CHARACTERIZING THE ROLE OF THE EST1 PROTEIN IN STIMULATING THE RECRUITMENT OF THE EST3 PROTEIN TO THE YEAST TELOMERASE COMPLEX

By

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CHAPTER I

INTRODUCTION

Significance of telomeres and telomerase

Telomeres are unique repetitive sequences at the ends of linear eukaryotic chromosomes. The strand running 5′ to 3′ (centromere to telomere) is TG-rich and ends in a 3′ overhang (reviewed in Ref. 1). Telomeric sequences provide binding sites for various proteins on both the double- and single-stranded DNA[1-4]. The main function of these protein/DNA complexes is to maintain genomic stability of the cell by capping chromosome ends, thereby protecting them from nucleolytic degradation, recognition as double-strand breaks, and end-to-end fusions. If the capping function of telomeres is lost, chromosome ends fuse, forming dicentric chromosomes that can be broken at random during anaphase, which result in gross genomic instability and usually cell death[1,5-7]. If checkpoint mechanisms are bypassed, this breakage-fusion-breakage cycle can continue, causing wide-spread, large-scale mutagenesis in an event known as crisis. Rarely, cells may activate a telomere maintenance pathway in this stage, halting the breakage-fusion-breakage cycle and establishing a new stable telomere length homeostasis (reviewed in Refs. 1,8; Figure 1). This event is just one hallmark of tumorigenesis. However, since telomere maintenance allows for perpetual self-renewal, these cells can divide indefinitely and need only to acquire mutations in certain other pathways to become cancerous[1,9-11].

Impaired telomere maintenance has also been associated with other disease states, the most common of which is dyskeratosis congenita[1,12]. This condition results from a number of inherited mutations[13,14], displays disease anticipation[15,16], and includes symptoms of nail dystrophy, abnormal skin pigmentation, premature aging, aplastic anemia, and increased susceptibility to cancer[1,12]. In aplastic anemia, proper telomere maintenance is disrupted in bone marrow stem cells, implicating telomeres in the aging process.

Interestingly, telomerase, the enzyme responsible for maintaining telomere length, is expressed in germ cells at relatively high levels throughout the human lifespan, ensuring that offspring are born with telomeres of sufficient initial length[17,18]. In stem cells, telomerase expression decreases over a lifetime, leading to limited self-renewal of stem cells and by extension, tissues that require replacement over time[6]. Somatic cells, in contrast, do not express telomerase, and telomeres in these cells shorten at a relatively fast rate until reaching the point known as the Hayflick limit when cell cycle arrest is triggered[9,19-21]. Cells that continue to divide past this point through the loss of checkpoint control enter crisis, described above (reviewed in Refs. 1,8; Figure 1). Normally, at the time of cell cycle arrest or certainly during crisis, old somatic cells are removed and replaced. It is the reduced renewal potential of stem cells that limits the body's ability to replace somatic cells, which has been linked to aging phenotypes[1].

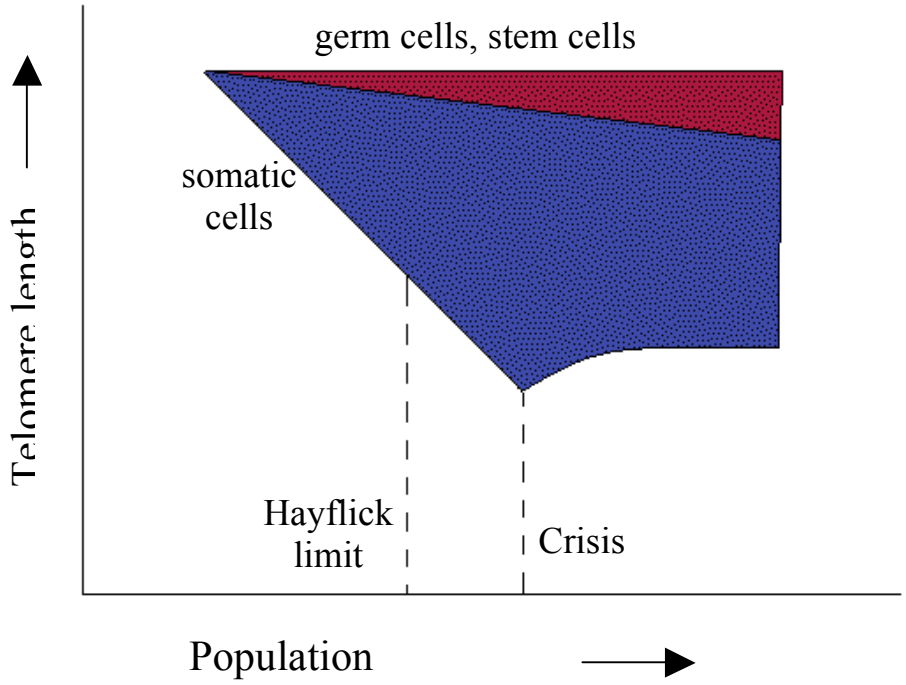

Figure 1. Telomere length in human cells. Germ cells and stem cells express telomerase and maintain relatively stable telomere length over the human lifespan (red). Somatic cells lack telomerase, so their telomeres shorten with cell divisions (blue). At the Hayflick limit, cellular machinery senses short telomeres and triggers cell cycle arrest. If cells bypass this arrest, telomeres continue shortening until the capping function is lost in crisis. Rarely, activation of a telomere maintenance pathway permits cells to exit crisis. If telomere maintenance is achieved by telomerase activity, telomeres are usually maintained at a short but stable length (Modified from Bryan and Cech, 1999) [8]

Considering the apparent importance of telomere maintenance to avoid genomic instability and disease states, significant research effort has been directed at understanding all aspects of telomere biology, from describing the telomere itself to telomerase function and regulation (reviewed in Ref. 1). Telomeres were originally described in the first half of the 20th century when Drs. Hermann Muller and Barbara McClintock independently demonstrated, in flies and maize, respectively, that the ends of linear chromosomes are unique regions of the chromosome, resistant to fusion and necessary for cell viability[22,23]. Muller termed the free ends of the chromosome

"telomeres" in 1938[22]. Years later, as more information was gleaned about the DNA double helix, speculations about telomere replication arose. In the early 1970s, Drs. Alexey Olovnikov and James Watson independently posed the end replication problem, a phenomenon describing the inability of the canonical DNA replication machinery to fully replicate the ends of linear chromosomes[24,25].

Classical DNA polymerases require a free 3′ hydroxyl group as a substrate for nucleotide addition, so an RNA primer is laid down to initiate DNA replication in a 5′ to 3′ direction. Following completion of replication, this RNA primer is removed from the daughter strand of lagging strand synthesis, recreating the single-stranded 3′ overhang required at the chromosome terminus. Leading strand synthesis results in a blunt-ended product that is processed by resection of the 5′ strand to regenerate the 3′ overhang (reviewed in Ref. 26; Figure 2). The double- and single-stranded portions of the telomere are then bound by various proteins to fulfill the protective capping function at chromosome ends[1-4]. However, processing of the 5′ strand results in degradation of terminal sequences and telomere shortening. Unchecked, this gradual shortening continues with successive rounds of DNA replication and leads to loss of telomeric sequences. Fortunately, the majority of the time, the cellular machinery that senses short telomeres inhibits further cell cycle progression, so genetic information is preserved. Additionally, to counteract this end replication problem, cells express the enzyme telomerase. Telomerase is a ribonucleoprotein (RNP) with a reverse transcriptase component that utilizes the internal RNA moiety as a template for nucleotide addition to the single-stranded 3′ overhang at chromosome termini[1].

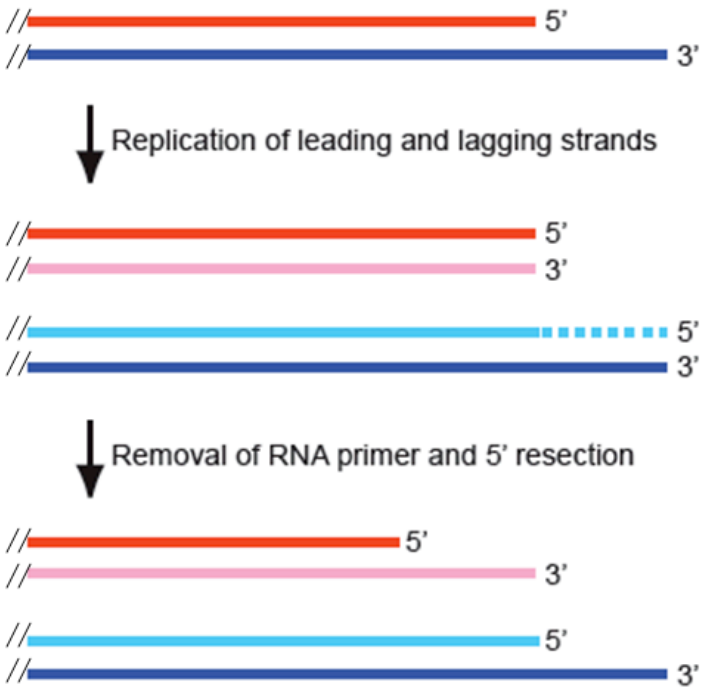

Figure 2. The end replication problem leads to loss of DNA sequence from the leading strand template and chromosome end shortening with successive rounds of DNA replication. An RNA primer is laid down to initiate DNA replication in a 5′ to 3′ direction. At the chromosome ends, the leading strand (red) and newly synthesized complement (pink) form blunt-ended double-stranded DNA, whereas the lagging strand (dark blue) and its complement (light blue) consist of template DNA and an RNA primer. To re-establish the necessary 3′ overhang, the 5′ end of the original leading strand (red) is resected and the RNA primer is removed from the lagging strand complement (light blue). These processing events result in shortened templates for leading strand synthesis in the following round of DNA replication. (Modified from Osterhage and Friedman, 2009)[26]

Telomerase in *Saccharomyces cerevisiae*

In *S. cerevisiae*, the telomerase RNP minimally consists of the TLC1 RNA and three Ever Shorter Telomere (EST) proteins (Figure 3)[27-29]. TLC1 contains the template region for nucleotide addition by the reverse transcriptase Est2p[27,30,31]. TLC1 and Est2p are the only components required for *in vitro* telomerase activity and comprise the

catalytic core of the enzyme[32]. Though not required for *in vitro* activity, the Est1 and Est3 proteins are necessary for *in vivo* telomere maintenance and are thought to serve regulatory functions in the cell[32-35]. Despite having been discovered over two decades ago, there are still many questions concerning precise assembly and function of each component of the yeast telomerase complex.

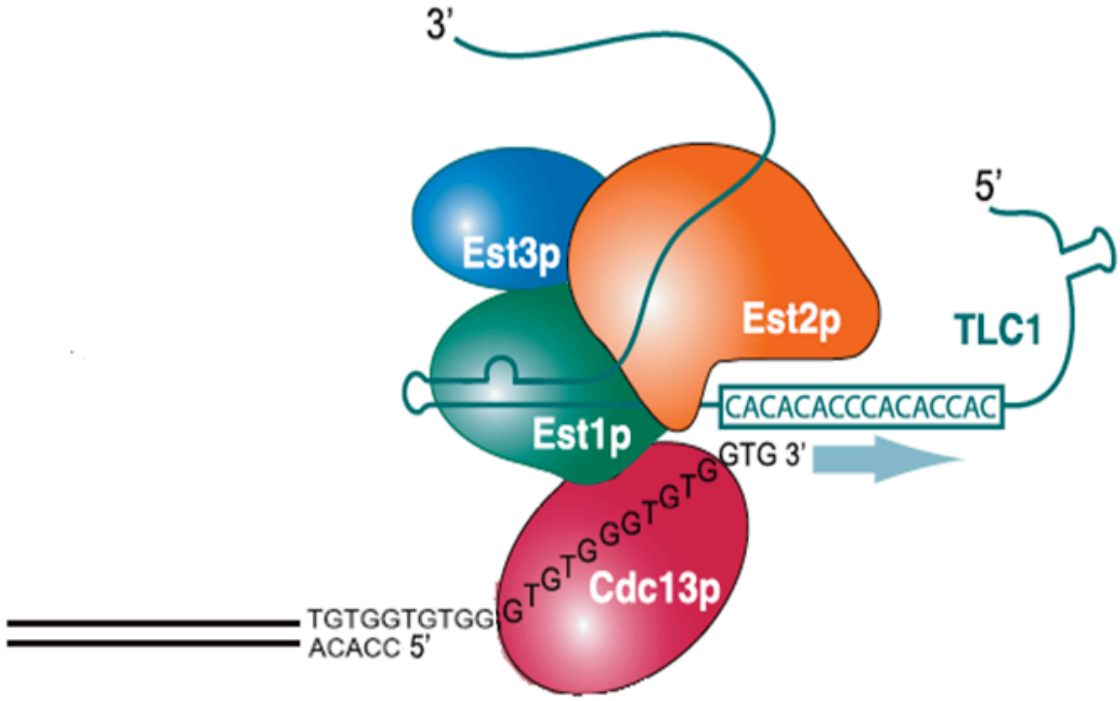

Figure 3. Telomerase complex in *Saccharomyces cerevisiae.* The reverse transcriptase subunit Est2p utilizes the template region of the TLC1 RNA (boxed) to add telomeric repeats to chromosomal termini (indicated by arrow). Est1p and Est3p are regulatory proteins necessary for *in vivo* telomere maintenance but dispensable for *in vitro* enzyme activity. Est1p recruits telomerase to the telomere via its interaction with the single-stranded DNA-binding protein Cdc13. (Modified from Smogorzewska and de Lange, 2004)[2]

The *EST1* gene was identified in a screen for mutant strains that display telomere shortening over time, coincident with reduced and variable growth rates, moderate temperature sensitivity, and increased chromosome loss and cell death[28]. These characteristics comprise the EST (Ever Shorter Telomere) phenotype that was used in a later screen to identify *EST2, EST3,* and *EST4*[29]. The *est4-1* allele was later determined to be an allele of *CDC13, cdc13-2,* disrupted for promoting telomerase access to the telomere[36] (separable from Cdc13p's essential DNA binding-dependent end protection function)[37]. Since Est1p was the first component of yeast telomerase identified, it has been extensively studied over the years. Recombinant Est1p has been shown to weakly bind G-rich oligonucleotides[33,38,39]. This activity requires a free 3′ end, is sequence-specific, and resides between amino acids 435 and 565 of the protein[38]. Est1p also binds a bulged stem loop of the TLC1 RNA in the absence of Est2p or Est3p via an RNA recognition motif between amino acids 456 and 515[33,40-43]. Est1p promotes single-stranded, guanine-rich DNA to form higher order G quadruplex (G quartet) structures *in vitro* and can unwind DNA/RNA heteroduplexes; an EF hand-like motif between amino acids 494 and 554 of the protein is important for these activities[39]. Addition of recombinant Est1p stimulates *in vitro* telomerase activity independent of its ability to bind TLC1 or DNA but dependent upon assembly of the holoenzyme[44]. Est1p has also been shown to interact with other non-telomerase proteins, including the Rfa2p component of Replication Protein A (RPA)[45] and Ies3p of the INO80 chromatin remodeling complex[46], which may serve regulatory functions at the telomere. The essential function of Est1p appears to be recruitment of the telomerase holoenzyme to the telomere terminus via a direct protein-protein interaction with the single-stranded

telomeric DNA-binding protein Cdc13[36,47-50]. This interaction is also needed to bring telomerase to double-stranded breaks for healing by *de novo* telomere addition[51]. Est1p's telomerase recruitment function can be bypassed by artificially bringing the enzyme to the telomere through direct fusion of Cdc13p and Est2p[48]. When the Cdc13p-Est2p fusion is expressed in an *EST1* strain, telomeres over-elongate with respect to wild-type telomeres. Remarkably, expression of Cdc13p-Est2p in an *est1*Δ strain rescues telomere maintenance, supporting the hypothesis that Est1p is required for the recruitment of telomerase to the chromosome end. However, telomere over-elongation no longer occurs in the absence of Est1p, suggesting that Est1p may contribute an additional function required for robust telomerase activity[48].

