLC1 RNA are localized to telomeres in G1 phase via an interaction with the yKu70/80 heterodimer [180, 181, 191]. Interestingly, Est1 protein levels were found to be much lower in G1 compared to G2/M; this observation led Jennifer Osterhage to hypothesize that perhaps a decrease in Est1 protein levels in G1 phase precludes telomerase assembly in this phase and results in an inactive complex in G1 [192]. Intrinsic to this hypothesis is the proposal that the EST1 RNA or protein is regulated differentially in G1 phase as compared to other phases of the cell cycle. To address this possibility, I determined the half-life for the Est1 protein in G1 and G2/M phases (Chapter II; [192]). I found that in G1 phase the stability of the Est1 protein is decreased compared to G2/M phase. Furthermore, I demonstrated that Est1 is degraded in a proteasome-dependent manner [192]. This finding allowed Jennifer to stabilize endogenous Est1 in G1 phase and assess both complex assembly and telomerase

activity (through proteasome inhibition by MG132). Under these conditions, the telomerase complex assembles and interestingly, telomerase is active *in vitro* even when partially purified using Est3 (a phenomenon not seen in untreated cells) [192]. Intriguingly, Est1 stabilization in G1 phase was not capable of extending telomeres *in vivo* [192]. These somewhat unexpected results suggest that there are mechanisms in addition to telomerase assembly that must regulate telomerase activity in G1 phase [192].

Arising from these findings are at least three additional questions: How is Est1 targeted to the proteasome in G1 phase of the cell cycle? Why is Est1 degraded in G1 phase? What other mechanisms regulate telomerase activity? The Anaphase Promoting Complex (APC), an E3 ubiquitin ligase, is an obvious candidate for what is targeting Est1 to the proteasome in G1 phase. Although, it is active during the time of Est1 degradation, the influence of the APC on Est1 stability remains in question (Chapter II-discussion). Another possibility is that there is a unique E3 ubiquitin ligase that recognizes Est1 in G1. There are a number of E3 ubiquitin ligases in yeast; therefore the most viable way to discover which E3 may target Est1 to the proteasome is a genetic screen. For example, *EST1-3xGFP* (**G**reen **F**luorescent **P**rotein) could be integrating into yeast strains deleted for different E3 ligases. These cells could be blocked in G1 phase and assessed for increases in GFP expression over wild-type cells. Any yeast strains that show an increase would be considered candidates for the E3 ligase that targets Est1 to the proteasome. The candidate(s) should be verified using immunprecipitation assays to determine if the E3 ligase and Est1 interact. In addition, showing that in G1 phase, in the absence of the candidate E3 ligase, Est1 stability is increased and the telomerase complex assembles would support its role as the targeting E3 ligase.

Est1 degradation could serve at least two functions. It could serve a redundant mechanism for preventing telomere elongation in G1 phase. Telomerase requires both Est1 and Est3 to function properly *in vivo* and since Est1 stimulates Est3 assembly with the complex, degradation of Est1 ultimately ablates Est1 and reduces Est3 assembly with telomerase in G1 resulting in an inactive complex [192, 209, 210]. Given the above observation, the fact that stabilizing Est1 in G1 is insufficient for telomere elongation *in vivo* suggests that there must be another mechanism(s) restricting telomerase function to S phase. Two of the more prominent thoughts on what is regulating telomerase activity in the cell cycle are the structure of the telomere (telomerase must be able to recognize the telomere) and that DNA replication and telomere elongation are coupled. For example, the length of the telomeric overhang is short in G1 phase, ~12-14 nucleotides, and longer in S phase, ≥30 nucleotides [25, 182]. The longer 3'-overhang in S phase binds Cdc13 molecules, which presumably permits telomerase recruitment, whereas the G1-telomere does not bind Cdc13p, as assessed by ChIP [190]. This finding predicts that a longer ssDNA telomeric overhang in G1 phase could recruit Cdc13 binding. Indeed, deletion of *Ku70*, a gene important both in NHEJ and protecting the telomere from resection, increases the length of the single-stranded 3'-overhang in G1 phase, resulting in increased Cdc13 binding in G1 [191]. Stabilizing the Est1 protein in G1 phase in this yeast strain, would address if the telomere structure in addition to telomerase assembly are the mechanisms restricting telomere elongation to S phase.