In 2002, a study of *est1* mutants created by scanning alanine block mutation revealed several classes of *est1* alleles[52]. The first class caused telomere shortening or senescence, but supported telomere over-elongation in the presence of a Cdc13p-Est2p fusion. This class most likely affects the telomerase recruitment function of Est1p. Another class displayed reduced association with telomerase, and some of those mutants fail to localize correctly to the nucleus (C. Hawkins and K. Friedman, in preparation). One class of mutants, including the *est1-Δ19* allele, was concluded to be disrupted for an additional, unknown function of Est1p. These mutants have similar telomere length and growth defects as the first group of mutants but do not display telomere over-elongation in the presence of the Est2-Cdc13 fusion protein. These data hint at another important role of Est1p not rescued by artificially tethering telomerase to the telomere, possibly an activation function. Data from *in vitro* studies using *Candida albicans* showed that EST1 was important for initiation of telomerase activity and elongation of primers[53], supporting

an activation function of the Est1 protein. Interestingly, apart from the conserved tetratricopepetide repeat (TPR) domain at the N terminus of the protein[35,54,55], all of these proposed functions map to fewer than two hundred of Est1p's 699 amino acids (Figure 4). The regions specified for different protein functions greatly overlap in the context of the amino acid sequence and add a significant layer of complexity as the field tries to identify separation-of-function alleles of *EST1* that each affects only one of its numerous functions in the cell.

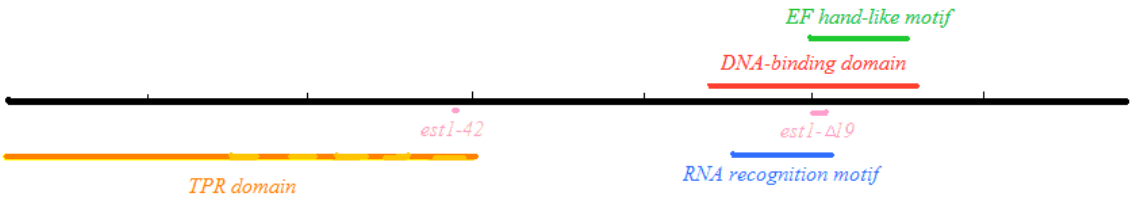

Figure 4. Domain map of Est1p suggests that a small region within the C terminus of the protein is involved in several biochemical activities. Shown are the DNA-binding domain (red): residues 435-565[38]; RNA recognition motif (blue): residues 456-515[33]; tetratricopeptide repeat (TPR) domain (orange; dashed region most conserved): residues 1-300[35,54]; *est1-Δ19* and *est1-42* (pink): residues 499-518 (deleted to create *est1-Δ19*) and 287, 290, 291, 292 (mutated to alanine to create *est1-42*)[52]; EF hand-like motif (green): residues 494-554[39].

The TLC1 RNA is approximately 1.3 kilobases in size, with the template for telomere elongation forming a single-stranded region surrounded by multiple stem-loop structures[27,56]. Est2p binds TLC1 outside the 3′ and 5′ template boundaries for synthesis of telomeric sequence[56]. Like other yeast telomerase RNAs, TLC1 contains several regions necessary for proper RNP assembly and structural stability outside the template sequence[57]. A conserved bulged stem loop from nucleotides 535 to 707 is required for Est1p association with the RNA, which is independent of the Est2p-TLC1 interaction[41,42].

Another stem loop is necessary for binding of the Ku70/80 heterodimer[58], and a single-stranded portion of the RNA is bound by the Sm proteins[59]. Data examining modifications to the placement of TLC1 structural elements indicate that this RNA molecule is a flexible scaffold for protein subunit binding; the long stem-loop structures can be swapped in position relative to the template while still retaining function[56]. Additionally, nearly two-thirds of this large RNA molecule are dispensable *in vivo* and *in vitro*[50].

Est2 is a 102 kDa protein with a conserved reverse transcriptase (RT) domain and less highly conserved extensions at both the N and C termini[30,31,61,62]. The RT domain contains the essential catalytic activity of Est2p[30,31], whereas the C-terminal extension is mostly dispensable in yeast (although it does contribute to normal levels of activity)[63]. The N-terminal extension (NTE), specific to telomerase reverse transcriptases, is important for interacting with the RNA component and the DNA substrate, as well as regulating the catalytic activity of the protein[61,63-69]. The NTE contains conserved motifs originally termed Regions I, II, and III[63]. Region I, the most N-terminal of these motifs, is now called the Telomerase Essential N-terminus (TEN) domain and has been implicated in protein-protein interactions[70,71]. There are also data suggesting that Est2p may serve a protective function by capping telomeres during part of the cell cycle[35,72,73].

Until very recently, little was known about Est3p. *EST3* was cloned by complementation and found that the functional, 181-amino acid gene product is produced by a stimulated translational frameshift[43,74,75]. Other data have suggested that recombinant Est3p dimerizes *in vitro,* and this activity may be important for *in vivo* telomere maintenance[76]. However, mutants compromised for dimerization have not been

thoroughly examined for disruption of more recently proposed roles of the Est3 protein, and phenotypes from this study may be attributable to protein misfolding. In the past year, Est3p has been shown to associate with both Est2p and Est1p *in vitro,* as well as stimulate *in vitro* primer extension by telomerase[77,78].

Assembly of the yeast telomerase complex/holoenzyme

Telomerase activity is regulated throughout the cell cycle. Telomere elongation is restricted to late S and G_2 phases[79,80] due in part to the temporal association of telomerase with the telomere, a phenomenon that has been extensively detailed by the Zakian lab using chromatin-immunoprecipitation (ChIP)[72,81,82]. To summarize, in G_1 phase, Est2p and TLC1 are associated with the telomere via the direct interaction between the double-stranded DNA-binding protein heterodimer Ku70/80 and TLC1 RNA[58,72,81,83]. As the cycle continues, Est2p and TLC1 association decreases but peaks again in late S phase, coincident with the time at which telomeres are elongated[72,79,80]. Cdc13p binding also increases in S phase, when the single-stranded 3′ overhang is longest[72,84]. Est1p's temporal association with the telomere is dictated by the cell cycle-dependent regulation of its protein levels. In G_1 phase, Est1p levels are very low due to proteasome-dependent degradation of the protein. As the cell cycle progresses into S phase, Est1 protein levels increase, allowing for association with the telomere and telomerase core[85]. Est3p telomere association generally follows the binding profile of Est1p, but is detectable to a slight degree in G_1 phase[78]. The manner in which Est3p assembles into the telomerase complex is currently a topic of debate in the field, one that I have chosen to address from the standpoint of the Est1 protein and how Est1p and Est3p interact.

The Lundblad group has published several experiments suggesting that Est3p can associate with the holoenzyme independently of Est1p. In each case, Est3p assembly with telomerase was assessed by its ability to co-immunoprecipitate (co-IP) the TLC1 RNA. Est3p co-immunoprecipitated TLC1 in the absence of Est1p but was unable to co-IP the RNA when *EST2* was deleted from the strain[34,43]. This association was also tested with different alleles of *TLC1*. Est2p cannot bind tlc1-59, while Est1p retains its ability to associate with this mutant RNA. In contrast, the *tlc1-47* and *tlc1-Δ535-770* alleles disrupt the Est1p-binding portion of the RNA, but interaction with Est2p is retained. Like Est2p, the Est3 protein could not co-IP the tlc1-59 mutant. In contrast, Est3p retained association with tlc1-47 and tlc1-Δ535-770 even though Est1p did not[34]. However, these results may represent background binding instead of a specific interaction. These data support the conclusion that Est3p assembly with the telomerase complex is dependent upon the Est2 protein but independent of Est1p.

Data from the Friedman lab suggest that Est1p is both necessary and sufficient for Est3p to associate with telomerase[85]. First, Est2p's ability to co-immunoprecipitate Est3p is drastically reduced in an *est1Δ* strain and in G_1-arrested cells, when Est1 protein levels are substantially reduced. Second, Est3p demonstrates greatly reduced binding with the tlc1-Δ535-770 RNA, an allele lacking the stem necessary for Est1p to associate with TLC1. The source of differences in results from those obtained by the Lundblad lab is unclear, though contradictions may be attributable to experimental design, including differences in protein tags, stringency and efficiency of immunoprecipitation, and use of certain controls. Finally, the association between Est2p and Est3p is greatly increased when Est1 protein is over-expressed or stabilized in G_1 phase by addition of the

proteasome inhibitor MG132. Supporting the notion that these proteins do interact *in vivo,* a recent study has shown that Est1p and Est3p interact directly *in vitro*[78].

One way to reconcile these results is to suggest that Est1p is not absolutely essential for Est3p to assemble with telomerase. Indeed, in experiments reported by the Friedman lab, Est3p retains a small amount of binding (7%) to the tlc1-Δ535-770 RNA[85]; and Est3p has been shown to directly interact with Est2p[77]. Each of these results indicates that Est3p can associate with telomerase without Est1p, though its assembly is severely reduced in the absence of Est1p *in vivo*. Therefore, I hypothesize that Est1p **stimulates** the recruitment of Est3p to the telomerase complex. In the hopes of potentially resolving this question in the field, I proposed to identify the residues in Est1p responsible for this stimulatory function using a combination of *in vivo* and *in vitro* analyses and genetic screens. To date, numerous tools have been developed to map these residues, and preliminary results are presented in this thesis.

CHAPTER II

MATERIALS AND METHODS

Primer sequences are listed in Table 1, yeast strains in Table 2, and plasmids in Table 3. Restriction enzymes were obtained from New England Biolabs (NEB).

Plasmids for over-expressing alleles of *EST1*

Plasmid pKF600 is the over-expression vector used for the *in vivo* assembly assay and details of its construction can be found in the Appendix. Briefly, *GALp-HA$_3$-EST1* was cloned from pRS423 (2µm *HIS3*) into pRS425 (2µm *LEU2*). The *EST1* open reading frame is flanked by an N-terminal *Bam*HI site and C-terminal *Sph*I site for cloning of previously constructed C-terminal deletion mutants (A. Sathiyakumar) from pRS423 into pKF600. These mutants included *EST1 CΔ100, EST1 CΔ115, EST1 CΔ136, EST1 CΔ150, EST1 CΔ200,* and *EST1 CΔ250. EST1 CΔ161* and *EST1 CΔ179* were amplified from pKF600 with 242bamhIfor paired with EST1del161 and EST1del179, respectively, and cloned into the *Bam*HI and *Sph*I sites of pKF600. The *est1-Δ19* allele was cloned from pVL1633 (2µm *TRP1*) into pKF600 using restriction sites *Nde*I and *Nru*I because the deletion in the *est1-Δ19* allele was marked with a *Bam*HI site.

Plasmids for screening alleles of *EST1*

Plasmids pKF610 and pKF611 were created as follows. pRS416 *EST1* and pRS416 *HA$_3$-EST1* (*CEN URA3*) were digested with *Kpn*I, *Sac*I, and *Alw*NI, and the

resulting 3.1 kilobase (kb) and 3.4 kb bands, respectively, were cloned into the *Kpn*I and *Sac*I restriction sites in pRS315 (*CEN LEU2*). To remove an additional *Bam*HI site in the multiple cloning site, pRS315 *EST1* and pRS315 *HA$_3$-EST1* were digested with *Pst*I and *Xba*I, which flank the extra *Bam*HI site. The ends were blunted by T4 DNA polymerase (NEB) and ligated, resulting in the final plasmid products. Both of these plasmids can be used for screening alleles of *EST1*, but pKF611 contains an N-terminal HA_3 tag for immunoprecipitation of Est1p as needed. The *est1-Δ19* allele was cloned from pKF600 *est1-Δ19* into pRS315 *EST1* and pRS315 *HA$_3$-EST1* using *Bsp*EI restriction sites.

Yeast strains for screening alleles of *EST1*[*]

All gene replacements were performed by transforming specified constructs into the appropriate strain by a standard lithium acetate protocol.

Integrating the Est2-Est3 fusion protein

EST2-G$_8$-EST3 was amplified from the pKF428 fusion plasmid (2µm *TRP1*) with the Xba2 Rev and Est3 Fusion Rev primer pair. The *NAT^R* drug resistance marker was amplified from pFA6a *NAT*-MX6 with primers Fusion NAT For and NAT Est2 Rev. These products were used as template to create a single PCR product using outside primers Xba2 Rev and NAT Est2 Rev. The final *EST2- G$_8$-EST3 NAT^R* product was transformed into the *EST2* locus of YKF122 + pKF431 and YKF122 + pKF441, creating YKF122 fusion Short and YKF122 fusion Long, respectively (C. Wiser and S. Lawrence).

[*] Work from this section was contributed by graduate rotation students as indicated in the text: Caroline Wiser, Stacey Lawrence, Derrick Cumberbatch.