DNA replication must occur once, faithfully and completely during each cell cycle and as such is a tightly regulated process (reviewed in [260]). Prevention of telomere elongation in G1 phase might be due in part to an overall inhibition of DNA

replication in G1 phase. Interestingly, the Diffley group has generated a yeast strain that replicates its DNA in G1 phase [261]. Investigating whether or not telomeres are elongated during G1 phase in this strain could address if Est1 protein degradation is a redundant mechanism preventing telomere replication in G1. If telomeres are replicated in G1 phase in this strain background while Est1 levels are low, then Est1 protein degradation is not a critical mechanism preventing telomere replication. Alternatively if telomeres are not elongated in G1 phase in this strain then Est1 could be considered an important regulator of telomerase activity in G1. One caveat to this experiment is that replication in the Diffley yeast strain may not be occurring normally or completely; therefore it would be important to verify that the telomeres are actually being replicated in this yeast strain under asynchronous (or wild-type) growing conditions.

Why is it important to prevent telomere elongation in G1 phase of the cell cycle? After the T/G-rich 3'-overhang is extended by telomerase in late S phase, it is thought that lagging strand polymerase (pol α) fills-in the C/A strand (reviewed in [183]). Consequently, if telomeres are elongated in G1 phase the long single-stranded T/G tracts generated by telomerase addition could encourage spurious recombination events (a common phenomenon in yeast) (reviewed in [262]) or induce a DNA damage checkpoint response (reviewed in [263]). Both of these events threaten the genomic stability of the yeast cell; therefore, coordinating DNA replication and telomere elongation may ensure proper genome maintenance.

Est1 degradation in G1 phase could also serve to "unmark" a recently extended telomere. Interestingly, only 6-8% of wild-type telomeres are elongated in each cell cycle and those that are replicated tend to be the shortest telomeres [196]. This finding suggests

that telomeres are "marked" for elongation during the cell cycle, most likely before late S phase. Inherent in this "marked-telomere" concept is that each "marked" telomere must be "unmarked" during the subsequent cell cycle to ensure that other telomeres (that would have been shortened in preceding rounds of cell division) have the chance to be elongated. Removing or disassembling telomerase from the telomere after replication would ensure that the newly elongated telomere would start the next cell cycle telomerase free. Since Est1 is important in both recruiting telomerase to the telomere and activating the complex, Est1 degradation could be an event that ensures the disassembly of the telomerase complex at the completion of telomere elongation [188, 224]. This hypothesis predicts that the same telomere could be elongated in successive cell cycles if the Est1 protein is not removed before the start of the subsequent S phase. Comparing WT cells to those constitutively overexpressing Est1 and assaying changes in the patterns of telomere addition in two consecutive cell cycles can begin to test this hypothesis. If cells overexpressing Est1 show a greater number of telomeric repeats added to a single telomere compared to WT cells then this would support the idea that degradation of Est1 is important in regulating telomerase action at a single telomere. Importantly, association of Est1 with the telomere would need to be assessed using ChIP to ensure that the Est1 protein is telomere-associated though out the cell cycle (Est1 is typically only associated in S phase).

The assembly and function of Est3 in the telomerase complex

The Est3 protein is abundant throughout the cell cycle, suggesting that unlike Est1, Est3 is not regulated in abundance in the cell cycle [192, 193]. Est1 is both

necessary and sufficient to stimulate Est3 assembly with telomerase, therefore the relatively low Est1 levels during G1 phase is one mechanism that precludes Est3 from complex formation in G1 [192]. Additionally, Est2 is critical for Est3 assembly since deletion of *EST2* prevents Est3 association with TLC1 RNA and Est1 [213]. Alternatively, deletion of *EST3* does not inhibit Est1, Est2 and TLC1 RNA from assembling *in vivo* [213]. Since deletion of either *EST1* or *EST2* precludes Est3 assembly with TLC1 RNA, it is widely accepted that Est3 does not, itself, independently interact with the RNA [213]. All of these data together suggest that Est1, Est2 and TLC1 RNA must be in complex before Est3 assembles. It is reasonable to hypothesize that Est3 could interact directly with Est1, Est2 or both.