Integrating the *TEF* promoter

The plasmid pKF408 trunc contains a C-terminally truncated version of *EST2* that was created to destroy the *Spe*I site within the C terminus of the *EST2* gene, leaving only the *Spe*I site in the promoter region for integration of the plasmid into the genome. pKF408 (integrating vector *URA3 EST2*) was partially digested with *Spe*I; the linear product was digested to completion with *Xba*I; and the resulting ends were ligated together, creating pKF408 trunc (S. Lawrence). A PCR product containing the *TEF* promoter and the first 12 amino acids of Est2p was created using pKF426 *TEF* as template and the primer pair of Est2 TEF For and Est2 TEF Rev (C. Wiser). This product was cloned into pKF408 trunc using the restriction enzymes *Spe*I and *Hind*III to create pKF408 trunc *TEF*. The sequence of the insert was confirmed by sequencing with the M13R universal primer. pKF408 trunc *TEF* was linearized with *Spe*I and integrated into YKF122 fusion Short and YKF122 fusion Long (S. Lawrence and D. Cumberbatch).

Two-step integration of the *TEF* promoter was confirmed by Southern analysis. Genomic DNA was digested with *Hinf*I and separated by gel electrophoresis on a 0.8% LE agarose (Lonza) gel with 0.003% ethidium bromide run at 80V for 6 hours. Primers H primer For and 3 rev were used to amplify an ~500 base pair (bp) portion of the N terminus of *EST2*, which was randomly-labeled to probe the membrane as previously described[70]. The endogenous *EST2* promoter yields a band at ~900 bp while the *TEF* promoter at the *EST2* locus yields a band of 1.2 kb.

Creating an *est1Δest2Δest3Δ* strain

To create the *est* triple mutant strain, pKF428 fusion replaced the pKF444 plasmid in YKF122 (*est3::KAN*^{R}) *est2::HYG*^{R} + pKF444 *EST3-EST2*. The endogenous *EST1* gene was replaced with *NAT*^{R} drug resistance amplified from pFA6a *NAT*-MX6 using primers EST1 TRP For (OP9806) and EST1 KO Rev2. YKF122 *est2::HYG*^{R} *est1::NAT*^{R} + pKF428 fusion was then complemented with pRS416 *EST1*, resulting the strain named 1,2,3Δ + 428 fus + 416 E1.

Increasing the number of HA tags on Est3p

Plasmid construction

EST3-HA$_3$ with a unique *Kpn*I restriction site between *EST3* and the triple hemagglutinin tag was cloned from pKF442 (*CEN URA3*) into pRS306 (integrating vector *URA3*) using *Pvu*II sites flanking the multiple cloning site of each vector. The oligos TopHA-Sfo-HA and BotHA-Sfo-HA were annealed and treated with T4 polynucleotide kinase (NEB). The double-stranded product of these reactions contains a *Kpn*I restriction site used to clone the *HA*$_2$ construct into pRS306 *EST3-HA*$_3$ one or more at a time. pRS306 *EST3-HA*$_7$ was the result of inserting two *HA*$_2$ constructs in the same orientation, into which another *HA*$_2$ construct was cloned, to create the final plasmid pRS306 *EST3-HA*$_9$. The sequence of *EST3-HA*$_9$ was confirmed with the Est3R primer (OP6589) to ensure that the *EST3* open reading frame (ORF) was frameshift-corrected for production of the full-length protein and that the nine hemagglutinin tags were all the correct sequence and orientation. Using *Pvu*II restriction sites, *EST3-HA*$_9$ was cloned

into the pRS416 vector (2μm *URA3*). The *NAT*^{R} drug resistance marker was amplified from pFA6a *NAT*-MX6 with the XbaI F1 and XbaI R2 primer pair and then cloned into pRS416 *EST3-HA*$_9$ using *Xba*I to make pRS416 *EST3-HA*$_9$ *NAT*^{R}.

Strain construction

The *bar1::KAN*^{R} construct was amplified from genomic DNA isolated from AVL78 *bar1::KAN*^{R} using the primer pair Bar1 For (BAR2529) and Bar1 Rev (BAR2530) and transformed into AVL78 *Myc*$_9$*-EST2* creating AVL78 *Myc*$_9$*-EST2 bar1::KAN*^{R}. pRS416 *EST3-HA*$_9$ was digested with *Eco*RV and *Nde*I. The *EST3-HA*$_9$ *NAT*^{R} portion was purified and transformed into AVL78 *bar1::KAN*^{R} and AVL78 *Myc*$_9$*-EST2 bar1::KAN*^{R} to make AVL78 *EST3-HA*$_9$ *bar1::KAN*^{R} and AVL78 *Myc*$_9$*-EST2 EST3-HA*$_9$ *bar1::KAN*^{R}.

Complementation analysis

Functional complementation of *EST3-HA*$_9$ was tested in YKF122 (*est3::KAN*^{R}). Loss of the complementing pKF441 plasmid was selected on 5-fluoroorotic acid plates, and pRS416 *EST3-HA*$_9$ was subsequently transformed using the standard lithium acetate method. Single colonies were passaged three times on media lacking uracil. Cell viability was assessed visually, and telomere length was determined by Southern blot as previously described[70,77]. Here, *Xho*I was used to digest genomic DNA, which was then separated on a 1.2% LE agarose (Lonza) gel run at 80V for 15.5 hours. Telomeres were detected with a randomly-labeled telomeric probe.

Immunoprecipitation and Western analysis

500mL or 1L cultures containing pKF600-bearing alleles of *EST1* were grown to an OD_{600} ~0.5 in 2% (w/v) raffinose media lacking leucine. Cells were arrested by addition of 1mM alpha factor (Zymo Research) for 3-4hrs, and cell morphology assessed by microscopy. At OD_{600} ~0.8, galactose was added to a final concentration of 0.2% for 1hr. Whole cell extract was prepared by glass bead lysis as previously described[70,85]. 500µL of protein extract were normalized to 20mg/mL and incubated with 20µL c-myc rabbit polyclonal antibody (A-14, Santa Cruz Biotechnology) at 4ºC for 1hr before addition of 60µL Protein G sepharose beads (GE Healthcare) prewashed four times with equilibration buffer 1 (10 mM Tris-HCl at pH 8, 1 mM $MgCl_2$, 10% glycerol) and once with equilibration buffer 2 [10 mM Tris-HCl at pH 8, 1 mM $MgCl_2$, 10% glycerol, 200mM NaCl, 0.05% Tween-20, RNasin (1:1,000 dilution; Promega)]. Samples were incubated with beads for 4hrs at 4ºC and then washed 5 times for 5 minutes with 0.5mL wash buffer 1 (10 mM Tris-HCl at pH 8, 1 mM $MgCl_2$, 10% glycerol, 200mM NaCl, 0.05% Tween-20) and once for 10 minutes with 0.5mL wash buffer 2 [10 mM Tris-HCl at pH 8, 1 mM $MgCl_2$, 10% glycerol, 50mM NaCl, RNasin (1:1,000 dilution; Promega)]. Washes with buffer 1 were performed such that 100µL of supernatant was left when pipetting off waste. Remaining beads were resuspended in 60µL of buffer 2, and 25µL were aliquotted for Western blotting and stored at -80˚C. For electrophoresis, the supernatant was removed from immunoprecipitation samples, and the beads mixed with 2X Laemmli loading buffer (125 mm Tris-HCl at pH 6.8, 4% SDS, 0.05% bromophenol blue, 20% glycerol, 5% β-mercaptoethanol). Samples were mixed gently two to three times while incubated at 100°C for 4min. The supernatant was immediately loaded on a

10% to 12% step gradient gel for separation by SDS-PAGE. Proteins were transferred to Hybond P membrane (GE Healthcare). The membrane was blocked with 5% milk/phosphate buffered saline at pH 7.4 with 0.05% Tween-20 (PBS-T) followed by incubation at 4ºC overnight with primary antibodies: murine monoclonal HA.11 (1:500 dilution; Covance) and murine monoclonal Myc Ab-1 (1:250 dilution; 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. ECL plus Western Blotting Detection system (GE Healthcare) was used for detection.

Table 1. Oligonucleotide primers

Primer name	Primer sequence 5´-3´
242 bamHI for	CCAAGCTTAAAAATGGGATCCTAATGGATAACGAAG
EST1del161	GAGTGCATGCTCATGGAGAAAGCATCTTAGTCAATG
EST1del179	GAGTGCATGCTCAGGGAGAATCATACACATAATCG
Xba2 rev	CATTCGTTCTTACAACGCATCATTGA
Est3 Fusion rev	TTAATTAACCCGGGGATCCGTCATAAATATTTATATACAA ATGGGAAAGT
Fusion NAT for	ACTTTCCCATTTGTATATAAATATTTATGACGGATCCCCGG GTTAATTAA
NAT EST2 rev	GCAATATTTTCCTTATCAGCATCATAAGCTGTCAGTATTTC ATGTATTATTAGTAGATATCATCGATGAATTCGAGCTC
Est2 TEF for	CTACTTTACTAGTTAGTTTACTTCCATGAATACAAAAGCAA AAATCATATACGGAGCTCATAGCTTCAAAATG
Est2 TEF Rev	CAATGTCAAGCTTGTCTTGAATGAACTCGAATAAGATTTTC ATGTTCTAGAAAACTTAGATTAGATTGC
M13R	CAGGAAACAGCTATGAC
H primer For	GAGTTCATTCAAGACAAGCTTG
3 rev	GAGGATGATCGTTGAGCCCATTTG
EST1 TRP For (OP9806)	GCAAACTTATCAGGGGAAAAAGTATATTCCATTAAATGA CACATGCCACCATAGATACGGATCCCCGGGTTAATTAA
EST1 KO Rev2	CGTCGTCATAATATATTTCATATTATGATTTTTTCCCTCAC CATTACTTGTTCTCGATATCATCGATGAATTCGAGCTCG
Top HA-Sfo-HA	CATGTACCCGTATGATGTCCCAGACTATGCCCCTGGCG CCCCCTATCCTTACGACGTTCCGGACTACGCATGTAC
Bot HA-Sfo-HA	ATGCGTAGTCCGGAACGTCGTAAGGATAGGGGGCGCCA GGGGCATAGTCTGGGACATCATACGGGTACATGGTAC
EST3R (OP6589)	CTTTATATATGTAGATAAACGAAG
XbaI FI	CATCATTCTAGACGGATCCCCGGGTTAATTAAGG
XbaI R2	ATGATGTCTAGACATCGATGAATTCGAGCTCG
Bar1 For (BAR2529)	GTTTATAGATAACGGCTCTTGC
Bar1 Rev (BAR2530)	CGTTTGGTTAGTTCAGCTAGG

Table 2. Yeast strains

Strain	Genotype	Source
AVL78	*MATa leu2 trp1 ura3-52 prb1 prc1 pep4-3*	V. Lundblad
YKF122	AVL78 *est3::KAN^R*	J. Talley
YKF122 *est2::HYG^R*	YKF122 *est2::HYG^R*	J. Talley/L. Maness
1,2,3Δ	AVL78 *est3::KAN^R est2::HYG^R est1::NAT^R*	This study
YKF110	AVL78 *EST3-HA$_3$*	K. Benton
AVL78 *Myc$_9$-EST2*	AVL78 *Myc$_9$-EST2*	M. Platts
AVL78 *bar1::KAN^R*	AVL78 *bar1::KAN^R*	G. Todd
AVL78 *Myc$_9$-EST2 bar1::KAN^R*	AVL78 *Myc$_9$-EST2 bar1::KAN^R*	This study
AVL78 *EST3-HA$_9$ bar1::KAN^R*	AVL78 *EST3-HA$_9$ bar1::KAN^R*	This study
AVL78 *Myc$_9$-EST2 EST3-HA$_9$ bar1::KAN^R*	AVL78 *Myc$_9$-EST2 EST3-HA$_9$ bar1::KAN^R*	This study
YKF122 fusion	AVL78 *est3::KAN^R est2::EST2-G$_8$-EST3*	This study

Table 3. Plasmids

Plasmid	Genotype	Source
pFA6a *NAT*-MX6	*NAT^R Amp^R*	T. Graham
pKF408	integrating vector *URA3 EST2*	K. Friedman
pKF408 trunc	integrating vector *URA3 EST2* minus C terminus from internal *Spe*I site	This study
pKF408 trunc *TEF*	integrating vector *URA3 EST2* minus C terminus and *EST2* promoter replaced with *TEF* promoter	This study
pKF428 fusion	2µm *TRP1 EST2-G$_8$-EST3*	J. Talley/L. Maness
pKF431	*CEN URA3 EST3* (ORF flanked by *Sac*I and *Kpn*I sites	M. Platts
pKF441	*CEN URA3 EST3* (no N-terminal *Sac*I site)	J. Talley
pKF442	*CEN URA3 EST3-HA$_3$* (no *Sac*I site)	M. Platts
pKF444	2µm *URA3 EST3-EST2*	J. Talley/L. Maness
pKF600	2µm *LEU2 GALp-HA$_3$-EST1*	This study
pKF610	*CEN LEU2 EST1* (no *Bam*HI site in MCS)	This study
pKF611	*CEN LEU2 HA$_3$-EST1* (no *Bam*HI site in MCS)	This study
pRS306	integrating vector *URA3*	T. Graham
pRS306 *EST3-HA$_3$*	integrating vector *URA3 EST3-HA$_3$*	This study
pRS306 *EST3-HA$_7$*	integrating vector *URA3 EST3-HA$_7$*	This study
pRS306 *EST3-HA$_9$*	integrating vector *URA3 EST3-HA$_9$*	This study
pRS315	*CEN LEU2*	T. Graham
pRS315 *EST1*	*CEN LEU2 EST1*	This study
pRS315 *HA$_3$-EST1*	*CEN LEU2 HA$_3$-EST1*	This study
pRS315 *est1-Δ19*	*CEN LEU2 est1-Δ19*	This study
pRS315 *HA$_3$- est1-Δ19*	*CEN LEU2 HA$_3$- est1-Δ19*	This study
pRS416	*CEN URA3*	T. Graham
pRS416 *EST1*	*CEN URA3 EST1*	J. Ferguson
pRS416 *HA$_3$-EST1*	*CEN URA3 HA$_3$-EST1*	J. Ferguson
pRS416 *EST3-HA$_9$*	*CEN URA3 EST3-HA$_9$*	This study
pRS416 *EST3-HA$_9$ NAT^R*	*CEN URA3 EST3-HA$_9$ NAT^R*	This study
pRS423 *GAL-HA$_3$-EST1*	2µm *HIS3 GALp-HA$_3$-EST1*	A. Sathiyakumar
pRS425	2µm *LEU2*	T. Graham
pVL1633	2µm *TRP1 est1-Δ19-Myc$_{18}$*	V. Lundblad

CHAPTER III

RESULTS AND DISCUSSION

Identifying a putative Est3p recruitment domain in Est1p

Previous analysis indicates that Est1p stimulates, but is not absolutely required, for the assembly of Est3p with yeast telomerase[85]. The goal of this study is to identify residues of the Est1 protein that are required for stimulating the recruitment of Est3p to the complex. I sought separation-of-function alleles of *EST1* disrupted solely for this stimulatory function by screening for Est1p mutants that retain association with the catalytic core (Est2p and TLC1 RNA) but lack the ability to stimulate Est3p recruitment. I hypothesized that such alleles of *EST1* could be isolated with the *in vivo* approaches described below and that characterization of these alleles might elucidate the regulation of Est3p assembly with telomerase.

Over-expression of Est1p increases the co-immunoprecipitation of Est3p with Est2p, an effect that is exaggerated in G_1-arrested cells. At this time in the cell cycle, endogenous Est1p is degraded in a proteasome-dependent manner, and Est3p association with the telomerase core is below the level of detection[85]. From these observations, it was concluded that the cell cycle-regulated reduction in Est1 protein levels temporally restricts telomerase assembly, with Est1p as the limiting factor in complex formation. Over-expression of Est1p in G_1 phase facilitates recruitment of Est3p to the telomerase holoenzyme, allowing me to detect differences in association of the telomerase components. I took advantage of this phenomenon to screen for Est1p alleles unable to

stimulate Est3p recruitment. Plasmid-born alleles of *HA_3-EST1* under control of a galactose-inducible promoter were transformed into strains containing integrated copies of *Myc_9-EST2* and *EST3-HA_3* (and expressing endogenous, untagged *EST1*). HA_3-Est1p over-expression was induced upon addition of galactose to G_1-arrested cultures. Myc_9-Est2p was immunoprecipitated from whole cell extract, and the co-immunoprecipitation (co-IP) of HA_3-Est1p and Est3p-HA_3 was monitored via Western blot.

While protein over-expression can lead to artifacts, it is often necessary due to the relatively low abundance of the four telomerase holoenzyme components[78,86]. The over-expression system used here has several advantages. First, it allows for simultaneous assessment of the ability of an Est1p variant to retain association with Est2p and its ability to stimulate the association between Est2p and Est3p. Second, untagged endogenous Est1p is present, so the effect of mutant alleles of *EST1* can be monitored without the concern of cellular senescence. Third, over-expression makes visualization and manipulation of the complex constituents easier. For example, over-expression of different alleles of *EST1* during G_1 phase (when the endogenous protein is degraded) forces the telomerase complex to assemble with the mutant Est1 proteins, ensuring that the effect of the mutant protein can be specifically examined.

Although the residues required for the interaction of Est1p with Est2p have not been specifically mapped, a former lab member had previously observed that degradation products of Est1p retained the ability to co-IP with Myc_9-Est2p (J. Osterhage, unpublished data). These variants were detected by the HA antibody and therefore corresponded to C-terminal degradation products of the N-terminally tagged HA_3-Est1p. These observations suggested that the C terminus of Est1p is not required for Est2p

association. It was reasoned that analysis of progressively larger deletions from the Est1p C terminus might serve as a method to identify a region of Est1p required to stimulate recruitment of Est3p to the telomerase complex. A series of truncation mutants was constructed with amino acids deleted from the C terminus in 50-amino acid blocks (i.e.: Δ50, Δ100, Δ150, …Δ500) and analyzed in the assembly assay described above for loss of co-IP of Est3p-HA_3. Another previous lab member preliminarily showed that deletion of the C-terminal 150 amino acids of Est1p resulted in loss of Est3p-HA_3 co-immunoprecipitation, whereas deletion of 100 amino acids from the Est1p C terminus retained the co-immunoprecipitation of Est3p-HA_3 (A. Sathiyakumar, unpublished data).

I extended these preliminary studies by creating additional truncation variants and by altering the experimental conditions to improve protein detection. Upon initial examination, I found that the Est1CΔ150 protein was not reproducibly co-immunoprecipitated by Myc-Est2p. Later sequencing revealed an unrelated mutation that likely disrupted protein folding such that it could no longer associate with the telomerase core. Analysis with other deletion mutants showed that Est3p-HA_3 co-IP was lost when the C-terminal 200 amino acids of Est1p were deleted but was retained when 136 amino acids were deleted from the C terminus of Est1p (Figure 5). While these data suggest that a putative Est3p recruitment domain lies between residues 499 and 563 within the Est1 protein, no single technical repeat with all the pertinent samples provided quantifiable signals for the tagged Est proteins.

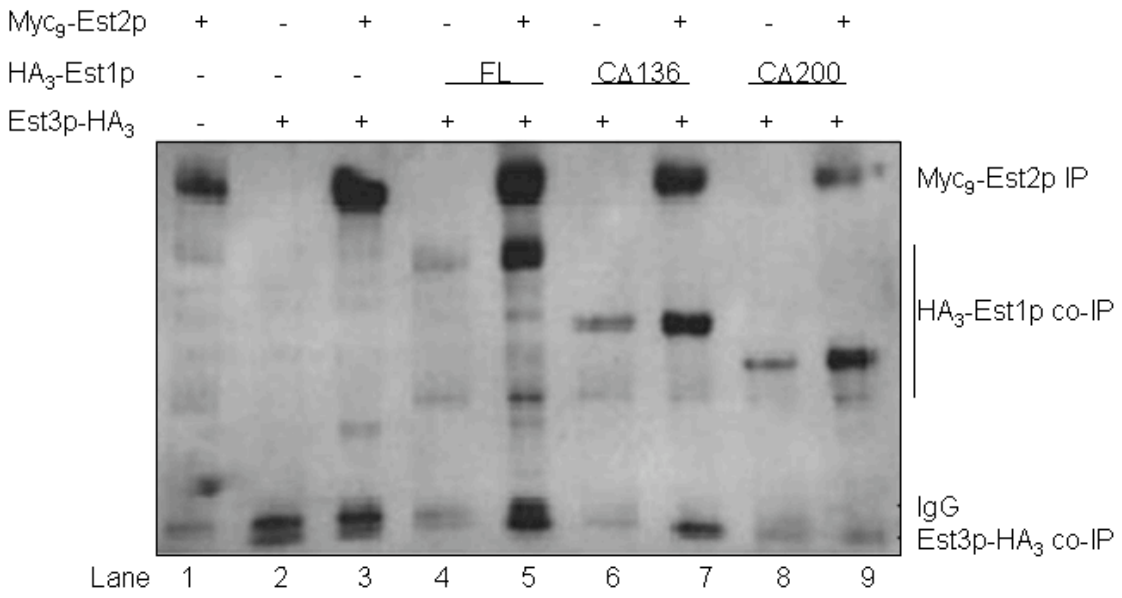

Figure 5. Co-immunoprecipitation of Est3p-HA$_3$ is lost when the C-terminal 200 amino acids of Est1p are deleted. Strains expressing Est3p-HA$_3$ or Est3p-HA$_3$ and Myc$_9$-Est2p were arrested in G$_1$ phase with alpha factor. Over-expression of the indicated alleles of *HA$_3$-EST1* was induced by addition of galactose. Myc$_9$-Est2p was immunoprecipitated from whole cell extract using Protein G sepharose beads bound to α-c-Myc. Proteins were separated by gel electrophoresis on a 10% SDS polyacrylamide gel. Western membrane was probed with α-c-Myc and α-HA antibodies.

Protocol development

Two issues prevented the acquisition of reproducible and quantifiable data. First, I observed background binding of Est3p to the beads, even in the absence of a Myc tag on Est2p. Second, background signal from the light chain of IgG interfered with the detection of Est3p-HA$_3$ because these two proteins migrate very similarly on the SDS-PAGE gels used here. Despite using antibodies from different sources for the immunoprecipitation and detection, IgG cross-reacts with the α-Myc primary antibody. Combined, these two issues made it difficult to identify specific co-immunoprecipitation of Est3p. Several approaches to circumvent these issues were pursued.

To address the background binding by Est3p, the extract concentration was reduced prior to immunoprecipitation (IP). I also determined a baseline of Est3p-HA_3 background binding to the beads. In these "beads only" samples, Protein G sepharose beads (without antibody) were added to protein extract. The volume of beads added to the extract was titrated to find a level that minimized the Est3p-HA_3 background signal. Increasing the detergent and salt concentrations in the wash buffers and using different detergents were methods also explored to reduce non-specific interaction of Est3p-HA_3 during the IP procedure.

To improve protein detection via Western blotting, primary antibody concentrations were altered in combination with changes to the concentration of milk and detergent in the blocking solution. Detergent concentration in the wash buffers was also changed to decrease interference.

The main approaches used to address the light chain interference were crosslinking and improving separation between IgG and Est3p-HA_3. The Pierce Crosslinking Immunoprecipitation Kit was utilized to crosslink the Myc probe to Protein G sepharose beads for immunoprecipitation of Myc_9-Est2p. Preliminary results suggested that while the IgG light chain signal was reduced by crosslinking, so was the efficiency of the immunoprecipitation (data not shown). However, there remain several options for potential optimization for using this kit. These include, but are not limited to, using a different combination of wash buffers (either from the kit and/or from lab immunoprecipitation protocols) and exploring other methods of elution and sample preparation for Western analysis, taking into consideration guidelines from the crosslinking kit.

Extensive work was done to resolve the issue of separation between the IgG light chain and Est3-HA_3 protein. Initially, the percentage of acrylamide in the gels and the duration of electrophoresis were altered, but with no success. Samples were then run on Invitrogen pre-cast gradient gels, which did improve detection of Myc_9-Est2p and HA_3-Est1p, but did not resolve the light chain interference. The best separation between the IgG background and Est3p-HA_3 was achieved when samples were separated on a 10% to 12% step gradient polyacrylamide gel, though even here, separation was variable and did not lend itself to consistently interpretable (quantifiable) results.

The sum of these efforts at optimizing the *in vivo* assembly assay allowed me to develop a protocol for the best quality results using the strains and tools available at the time. The specifications of this protocol are described in Materials and Methods, and the best results are shown in Figure 6.

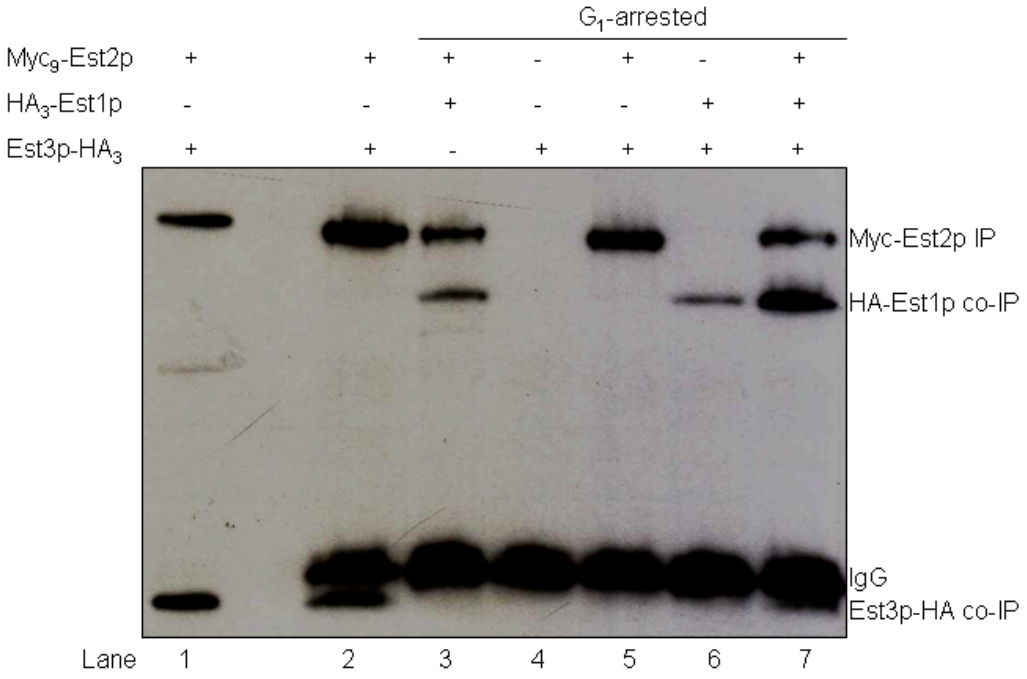

Figure 6. Est3p-HA$_3$ and the light chain of IgG are best separated on a step gradient gel. Strains expressing Est3p-HA$_3$ or Est3p-HA$_3$ and Myc$_9$-Est2p were arrested in G$_1$ phase with alpha factor as indicated. Over-expression *HA$_3$-EST1* was induced by addition of galactose. Whole cell extract was made by glass bead lysis. Myc$_9$-Est2p was immunoprecipitated using Protein G sepharose beads. Proteins were separated by gel electrophoresis on a 10% to 12% step gradient SDS polyacrylamide gel. Western membrane was probed with α-c-Myc and α-HA antibodies. Lane 1 is input from asynchronously growing cells in rich media. Lanes 2-7 are Myc IP samples; the sample in Lane 2 was immunoprecipitated from the extract shown in Lane 1. The higher molecular weight band in Lane 1 is a background band detected by the α-HA antibody. Myc-Est2p is not detected under these conditions in whole cell extract (data not shown).

Increasing the epitope size on Est3p

Because all attempts to eliminate interfering signal from the IgG light chain were unsuccessful, I decided the best method to clearly detect differences in the co-immunoprecipitation of Est3p was to increase the size of the epitope on the protein. I hypothesized that increasing the tag on Est3p from three to nine hemagglutinin molecules

would both allow for a stronger signal from Est3p on the Western blot and result in an Est3 protein that runs at a higher molecular weight than the IgG light chain for substantial separation of these proteins during gel electrophoresis. *EST3-HA$_9$* was integrated at the endogenous *EST3* locus in strains with and without endogenously-tagged *Myc$_9$-EST2*.