To begin to understand what interactions Est3 could have within the telomerase complex, I chose to look at a potential interaction between Est3 and Est2. Genetic evidence demonstrates that overexpression of *EST3* specifically suppresses the temperature-sensitive phenotypes of alleles within a defined region of Est2 (termed the TEN domain; amino acids 1-162) [245]. After developing methods to purify recombinant Est3 and $Est2^{TEN}$ protein from *Escherichia. coli* and assessing a number of different mutants for both proteins (Chapter III and data not shown), I found that Est3 interacts directly with the TEN domain of Est2 in *Saccharomyces cerevisiae* and that this interaction depends upon at least three residues within the central region of Est3 (glutamate 114, threonine 115, and asparagine 117) [242]. Interestingly, using complementary approaches (*in vitro* co-purification and *in vivo* co-immunoprecipitation assays) I determined that *EST3* mutants that disrupt telomerase assembly *in vivo* do not necessarily have the same effects in a direct binding assay. For example, mutation of Est3

aspartate 166 and glutamine 167 ($Est3^{DQ}$) resulted in a dramatic decrease in telomerase assembly *in vivo*, but only modestly reduced interaction with the $Est2^{TEN}$ domain *in vitro* (Chapter III-Figures 3.3 and 3.5). This result suggests that the assembly defect *in vivo* is due to perturbation of interactions that lie outside of the Est2 TEN domain [242]. These data also demonstrate that interaction with the TEN domain is insufficient for complex assembly *in vivo*. Mutation of lysine 71 to alanine resulted in the least severe assembly defect *in vivo*, but abrogated binding to the TEN domain *in vitro* (this could be due to a minor disruption in tertiary structure), suggesting that interaction with the Est2 TEN domain is not necessary for assembly *in vivo* and that additional interactions must exist [242]. Combining both *in vivo* and *in vitro* techniques, I have created assays to better determine the individual contributions of Est3 and Est2 to assembly of the telomerase complex.

Making more mutations within each protein and determining their binding phenotypes both *in vitro* and *in vivo* could further characterize the interaction(s) between Est3 and $Est2^{TEN}$. I have attempted to purify other N-terminal regions of Est2 (Regions II and III). Neither of these proteins expresses well in *E. coli*, nor are they soluble in any conditions I have tried. Interestingly, although a small amount of the N-terminal half of Est2 (comprising the TEN domain and Regions II and III) can be expressed, it is rapidly degraded to generate a fragment corresponding to the TEN domain (Kanika Pulliam, unpublished data). This result suggests that Regions II and III may be unstable outside the context of the full-length protein. It is unlikely that either of these regions interacts directly with Est3 since the temperature-sensitive alleles within Regions II and III were not suppressed upon overexpression of Est3 (as was seen for the TEN domain) [245].

Even so, assessing if these regions do have direct interactions with Est3 will be difficult if they cannot be purified. To determine their contribution, the full-length protein would need to be expressed and purified and point mutations made within either Region II or Region III.

Where within the TEN domain of the Est2 protein does Est3 interact? In an attempt to better understand the function of Est2's N-terminus, large alanine block mutations of 10 consecutive amino acids changed to alanine were created within each region (TEN and Regions II and III) of Est2. $Est2^{ala1}$, which is in the TEN domain, retains its ability to extend a primer *in vitro* and bind RNA. However, a strain expressing this allele at WT levels still senesces [98]. This mutant phenocopies strains that are deleted for *EST1* and *EST3*. Co-immunoprecipitation (coIP) experiments revealed that $Est2^{ala1}$ failed to pull-down Est1 or Est3. [245]. Intriguingly, another alanine block mutation within the TEN domain, $Est2^{ala4}$, has preliminarily shown a specific loss of Est3 binding, while Est1 binding is maintained, albeit at a lower level than WT cells (K. Friedman and J. Talley, unpublished data). These results need to be repeated, but suggest that $Est2^{TEN}$ and Est3 may interact within or near the residues mutated in the $Est2^{ala4}$ allele (amino acids 110-119). It is important to note that the ala4 mutant protein is somewhat unstable and supports reduced *in vitro* telomerase activity, most likely explained by a concomitant reduction in TLC1 RNA binding. These phenotypes are probably due to gross changes in protein structure, therefore generating less disruptive mutations is critical for analyzing what contribution the ala4 region of Est2 may have in binding Est3.