EST3-HA$_9$ complements the growth and telomere length defects of a strain lacking endogenous *EST3* when expressed from a low copy vector (Figure 7). Strains with integrated *EST3-HA$_9$* have shorter telomeres than a wild-type strain, similar to the shortening observed in a *Myc$_9$-EST2* strain. Telomere shortening was exacerbated when both *EST3-HA$_9$* and *Myc$_9$-EST2* were expressed in yeast (Figure 8). Est3p-HA$_9$ was confirmed to migrate at a clearly distinguishable molecular weight from the light chain of IgG and at a lower molecular weight than the Est1CΔ250 protein, the smallest of the Est1p deletion mutants analyzed with the assembly assay (Figure 9). Myc$_9$-Est2p co-immunoprecipitated Est3p-HA$_9$ at levels clearly above background in asynchronously growing cells (Figure 10).

These data indicate that the Est3-HA$_9$ protein is a promising tool for assessing the ability of mutant Est1 proteins to stimulate the co-immunoprecipitation of Est3p. The Est3-HA$_9$ protein product gives a stronger signal by Western blotting than the original allele (HA$_3$) and migrates considerably more slowly in a gel than the light chain of IgG (Figure 9), negating the concern for interference from IgG as separation of these two proteins is easily accomplished. This latter observation may also prove useful in the *in vitro* binding assay optimization described later. Notably, a strain expressing the new *EST3-HA$_9$* allele has been utilized in the lab to probe the interaction between Est3p and Est2p. In this instance, ProA-tagged alleles of *EST2* are expressed in the strain with

integrated *EST3-HA*$_9$; ProA-Est2p is immunoprecipitated from whole cell extract; and the co-immunoprecipitation of Est3p-HA$_9$ is determined by Western analysis. Encouragingly, the signal of Est3p-HA$_9$ is quantifiable in these experiments and has been used to determine quantitative differences in the ability of various Est2p mutants to co-immunoprecipitate Est3p-HA$_9$ (L. Bechard, unpublished data).

Unfortunately, co-expression of Est3p-HA$_9$ and Myc$_9$-Est2p enhances the telomere shortening effect that each epitope confers individually (Figure 8). Such short telomeres at the start of further manipulations to the strain could lead to senescence before whole cell extract can be harvested. Specifically, cells expressing both tagged proteins seem to grow poorly when the *GALp-HA*$_3$*-EST1* over-expression vector is introduced (data not shown), perhaps due to the requirement for growth in selective media. Further adjustments to improve the growth of this strain are needed to fulfill the potential of using Est3p-HA$_9$ for these studies. One possibility is to incorporate a linker between Est3p and the series of HA tags, a strategy that has been shown to improve telomere length maintenance upon tagging of other yeast telomerase components[78,87].

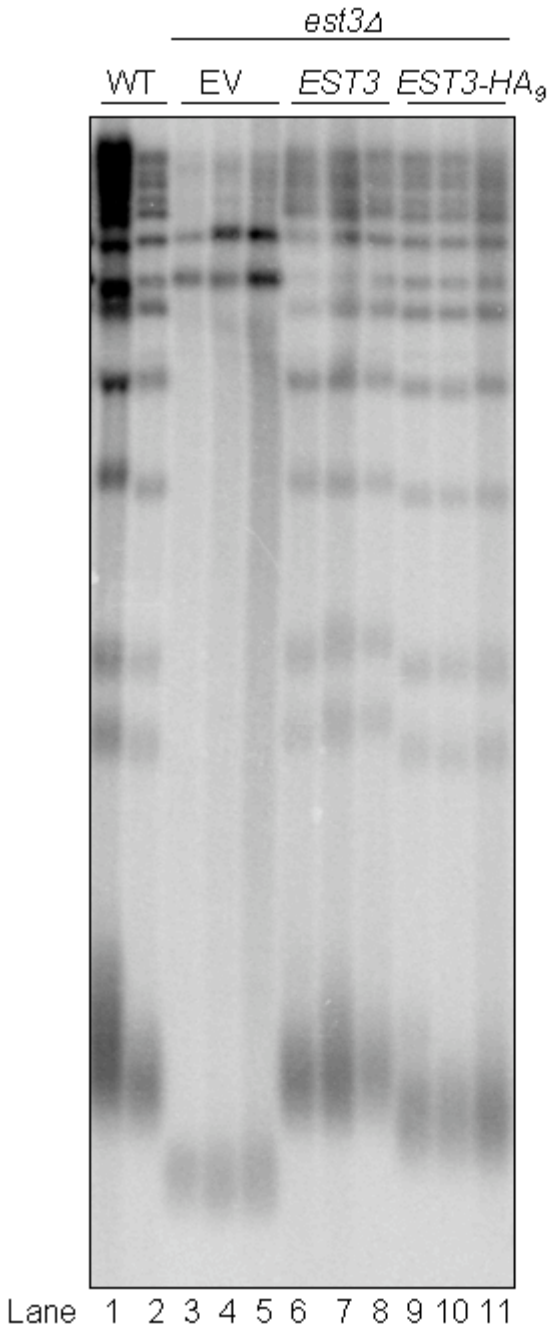

Figure 7. Est3p-HA$_9$ complements the telomere length defects of a strain lacking endogenous *EST3*. Genomic DNA was isolated by glass bead lysis and phenol-chloroform extraction, digested with *Xho*I, and separated by gel electrophoresis. Telomeres were detected by a randomly-labeled probe. Lanes 1 and 2: wild-type AVL78 cells grown in rich media. For lanes 3-11, cells were passaged three times on media lacking uracil to maintain the specified plasmid; all vector backbones are pRS416 (*CEN URA3*). Lanes 3-5: three independent transformants containing empty vector (EV); lanes 6-8: three independent transformants containing an *EST3* complementing plasmid; lanes 9-11: three independent transformants containing a plasmid expressing *EST3-HA$_9$*.

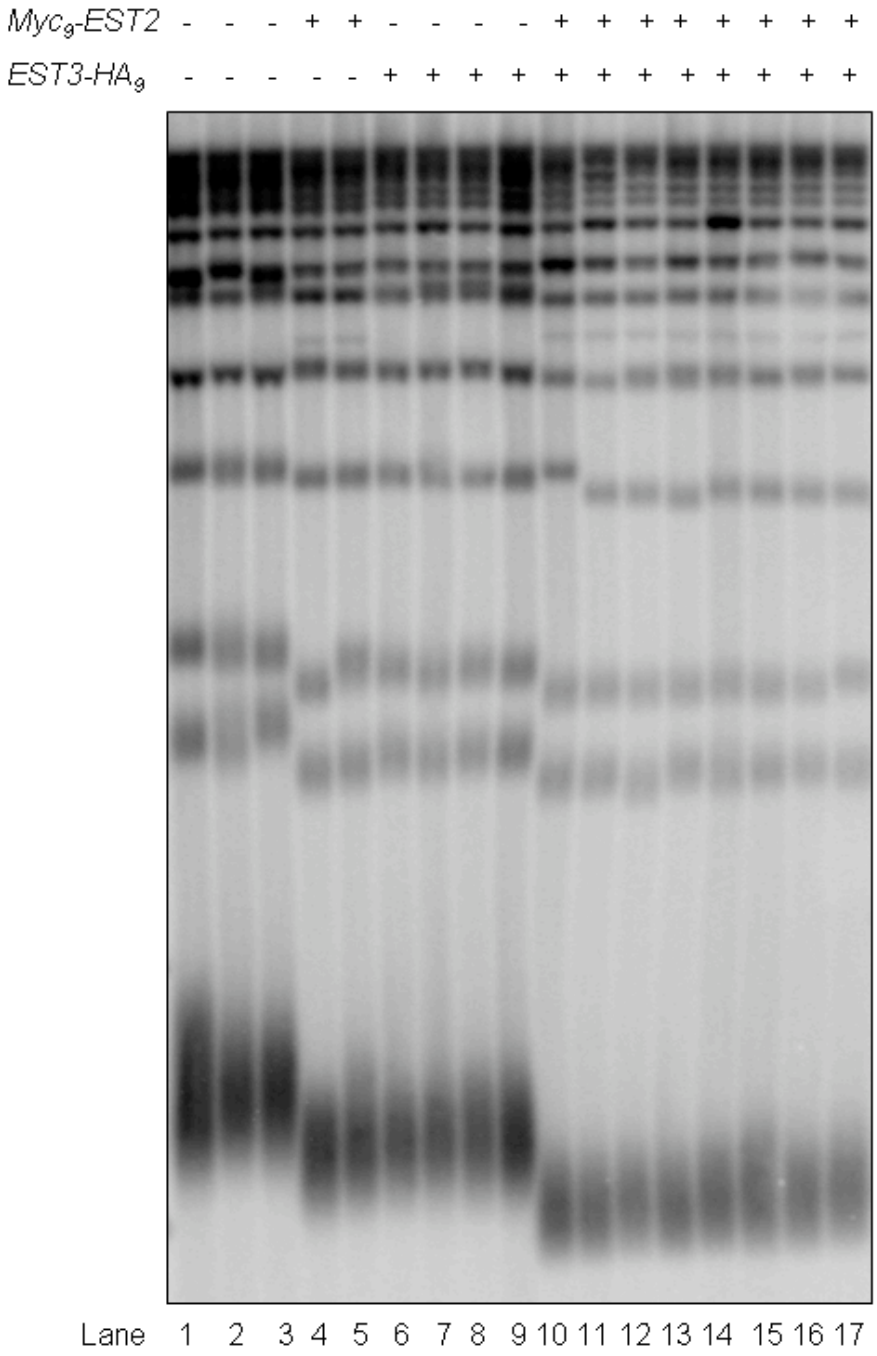

Figure 8. Epitopes on telomerase proteins cause telomere shortening. Each lane represents an independent colony that was passaged three times on rich media. Genomic DNA was isolated by glass bead lysis and phenol-chloroform extraction, digested with *Xho*I, and separated by gel electrophoresis. Telomeres were detected by a randomly-labeled probe. Tagged alleles were integrated at the endogenous locus; strains without tags retained endogenous genes. Lane 1: wild-type AVL78; Lanes 2 and 3: AVL78 *bar1::KAN*^{R}; Lanes 4 and 5: AVL78 *Myc*$_9$*-EST2 bar1::KAN*^{R}; Lanes 6-9: AVL78 *EST3-HA*$_9$ *bar1::KAN*^{R}; Lanes 10-17: AVL78 *Myc*$_9$*-EST2 EST3-HA*$_9$ *bar1::KAN*^{R}.

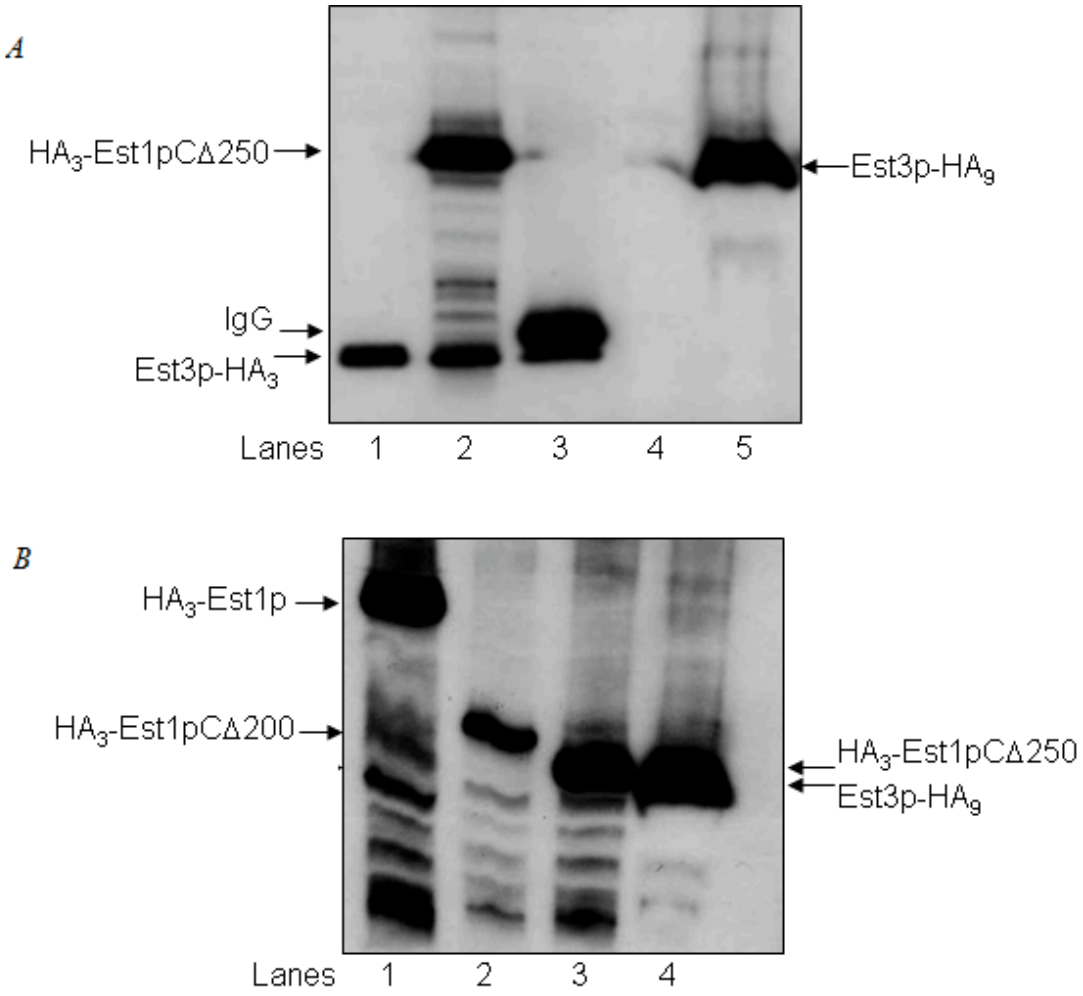

Figure 9. Est3p-HA$_9$ migrates slower than the IgG light chain but faster than Est1pCΔ250. Proteins were separated on a 10% SDS polyacrylamide gel and detected with α-HA. **A.** Lanes: 1) Extract from a strain with endogenously-tagged *EST3-HA$_3$* arrested in G$_1$ phase, 2) Extract from a strain with endogenously-tagged *EST3-HA$_3$* and plasmid overexpressing *HA$_3$-EST1Δ250,* arrested in G$_1$ phase, 3) Myc IP from a strain with endogenously-tagged *Myc$_9$-EST2* and *EST3-HA$_3$* grown asynchronously, 4) Protein isolated by trichloroacetic acid (TCA) precipitation from an *est3Δ* strain expressing plasmid-born, untagged *EST3*, 5) TCA prep from the same *est3Δ* strain expressing plasmid-born *EST3-HA$_9$*. **B.** Lanes 1-3: Extract from strains over-expressing plasmid-born *HA$_3$-EST1, HA$_3$-EST1CΔ200,* or *HA$_3$-EST1CΔ250*, respectively. Lane 4: TCA prep from *est3Δ* strain expressing plasmid-born *EST3-HA$_9$* (same as in Panel **A**, Lane 5). **Note:** The gels here were run for the same duration, but in future analyses, electrophoresis time will be increased to run the IgG light chain off the gel for greater separation between Est3p-HA$_9$ and Est1pCΔ250.

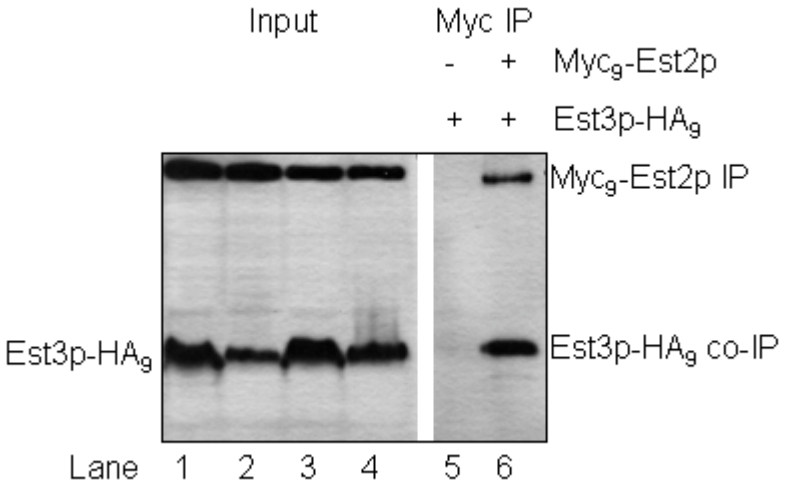

Figure 10. Myc$_9$-Est2p specifically co-immunoprecipitates Est3p-HA$_9$. Strains expressing Est3p-HA$_9$ or Est3p-HA$_9$ and Myc$_9$-Est2p were grown asynchronously in rich media. Whole cell extract was made by glass bead lysis. Myc$_9$-Est2p was immunoprecipitated using Protein G sepharose beads. Proteins were separated by gel electrophoresis on a 10% SDS polyacrylamide gel. Western membrane was probed with α-c-Myc and α-HA antibodies. Lanes 1 and 2 are extract samples obtained before (lane 1) and after (lane 2) incubation with the beads from the sample in Lane 5. Lanes 3 and 4 were taken before (lane 3) and after (lane 4) incubation with the beads from the sample in Lane 6. The higher molecular weight band in Lanes 1-4 is a background band detected by the α-HA antibody.

Screening for *est1* alleles disrupted specifically for stimulating Est3p recruitment[†]

As development of the assembly assay continues, screening for *est1* alleles unable to stimulate Est3p recruitment is a highly complementary approach. I have designed two genetic screens to identify alleles of *EST1* disrupted solely for the ability to stimulate recruitment of Est3p to the telomerase complex. The first of these screens utilizes an Est2-Est3 fusion protein expressed from the *EST2* locus in a strain lacking endogenous *EST3* and the other uses a galactose-inducible allele of *EST3* integrated at the *EST1* locus.

[†] Work from this section was contributed by graduate rotation students as indicated in the text: Caroline Wiser, Stacey Lawrence, Derrick Cumberbatch.

In both cases, *est1* alleles generated by random or directed mutagenesis are screened for suppression of growth defects by expression of the Est2p-Est3p fusion or by over-expression of Est3p. Alleles of *EST1* lacking the ability to stimulate Est3p recruitment are expected to display telomere loss and senescence in the presence of endogenously expressed Est2p and Est3p, but growth and telomere length are expected to be rescued by co-expression with the Est2-Est3p fusion protein and/or over-expressed Est3p.

We reason that expression of Est2p and Est3p as a single protein will eliminate the requirement for Est1p in recruiting Est3p to telomerase. This fusion protein is not expected to bypass any other function of the Est1 protein, and we predict that Est1p has other essential functions. Therefore, alleles isolated here are expected to be separation-of-function alleles specifically disrupted for stimulating Est3p recruitment. Importantly, the Est2p-Est3p fusion containing an eight-glycine (G_8) linker between the individual polypeptides is competent for telomere maintenance; it complements the growth and telomere length defects of cells lacking endogenous *EST2* and *EST3* when expressed from a high copy number plasmid. Note that the telomere length is almost fully rescued, but not completely to wild-type length (J. Talley and L. Maness, unpublished data).

For use in my screen, the Est2p-Est3p fusion needed to be the only version of the Est2 and Est3 proteins expressed in the cell. Endogenous Est2p and Est3p may compete with the fusion protein for interaction with Est1p mutants, decreasing the chance of suppression. To create the appropriate strain, *EST2-G_8-EST3* was integrated into the endogenous *EST2* locus of a strain lacking *EST3* (C. Wiser). The resulting strain expresses only fused Est2p and Est3p driven by the *EST2* promoter. However, Southern

blot analysis revealed that this strain maintains very short telomeres (C. Wiser, S. Lawrence, unpublished data).

With such short telomeres, we hypothesized that rescue of any defect due to Est1p mutants by the Est2p-Est3p fusion would have to be complete, or nearly so; otherwise the cells would senesce and form survivors (which maintain telomeres via a recombination-based mechanism)[88]. While this strain background could provide a very stringent test for rescue by the fusion protein, I was also interested in finding mutants of Est1p that may not be fully rescued by the Est2-Est3 fusion protein. To allow for the possibility of isolating such alleles of *EST1,* we sought to increase the steady-state telomere length in the Est2-Est3 fusion strain and simultaneously pursued the approaches below.

First, we reconstructed the fusion-expressing strain by integrating *EST2-G_8-EST3* into another *est3Δ* strain containing a different *EST3* complementing plasmid that permits maintenance of nearly wild-type length telomeres. The final fusion-containing strain maintains telomeres near wild-type length (S. Lawrence, unpublished data).

Second, we speculated that driving higher expression of the Est2-Est3 fusion protein may permit the cell to maintain longer telomeres. This idea was based on the observation that the Est2p-Est3p fusion complemented the telomere length of an *est2Δest3Δ* strain when expressed from a high copy number vector better than when expressed from a centromere plasmid (J. Talley and L. Maness, unpublished data). To increase Est2-Est3 protein levels, we designed a method to replace the *EST2* promoter with the constitutively active *TEF1* promoter directly upstream of the *EST2-G_8-EST3* fusion by two-step integration. To date, the first step of integration of the *TEF* promoter at the *EST2-G_8-EST3* locus has been confirmed by Southern blot (D. Cumberbatch,

unpublished data); however, strains in which recombination has replaced the *EST2* promoter with the *TEF* promoter have not yet been isolated.

The last step in creating the strains for this screen is to replace endogenous *EST1* with the hygromycin-resistance gene (HYG^R). The resulting strains will express only fused Est2p and Est3p and express *EST1* from a *URA3*-marked complementing plasmid that can be selected against when mutagenized *est1* alleles are introduced into the cell on a *LEU2*-marked plasmid (Figure 11). I first want to test the deletion alleles created for the *in vivo* assembly assay in this fusion-expressing strain. Because the C-terminal truncation mutants were originally constructed in an over-expression vector and driven by the *GAL1* promoter, I have constructed a centromere (low copy) vector with compatible restriction sites that will allow easy transfer of these alleles to a vector suitable for my genetic screens.

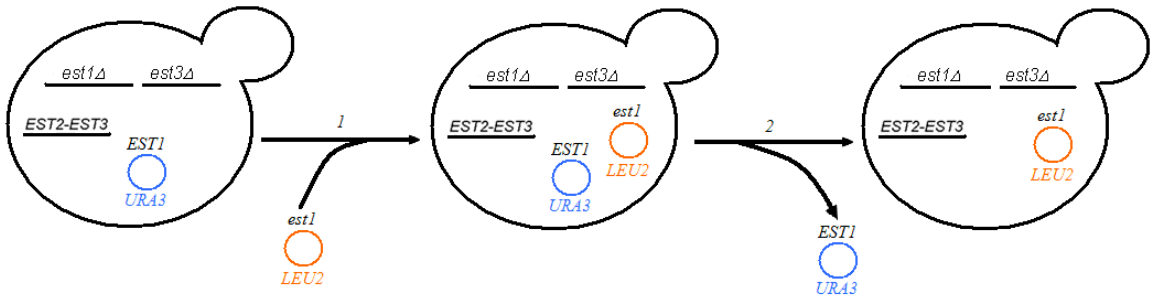

Figure 11. Schematic of plasmid shuffle to screen plasmid-born alleles of *EST1* for rescue by the Est2-Est3 fusion protein. A strain lacking endogenous *EST1* and *EST3* with the *EST2-G$_8$-EST3* fusion integrated into the genome at the *EST2* locus contains a *URA3*-marked *EST1* complementing plasmid that is maintained by growth on media lacking uracil. Alleles of *EST1* contained on a *LEU2*-marked centromere plasmid are transformed into this strain (1), and both plasmids are maintained by plating on media lacking uracil and leucine. Loss of the complementing plasmid is selected by plating on 5-fluoroorotic acid (2). Surviving cells are passaged on media lacking leucine for observation of normal growth or senescence.

I have created a strain lacking endogenous *EST1, EST2,* and *EST3,* but while working through that process, I tested the feasibility of using the Est2-Est3 fusion protein as a tool for screening *est1* mutants with growth and telomere length complementation analysis. The caveat of this particular experiment is that the fusion protein is present in addition to endogenous *EST2* and *EST3*. Specifically, a *TRP1*-marked empty vector or high copy number vector with *EST2-G$_8$-EST3* was co-transformed into an *est1Δ* strain with a *LEU2*-marked *EST1* complementing plasmid or low copy number plasmid containing the previously published *est1-Δ19* allele[52]. Cells were then passaged on media lacking tryptophan and leucine for observation of growth phenotypes before determining telomere length by Southern blot. The Est2p-Est3p fusion was unable to rescue the senescence and telomere loss of a strain completely lacking *EST1*. This result was expected, since this fusion protein should not be able to bypass Est1p's other functions, such as recruitment of telomerase to the telomere. In contrast, when the Est2p-Est3p fusion was co-expressed with wild-type Est1p, cells continued to grow normally and telomeres were maintained at nearly wild-type length. Similar to the empty-vector control, cells co-expressing *EST2-G$_8$-EST3* and *est1-Δ19* senesced and formed survivors (Figure 12).

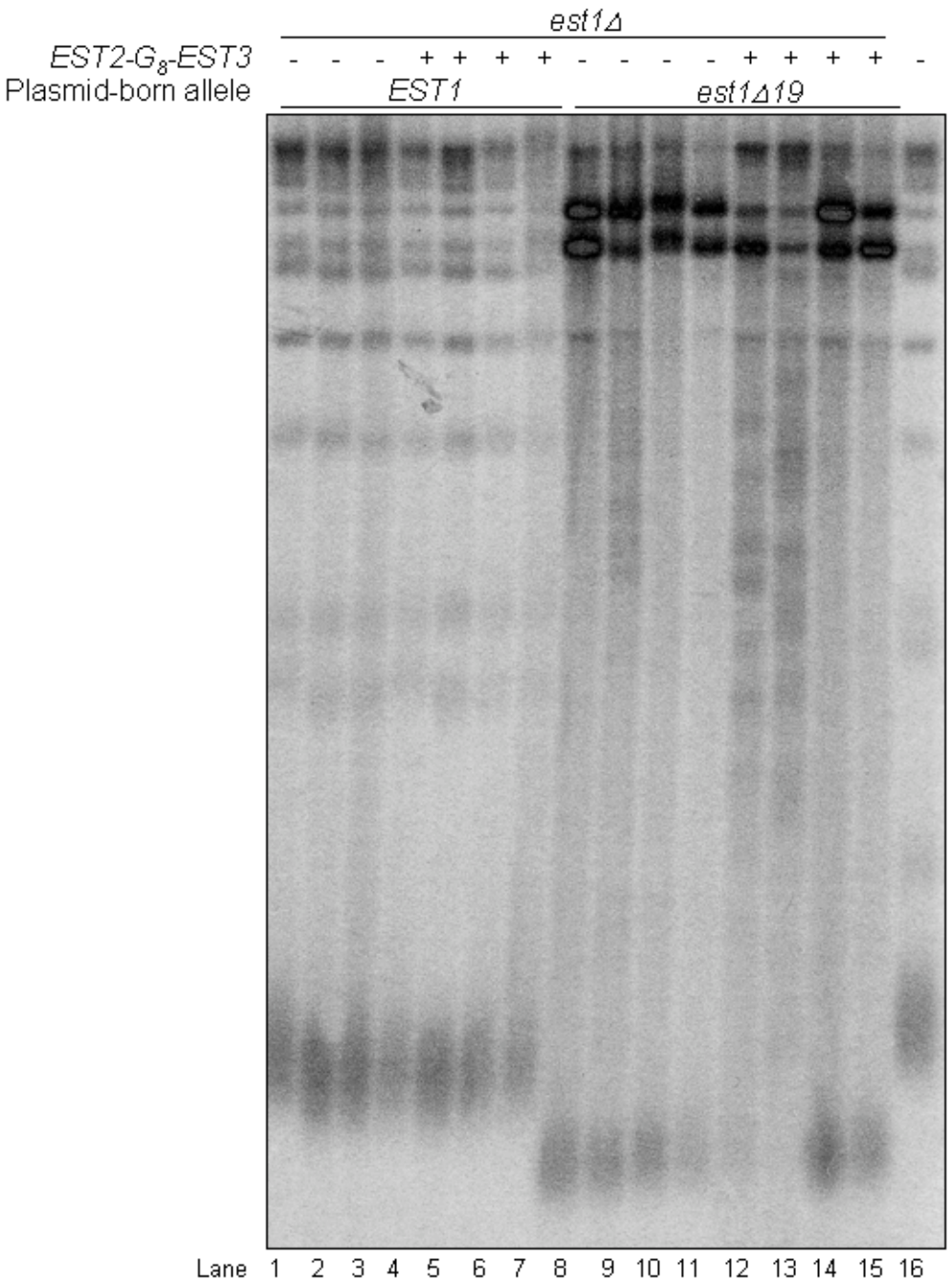

Figure 12. *EST2-G$_8$-EST3* does not suppress the telomere shortening of the *est1-Δ19* allele. Plasmid-born *EST2-G$_8$-EST3* and either *EST1* or *est1Δ19* were co-transformed into a strain lacking endogenous *EST1*. Each lane represents a single transformant that was passaged five times on media lacking tryptophan and leucine. Genomic DNA was isolated by phenol-chloroform extraction, digested with *Xho*I, and separated by gel electrophoresis. Telomeres were detected by a randomly-labeled probe.