Our lab worked with the Center for Structural Biology at Vanderbilt to generate a 3-dimensional model of the *S. cerevisiae* Est2 TEN domain based upon the crystal

structure of the *Tetrahymena thermophila* TEN domain [217]. Based on this model, there are three positively charged residues (lysine 111, lysine 116 and histidine 119) within Est2^{ala4} that are predicted to be surface-exposed and therefore, good candidates for sites of interaction with Est3. Additionally, there are three other alleles already available in the lab: glycine 112 to alanine, alanine 113 to aspartate and the tryptophan 115 to alanine. These mutations can be tested for complex assembly with TLC1 RNA, Est1 and Est3 *in vivo* and recombinant protein generated to test His_6-Est3 binding *in vitro*. Alleles that retain assembly with TLC1 RNA and Est1 while losing Est3 binding *in vivo* would be great candidates to test in the *in vitro* binding assay.

Since it does appear that Est3 makes multiple contacts with telomerase components, the development of additional *in vitro* assays could aid the dissecting of this seemingly complex set of interactions. Full-length Est2 and Est1 can be expressed using rabbit reticulocyte lysate ([212, 224] and J. Ferguson, unpublished data). Therefore, the interaction of Est3 with Est2 and/or Est1 could be assessed using an *in vitro* transcription/translation system. The rabbit reticulocyte lysate system has several advantages: it allows more rapid protein production than expression and purification from *E. coli* (1 day vs. 5-7 days) and the addition of radiolabel is facile (potentially eliminating the need for epitope tags). Single point mutations or large deletions within either Est2 or Est1 could be made and incubated with recombinant His_6-Est3 from *E. coli*. The His-tagged Est3 could be isolated using affinity purification and Est2 or Est1 could be detected using a PhosphoImager. Alternatively, an epitope tag could be cloned on either *EST2* or *EST1* (negating the need for radioactivity) and the reciprocal purification could be attempted and detected using traditional Western blotting techniques.

Another possible means of determining how Est3 is assembling with telomerase is using proteins purified from yeast extract. For example, to understand how Est3 and Est1 might be interacting in an *in vivo* context, yeast protein extract could be made from yeast containing an epitope-tagged version of Est1. His_6-Est3 could be added and then Est1 could be immunopurified from the yeast extract. If recombinant Est3 is co-immunoprecipitated then mutations in either protein could be assessed for their ability to interact with each other. Abigail Riddle, a graduate student in the lab is attempting this type of experiment and having variable success. The major caveat to this experiment is that since other telomerase complex components are present in the yeast extract, determining the specificity of this interaction is difficult. Additionally, because telomerase assembles Est3 in S phase and not earlier, it might be important to enrich for an Est3-competent complex by blocking cells in S or G2 phase. Alternatively, blocking cells in G1 phase using alpha factor and then releasing them to grow synchronously until they reach mid-late S phase and then harvesting them could also enrich for an Est3-competent telomerase.

Est3 as a TPP1 homologue

Though *EST3* was discovered in a genetic screen in 1996 its function within the complex has remained unknown [210]. It has been suggested to unwind DNA/RNA duplexes *in vitro* in a non-sequence specific manner [228]. However, no mutation created within Est3 decreased or changed this function, suggesting that this activity could be due to a contaminant or an indirect mechanism [228]. Recently, it has been demonstrated with NMR that titration of DNA into an Est3 sample does not shift Est3 crosspeaks,

suggesting that Est3 from *S. cerevisiae* is unable to bind DNA directly (or at least Est3 does not bind the DNA primers used in this experiment) [194]. Est3 in *S. castelli* and *C. albicans* influence telomerase activity *in vitro*, though no change in telomerase activity upon loss of the Est3 protein was previously documented in *S. cerevisiae* (the more widely studied yeast species) [194, 248].

When the amino acid sequence of Est3 from *C. albicans* and *S. cerevisiae* was queried against different structural prediction servers, the OB fold domain of TPP1 was the highest-ranking hit, suggesting that the mammalian shelterin protein, TPP1, could be a homologue of Est3 [226, 227]. TPP1 consists of at least three structural domains, one that binds to POT1, another that binds to TIN2, and an N-terminal domain that adopts an OB fold [37]. TPP1 has at least three important functions: (1) it "shelters" the telomere from nucleolytic degradation as a member of the shelterin complex [37]; (2) it works with POT1 to protect the telomere and increase telomerase processivity [33, 264] and (3) it helps localize telomerase to the telomere [252, 265]. Since TPP1 influences processivity and telomerase localization in mammalian cells and the Est3 protein from other yeast species affects telomerase activity, I was interested in determining if Est3 in *S. cerevisiae* influences telomerase function [194, 248, 253]. To address this possibility, I initiated a collaboration with Brian Freeman's lab at the University of Illinois. Since all that is required to do Dr. Freeman's telomerase assay is recombinant protein (such as the Est3 protein I generated for *in vitro* binding assays), I felt that using his techniques could easily assess an influence, if any, Est3 might have on telomerase activity. Strikingly, addition of Est3 to partially purified telomerase from yeast extract caused a robust stimulation of overall telomerase activity in the primer extension assay (Chapter IV). It is