As described above, the *est1-Δ19* allele is hypothesized to be defective for an activation function of Est1p. *est1-Δ19* contains a deletion of amino acids 499 to 518, a region within Est1p's proposed DNA- and RNA-binding domains and the EF hand-like motif needed for G quadruplex formation (Figure 4). Because this deletion is predicted to compromise several functions of Est1p, it is not surprising that *EST2-G_8-EST3* fails to rescue the growth and telomere length defects of *est1-Δ19*. Therefore, the results shown in Figure 12 support the notion that the Est2-Est3 fusion protein does not bypass functions of Est1p other than stimulating Est3p recruitment. However, until an allele of *EST1* that is rescued by the fusion protein is isolated, we cannot be sure that the Est2p-Est3p fusion can suppress any function of Est1p.

The second screening method tests whether *est1* phenotypes can be rescued by *EST3* over-expression. Here, alleles of *EST1* disrupted for stimulating Est3p recruitment are hypothesized to grow normally and maintain telomeres when Est3p is over-expressed, but senesce when Est3p is expressed at its endogenous levels. Strain construction for this screen is straightforward. Briefly, the *GAL1* promoter, *EST3* open reading frame (ORF) and terminator, and NAT^R gene will be amplified as a single PCR product with flanking homology to the *EST1* locus. Integration of this construct into the yeast genome will simultaneously remove endogenous Est1p and introduce an inducible allele of *EST3* (in addition to the endogenous *EST3* gene). Combining these steps reduces the required number of nutritional or drug-resistance markers and circumvents the need for additional plasmids (in contrast to the experiment shown in Figure 12). The resulting strain will be complemented with a *CEN URA3 EST1* plasmid. For this screen, the same *est1*-bearing plasmids mentioned above will be transformed on media containing galactose. Loss of

the *EST1* complementing plasmid will be selected on 5-FOA. Single colonies will be restreaked onto selective media containing galactose. Surviving colonies will then be passaged on selective media containing glucose, inhibiting over-expression of Est3p. Those strains that survive upon galactose but undergo senescence on glucose may contain alleles of *EST1* that are specifically defective in Est3p recruitment to the telomerase complex.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

In this study, I have pursued a variety of approaches to address the role of the Est1 protein in the assembly of Est3p with the telomerase holoenzyme. The Friedman laboratory has published that Est1p is both necessary and sufficient for Est3p assembly with the complex[85], and the Zakian group has obtained chromatin immunoprecipitation data consistent with this model[72,81]. However, the Lundblad lab argues that Est3p can interact with telomerase in the absence of Est1p[34,43]. Based on the observation that Est2p and Est3p directly interact[77], I hypothesize that Est3p has a low basal ability to interact with the telomerase holoenzyme and that Est1p stimulates this recruitment during S phase. I have applied an *in vivo* co-immunoprecipitation assay and two genetic screens to identify alleles of *EST1* disrupted for this stimulatory function. The mechanism through which Est1p recruits Est3p to the telomerase complex may be clarified through the identification of *EST1* alleles that retain other essential functions but specifically lose the ability to recruit Est3p.

Putative Est3p recruitment domain

Over-expression of Est1p increases the co-immunoprecipitation of Est3p with Est2p compared to wild-type cells[85]. Using an *in vivo* assembly assay, I tentatively determined that the region of Est1p responsible for stimulating the recruitment of Est3p lies between amino acids 499 and 563 (Figure 5). Other deletion mutants were designed

based on sequence conservation within this domain and will be analyzed with the intention of further dividing this putative Est3p recruitment domain into groups of 11 to 21 amino acids. Unfortunately, as described earlier, satisfactory resolution of this question awaits the development of an improved method to detect co-immunoprecipitation of Est3p.

Once the deletion mutants have been used to define a small region of Est1p required for Est3p recruitment, single amino acid (or point) mutations can be made within that region to identify residues responsible for this function of Est1p. Importantly, point mutations should not be limited to alanine, and sequence conservation and proposed structural elements of Est1p should be taken into consideration when determining which mutant alleles are to be introduced into the assembly assay. In particular, the choice of mutations within the EF hand-like motif important in Est1p's G quadruplex-promoting activity[39] should be informed by the potential for interrupting the motif, which could lead to general protein instability instead of disruption of a specific function. For example, within the ion-coordinating residues of the EF hand-like motif, mutation of larger charged residues to alanine will likely produce different phenotypic manifestations than if those same residues were mutated to the oppositely-charged or a neutral amino acid with a side chain of similar size and shape. Therefore, various types of mutations will be examined because differences between mutations of the same amino acid may hint at the mechanism by which Est1p stimulates Est3p's assembly with telomerase. For instance, if a charge reversal mutation loses the ability to stimulate the co-immunoprecipitation of Est3p, then the mutated residue may more likely be involved in a direct protein-protein interaction with Est3p. If such an allele of *EST1* were identified as a separation-of-

function allele (discussed below), then a series of experiments to identify the reciprocal residue in Est3p would be useful for clarifying the current model of yeast telomerase assembly.

Screening for Est1p mutants disrupted for stimulating recruitment of Est3p

I have designed two genetic screens for isolating alleles of *EST1* specifically disrupted for stimulating the recruitment of Est3p to the telomerase complex. The first uses an Est2-Est3 fusion protein and the second utilizes over-expressed Est3p to bypass the Est3p recruitment function of the Est1 protein. Strain and plasmid construction for both screens is underway.

A final topic I addressed while designing these screens concerns how mutagenized alleles of *EST1* will be introduced into the yeast strains. Randomly mutagenized alleles will be generated via PCR mutagenesis and/or passage through a mutagenic strain of *E. coli*. If gap repair is used to introduce *est1* alleles into the strains, pKF610 *est1CΔ250* may serve as appropriate vector. Gap repair relies on the cell's ability to perform homologous recombination (HR), and therefore requires the HR protein Rad52. Unfortunately, the presence of *RAD52* can confound the interpretation of genetic screens involving telomerase function. If cells lose the ability to maintain telomeres with telomerase, a recombination-based method dependent on Rad52p is utilized to amplify sub-telomeric regions to retain the telomere capping function[88]. In this instance, the role of telomerase in telomere maintenance can no longer be examined. For example, if the Est2p-Est3p fusion fails to rescue defects of a mutant Est1 protein, *RAD52* cells will undergo senescence and form survivors, which can be difficult to

discern as it occurs on solid agar media and can only be confirmed with Southern blot analysis of telomere length. In contrast, cells lacking *RAD52* will eventually fail to divide, a phenotype much more evident on solid media. The fusion-expressing and Est3p over-expression strains were intentionally constructed to allow for the deletion of endogenous *RAD52*. Another option is to introduce mutagenized *EST1* using a plasmid library, which does not require *RAD52*. One potential drawback to creating a plasmid library is that sufficient complexity requires very high ligation efficiency of mutagenized gene inserts into the vector backbone, which can be difficult to achieve. Due to the nature of my design, though, either of these approaches is possible for executing these screens for mutant alleles of *EST1* incapable of stimulating the recruitment of Est3p.

Alternative approach

To perform the screen with the Est2-Est3 fusion protein, mutagenized alleles of *EST1* will be expressed in two strains. The control strain will only lack endogenous *EST1* and contain wild-type *EST2* and *EST3*. The experimental strain will lack endogenous *EST1* and *EST3,* while the endogenous *EST2* gene is replaced with the *EST2-G_8-EST3* fusion construct. Plasmids containing mutant *est1* alleles (obtained through either random or directed mutagenesis) can be transformed into these strains and the complementing plasmid selected against by plating cells on 5-fluoroorotic acid (5-FOA). This approach will be most easily applied with specific mutants that are isolated from the *in vivo* over-expression/co-immunoprecipitation assay described above because interpretation of the results requires that growth of a specific mutant *est1* allele be monitored separately in the control and fusion strains.

A more efficient way to examine rescue by the fusion protein is to have the ability to turn expression of Est2p-Est3p on and off within the same cell, but without competition from free Est2p and Est3p. This experimental design would allow for selection of *est1* alleles that are suppressed in the presence of the fusion protein, but not in its absence. One approach to create an "inducible fusion" strain would be to express versions of Est2p and Est3p fused to protein domains that dimerize when in the presence of the drug rapamycin[89]. Transformants with plasmids bearing alleles of *EST1* would be plated on media containing and lacking rapamycin, and their growth tracked on each medium over time, followed by telomere length analysis. The constructs required for this screen are available in the Friedman laboratory; however, time did not permit me to explore the various options that may have allowed me to use the "inducible fusion" for my studies. One issue to be considered if this approach is undertaken: the placement of epitopes, including these dimerization domains, on telomerase components can be extremely important in terms of allowing the complex to maintain telomeres (L. Bechard, unpublished data). Therefore, the protein domains may need to be tested at each terminus of both Est2p and Est3p to determine the optimal configuration for this assay. Another caveat to this system is that it relies upon use of a rapamycin-resistant yeast strain.

Interpretation: Mechanism of assembly

The Est2p-Est3p fusion is predicted to bypass the need for Est1p to stimulate Est3p association by constitutively tethering Est3p to the telomerase core (but is not expected to rescue other functions of Est1p). Over-expression of Est3p might permit more efficient assembly with the complex due to higher protein abundance, thus

bypassing the need for Est1p in Est3p recruitment. Each of these screening methods has its advantages. Excess levels of Est3p due to over-expression could lead to isolation of false positives because such high protein levels may also allow for non-specific interactions to occur within the complex or alterations in other cellular processes that somehow rescue defects of the *est1* allele in the cell. Use of the Est2-Est3 fusion protein does not introduce this variable into the system. However, the fusion protein does present one considerable limitation: the protein conformation and potential interaction surfaces of Est2p and Est3p are restricted when expressed as a fusion. Presumably, Est2p and Est3p are in approximately the correct configuration for telomere maintenance because the fusion protein complements the absence of endogenous *EST2* and *EST3*. Though the eight-glycine linker was determined to be ideal for telomere maintenance (Friedman lab, unpublished data), there is still limited flexibility of proteins to fold and move and be accessible to binding partners when they are joined together. Another possibility is that simply getting Est3p into the complex is insufficient for function and that Est1p is required to induce a specific interaction not bypassed by the fusion alone. This particular caveat is not an issue when over-expressing Est3p, because in these cells, the free telomerase proteins are translated individually as usual and have the opportunity to be in any conformational state they might normally take. In view of these differences, it is important to utilize both of these screening methods to isolate and test alleles of *EST1* that are disrupted for stimulating recruitment of Est3p to the telomerase complex. In particular, the strains created for isolating new *est1* alleles for analysis in the assembly assay can also be used to analyze *est1* mutants that display reduced or absent Est3p co-immunoprecipitation as a form of confirmation of the validity of the assembly assay.

Additionally, as suggested, using both screening methods may allow for interpretation of potential mechanisms through which Est1p stimulates Est3p recruitment, particularly if an *est1* mutant is rescued by either the fusion protein or over-expression of Est3p but not both. Specifically, I hypothesize that Est1p mutants disrupted for a direct protein-protein interaction with Est3p would be rescued by co-expression with the Est2p-Est3p fusion but not necessarily by over-expressing Est3p. If the defect in recruiting Est3p were not profound, over-expressing Est3p might also rescue the growth and telomere phenotypes of such mutants. And I would expect mutants of Est1p lacking the ability to induce a conformational change in the complex for Est3p association to be rescued by Est3p over-expression but not by the presence of the Est2-Est3 fusion protein, which is likely unable to take all conformations the free telomerase proteins are capable of forming. Isolating *est1* alleles rescued by the fusion protein (and possibly over-expression of Est3p) would support a model in which Est1p recruits Est3p to telomerase via a direct protein-protein interaction, as the Zakian lab has suggested[78]. Another, not necessarily mutually exclusive, possibility is that Est1p confers a conformational change within the holoenzyme that increases the affinity of Est3p and Est2p. This mechanism would be indicated by rescue in the presence of over-expressed Est3p but not necessarily by the Est2-Est3 fusion protein, again, due to the restriction of the fusion protein's conformational state and ability to access binding partners. This model is supported by data showing a direct interaction between the Est2 and Est3 proteins[77]. Alternatively, since the function of Est3p is presently unknown, if its precise localization within the complex is critical for its *in vivo* function, the Est2-Est3 fusion protein may not be able to appropriately bypass the Est3p recruitment function of Est1p. So even if no alleles of

EST1 are rescued by co-expression with the fusion protein, conclusions may still be drawn about the regulation of telomerase holoenzyme assembly and the specific role(s) for each component within the complex, though conclusions based on negative results should always be considered critically. Therefore, nearly any finding from these screens would be important for the field in resolving the debate concerning precise assembly of telomerase, particularly as to the mechanism by which Est3p is assembled into the telomerase complex.

In vitro binding assay

The Friedman laboratory has demonstrated that purified, recombinant Est2p and Est3p interact directly[77]. However, the role of Est1p in modulating this interaction has not been explored. One approach to this question is to assay the ability of recombinant Est3p to interact with telomerase in extract derived from strains expressing different alleles of *EST1*. If the recombinant protein recapitulates the endogenous situation, we would predict that the interaction of Est3p with telomerase would be increased when Est1p is over-expressed in whole cell extract and decreased in the absence of Est1p or in the presence of Est1p variants that lack the ability to stimulate Est3p recruitment *in vivo*. The Zakian lab has recently shown that recombinant Est1 and Est3 proteins interact *in vitro*[78], presenting a potential opportunity for collaboration to investigate the Est1p mutants I isolate *in vivo* in their *in vitro* assay.

The *in vitro* binding assay uses purified, recombinant His_6-Est3p mixed with whole cell extract lacking endogenous Est3p from which Myc-tagged Est2p is immunoprecipitated and probed for pull down of Est3p via Western analysis. Different

aspects of this assay were briefly targeted for optimization, with the most focus being on incubation time of Est3p with the extract and detection of all pertinent proteins. Unfortunately, as with the *in vivo* assembly assay, clear interpretation of differences in Est3p pull down was confounded by high interference from the light chain of IgG. While the step gradient gels mentioned earlier did allow for some separation between IgG and His_6-Est3p (data not shown), changing the tag on Est3p would be the best method to resolve this issue. There are several options for tagging Est3p with different epitopes, including MBP and GST tags previously used in the lab, in addition to the Est3-HA_9 protein discussed earlier, or epitopes that can be cleaved from Est3p following purification (J. Talley, personal communication). Changing the epitope tag would likely require re-optimization of conditions to purify useful concentrations of quality protein.