important to note that addition of Est3 did not increase the number of repeats added to the primer (called repeat addition processivity) or change the pattern of telomere addition. These data suggest that Est3 may act at an early step of telomerase activity, by influencing the probability that a primer will be elongated. For example, Est3 may influence appropriate positioning of telomerase on the DNA substrate or may increase telomerase's affinity for the substrate through conformational changes.

Mutations within Est3 that reduced or completely disrupted binding to $Est2^{TEN}$ *in vitro* also negatively affected telomerase stimulation, supporting the idea that assembly of Est3 into the telomerase complex is a prerequisite for Est3 stimulation. Unfortunately, I was unable to identify Est3 variants that retain binding *in vitro* but disrupt stimulation. Identification of such alleles is critical to an understanding of the molecular basis for stimulation and to verify that the stimulatory function observed *in vitro* contributes to telomerase function *in vivo*. Although our lab is very interested in finding this type of mutation, it is possible that no such region or mutation exists within Est3. For example, if assembly of Est3 into the complex induces a conformational change within Est3 that is critical for its stimulation function, then complex assembly and telomerase activation cannot be separated.

I can envision two primary methods of defining the "stimulatory" region of Est3: (1) using available mutation information to generate a number of recombinant Est3 mutants that are tested both for their ability to bind $Est2^{TEN}$ and to stimulate telomerase activity *in vitro* and (2) designing a genetic screen to determine a minimal stimulatory Est3 fragment. The former would require a considerable amount of time and energy and is dependent upon choosing the correct alleles to study. In contrast, the second approach

might allow a larger number of random mutants to be screened. An undergraduate, Leslie Maness, and I worked on the development of a genetic strategy to define a minimal stimulatory Est3 fragment by constitutively fusing Est3 to Est2. After considerable trial and error, we identified an Est2-Est3 protein fusion construct that restores nearly normal telomere length in an *est3*Δ *est2*Δ yeast strain, demonstrating that the fusion protein is functional (L. Maness, unpublished data). We reasoned that forced assembly of Est3 into the complex by fusion with Est2 would bypass mutations that specifically disrupt Est3 assembly, while those that affect stimulation would not be bypassed. Laura Bechard, a post-doctoral fellow in the lab, is currently pursuing this idea by selecting functional alleles from a library of Est2-Est3 fusion proteins containing random deletions within the *EST3* coding sequence. This approach should help determine if there is any region or domain of Est3 that is solely responsible for its stimulation activity.

Implications

In 2009, Drs. Elizabeth Blackburn, Jack Szostak, and Carol Greider won the Nobel Prize in Medicine for their work in understanding how chromosomes are protected by telomeres and the discovery of the enzyme telomerase. Their initial discoveries of telomeres and telomerase have allowed researchers to gain greater insight into how this important and fundamental process of DNA maintenance works. Through the study of both mouse models and human genetics, it has become increasingly apparent that these dynamic DNA structures are critically important during cellular and organismal aging and during development and continued proliferation of cancer cells. [168]. Since telomeres and telomerase are highly conserved in most eukaryotic organisms, the study

of telomerase regulation in yeast has implications for the understanding of similar processes in human cells.

Prior to the work presented in this thesis, little was known regarding the assembly and function of the telomerase component Est3. While this work was in progress, TPP1 was proposed to be an Est3 homologue in higher eukaryotes. However, there was little evidence, beyond structural modeling, that the proteins were functionally related. Through my work, I have determined that Est3 interacts with the catalytic protein subunit of telomerase through its N-terminal TEN domain and have demonstrated that Est3 can stimulate telomerase activity. Both of these functions may be analogous to those of TPP1: TPP1 and TERT (the Est2 homologue in humans) interact and this interaction stimulates the association of TERT with as well as telomerase processivity [252, 253, 265]. These findings lend more credence to the idea that Est3 and TPP1 serve analogous functions.

CHAPTER VII

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