To further examine the role of Est1p in Est3p's assembly with telomerase, different alleles of *EST1* need to be analyzed with this assay. One possibility for performing these experiments is to purify recombinant, epitope-tagged variants of Est1p and mix them in combination with purified Est3p and extract from strains lacking endogenous *EST1* and *EST3*. However, it is technically difficult to purify even wild-type Est1p from *E. coli* (J. Talley, personal communication), so another source of recombinant protein should be considered, such as wheat germ extract or rabbit reticulocyte lysate (J. Ferguson and K. Friedman, in preparation).

An alternative approach is to express the mutant alleles of *EST1 in vivo* and to assay the effect of these mutations on the interaction of recombinant Est3p with the telomerase complex in extract. Toward this end, I have constructed the plasmid pKF611 to allow the expression of HA-tagged alleles of *EST1* from a centromere plasmid with the

endogenous *EST1* promoter and terminator. This plasmid could replace the complementing plasmid in cells lacking endogenous *EST1,* but whole cell extract would need to be harvested before senescence. Another option is to over-express tagged alleles of *EST1* in G_1-arrested cells (as in the *in vivo* assembly assay) or cells lacking endogenous *EST1* (again harvesting extract before senescence). Myc_9-Est2p would be immunoprecipitated as before, and the pull down of His_6-Est3p from each strain extract compared. This method assumes that the variants of Est1p retain association with telomerase, a requirement for separation-of-function alleles as mentioned above. While there are several technical difficulties to consider when pursuing the *in vitro* binding assay, it is a valuable tool for addressing my hypothesis.

In vitro telomerase activity assay

Independently, addition of recombinant Est1p or Est3p has been shown to enhance nucleotide addition by telomerase *in vitro*[44,77]. This stimulation assay entails incubating telomerase partially purified from yeast extract with a biotinylated telomeric primer conjugated to magnetic streptavidin beads and determining the enzyme's ability to add radio-labeled nucleotides to the primer by separating products on a urea/polyacrylamide gel. A preliminary experiment from the Freeman lab suggested that Est1p is not required for the stimulation of telomerase activity by Est3p (B. Freeman, unpublished data). However, simultaneous addition of both proteins has not been studied, and the potential interaction between the Est1 and Est3 proteins may also affect the enzymatic activity of telomerase.

One caveat of this assay is that it is not clear which telomerase components are present when the complex is purified from yeast. While Est2p and TLC1 RNA must be present for catalytic activity to be retained, other components may not be purified at stoichiometric levels. To ensure that wild-type Est1p is not present in the telomerase extract, strains lacking endogenous *EST1* have been used as the source of telomerase for this assay[44]. In the case of adding both recombinant Est1 and Est3 proteins, the suggested strain background to avoid any interference from wild-type proteins would be one lacking endogenous *EST1* and *EST3*. However, it may be difficult to obtain sufficient amounts of telomerase extract from yeast undergoing senescence due the lack of two telomerase components. If it becomes necessary, options for obtaining individually purified telomerase components should be explored, as mentioned for the *in vitro* binding assay (above), including *in vitro* transcription and translation methods.

If addition of Est1p and Est3p additively or synergistically increases *in vitro* telomerase activity, then this assay could be used to examine how interactions between the telomerase complex components contribute to the enzyme's activity. For example, if a direct interaction between the Est1 and Est3 proteins affects enzyme activity, mutants of Est1p disrupted for stimulating Est3p recruitment may be predicted to lack the ability to stimulate *in vitro* telomerase activity. But, as with any *in vitro* experiment, the caveat must be noted that negative results could be attributed to the nature of the *in vitro* system, without post-translational modifications or potential telomerase-interacting factors, and may not reflect the role of telomerase components in the cell.

Separation of function within the Est1 protein

To demonstrate that alleles of *EST1* identified by the experiments presented here are true separation-of-function alleles, representing residues only involved in stimulating the recruitment of Est3p, other studies must also be completed. Variants of Est1p expressed from a centromere plasmid and driven by the endogenous *EST1* promoter (pKF610; Table 3) will be examined for complementation of growth and telomere length defects of an *est1*Δ strain. I predict that mutants defective for Est3p recruitment will fail to maintain telomere length. In addition to stimulation of Est3p recruitment, Est1p's ability to associate with telomerase is demonstrated by co-IP with Myc_9-Est2p in the *in vivo* assembly assay. The ability of the Est1 protein to specifically bind TLC1 will be assayed by examining the ability of HA_3-Est1p to co-IP the TLC1 RNA as detected by Northern blotting. The stimulation assay will determine whether the Est1p mutants retain the ability to enhance *in vitro* telomerase activity. DNA binding can be assessed by electromobility shift assays with radio-labeled substrates. Using the same *in vitro* assay as the Zhou group[33], my Est1p mutants can also be tested for the ability to promote G quadruplex structures. If any mutants isolated for the inability to stimulate Est3p recruitment **retain** the ability to perform all of these functions of Est1p, they would be considered true separation-of-function alleles of *EST1*.

The stimulation assay could also be used to probe the relationship of Est1p's various functions. For instance, the Est1 protein has been shown to bind DNA substrates[38]; if this function of Est1p is important for telomerase activity, Est1 mutant proteins disrupted for binding DNA are predicted to not stimulate *in vitro* telomerase activity. Notably, the G quadruplex (or G quartet)-promoting function of Est1p could be

tested by introducing Est1p mutants into the assay or by using different telomeric primers as substrates. In particular, two types of primers should be compared in the stimulation assay: one that is incapable of forming higher order G quartets and one that can form G quadruplexes. If the formation or stabilization of G quartets by Est1p is necessary for the protein's contribution to telomerase activity, then the primer incapable of forming these DNA structures may not be elongated. The hypothesis for any function of Est1p is: if the specified function is necessary for Est1p to affect telomerase activity, then mutants for that function will not show stimulation of primer extension above background.

One question remains: what if no separation-of-function allele of *EST1* disrupted for stimulating recruitment of Est3p is identified? This outcome is possible, if not likely, due to the fact that many of Est1p's functions lie in essentially the same region of the protein. If any Est3p recruitment mutants were also disrupted for even one other function of Est1p, it could be considered evidence of the significance of the region spanning approximately residues 450 to 550 in contributing to Est1p's role in *in vivo* telomere maintenance. Specifically, such findings could indicate that this region is necessary for the protein's tertiary structure and a broad function within the holoenzyme. For instance, Est1p may induce a conformational change or stabilize overall architecture of the complex via several interactions with other components of telomerase, minimally including TLC1 and Est3p and possibly Est2p.

Conservation of telomerase from yeast to humans

As with all basic science research, findings of this study must be considered in the context of human telomere maintenance. While connections between different model systems can be quite obvious, potential correlations between *S. cerevisiae* and *H. sapiens* telomerase protein interactions are less clearly defined due to substantial divergence between these two species, especially in the case of the Est1 and Est3 proteins.

Humans have three EST1 proteins with similar sequence and domain structures as *Sc*Est1p[35,54,55]. Human EST1A and EST1B have been shown to associate with telomerase. hEST1A also has a single-stranded DNA binding activity and plays a role in telomere capping[90]. The functions of hEST1B and hEST1C have not been characterized to date. One feature that the human EST1 proteins and *S. cerevisiae* Est1p have in common is a tetratricopeptide repeat (TPR) domain in the N terminus of the protein[35,54,55]. TPR domains are most often implicated in protein-protein interactions, and it was very recently published that hEST1A interacts with the reverse transcriptase of human telomerase (hTERT) *in vitro* via its TPR domain[91]. Intriguingly, the residue of *Sc*Est1p required for interaction with Cdc13p lies outside the TPR domain, contrary to what may be expected considering the main function of tetratricopeptide repeats in the majority of other proteins. And based on the position of Est1p's putative Est3p recruitment domain, any amino acids with a contribution to this stimulatory function of Est1p will also lie outside the TPR domain.

These observations call into question the purpose of the TPR domain in *Sc*Est1p. With little data examining potential functions of this region, one possibility is that Est1p interacts with an as-of-yet unidentified partner, likely another protein, via its TPR

domain. Evidence supporting this speculation showed that mutations in the Est1p TPR domain disrupted the interaction of Est1 and Ies3 proteins as assessed by yeast 2-hybrid and immunoprecipitation[46]. This partner may regulate Est1p's actions in telomere maintenance, such as restricting its association with the holoenzyme for proper timing of telomere elongation or directing Est1p to short telomeres in need of elongation as a way to target telomerase to the correct substrates. Considering other unpublished data from the Friedman lab, the TPR domain of Est1p may be partly responsible for nuclear localization of the protein by serving as a platform for interaction of Est1p with a component(s) of an import pathway, such as an importin. Currently under examination is a bipartite nuclear localization sequence within the TPR domain that partially disrupts proper nuclear localization of Est1p when basic residues in the sequence are mutated to alanine (C. Hawkins and K. Friedman, in preparation), potential preliminary support for a specific role of Est1p's TPR domain.

Similar to the *S. cerevisiae* proteins, human EST1 may not interact with its Est3p counterpart via the TPR domain, if these proteins interact at all. The proposed homologue of *Sc*Est3p is the oligonucleotide/oligosaccharide binding fold domain of the shelterin component TPP1, although the direct evolutionary relationship between these proteins is debatable[92]. As a member of the shelterin complex, TPP1 functions in telomere capping by bridging double- and single-stranded DNA-binding proteins[93]. Since hEST1A has been shown to interact with hTERT[91], it might be localized to the terminus of the telomere, similar to where *S. cerevisiae* Est1p and Est3p are predicted to associate. However, having been shown genetically to affect telomere capping[90], hEST1A may also be able to interact with TPP1 for this protective function, perhaps with

its localization regulated temporally, like the yeast Est1p. Additionally, because so little is known about the human EST1B and EST1C proteins, further study of these homologues may reveal distribution of *Sc*Est1p functions to the different human EST1 proteins and potentially novel roles for conserved regions of Est1p in human telomere maintenance or other cellular pathways.

Summary

In this study, I have shown that the region of Est1p between amino acids 499 and 563 is important for stimulating the recruitment of Est3p to the yeast telomerase complex using an *in vivo* assembly assay. Strains and plasmids for two genetic screens will yield tools for testing existing alleles for their role in Est3p recruitment and isolating new alleles of *EST1* disrupted for this stimulatory function. Proposed *in vitro* studies can be utilized to support findings from these *in vivo* experiments, potentially determine whether isolated alleles are true separation-of-function alleles, and probe interplay between Est1p's various functions in the cell. These analyses will provide insight into the mechanism of recruitment of the Est3 protein to the holoenzyme and thereby clarify the role of Est1p in regulating complex assembly, a debated topic in the field of yeast telomerase. Additionally, understanding of *S. cerevisiae* telomerase components' functions, interactions, and regulation can provide a basis for pursuing examination of human telomerase and potentially effect change in the treatment of certain disease states.

APPENDIX

PLASMID CONSTRUCTION: pKF600 SERIES

The Friedman lab has previously used two high copy number plasmids containing *EST1* under control of the *GAL1* promoter. The first of these plasmids, pRS423 *GALp-HA$_3$-EST1*, produced Est1p that is subjected to the normal cell cycle regulated degradation of the WT protein (J. Ferguson, unpublished data). The second plasmid, pVL242 R→A, does not have restriction sites for easily cloning other alleles of *EST1* into this plasmid. Therefore, with the guidance of fellow graduate student Jenifer Ferguson, I pieced together a high copy number plasmid containing galactose-inducible *EST1* and convenient restriction sites.

To begin, the *Sph*I restriction site in pVL242 R→A was destroyed by digesting with *Sph*I, blunting with T4 DNA polymerase (NEB), and ligating the ends together. The *GAL* promoter was amplified with primers 242bgl2for and 242bamhIrev and the *EST1* N-terminus was amplified using primers 242bamhIfor and E1DB3RtoKrev (both using pVL242 R→A as template). These products were then amplified as a single *GALp-HA$_3$-EST1^{Nterm}* product using 242bgl2for and E1DB3RtoKrev. The end result was the creation of a unique *Bam*HI restriction site immediately upstream of the START codon of the *EST1* open reading frame (ORF). The *GAL* promoter was cloned from pVL242 R→A into pRS416 with *Eco*RI and *Bam*HI. The *EST1* ORF was amplified using the primer pair Gal1-2for (OP7753) and 242sphI/sacIrev from pVL242 R→A. The latter of these primers introduced an *Sph*I restriction site at the end of the open reading frame and a *Sac*I

site further downstream. This PCR product was cloned into pRS416 *GALp* using *Bam*HI and *Sac*I. The *GALp-HA$_3$-EST1^{Nterm}* PCR product was digested with *Bgl*I and *Pflm*I and cloned into the *Bam*HI and *Pflm*I sites of pRS416 *GALp-HA$_3$-EST1*, making pRS416 *GALp-HA$_3$-EST1* fixed *Bam*HI. Inserting the PCR product into this vector moved the *Bam*HI site immediately upstream of *EST1* and destroyed the upstream *Bam*HI site. *Pvu*II was then used to move *GALp-HA$_3$-EST1* from the pRS416 vector to the pRS425 vector backbone. Next, a portion of *EST1* was replaced with sequence that had not been PCR-amplified by digesting pVL242 R→A with *Nde*I and *Nru*I and cloning that portion into pRS425 *GALp-HA$_3$-EST1*. Finally, the *EST1* termination sequence was amplified from pVL242 R→A with the primers 423terminatorFor and 423terminatorRev and cloned into pRS425 *GALp-HA$_3$-EST1* using *Sac*I. This plasmid, pRS425 *GALp-HA$_3$-EST1* + terminator, was sequenced with M516, E1DB3RtoKrev, E1DB2RtoKrev, and M13F before being renamed as pKF600.

Table A1. Oligonucleotide primers

Primer name	Primer sequence 5´-3´
242 bgl2 for	GAGAAAAAACCCCAGATCTATGTACCCGTATG
242 bamHI rev	CTTCGTTATCCATTAGGATCCCATTTTTAAGCTTGG
242 bamHI for	CCAAGCTTAAAAATGGGATCCTAATGGATAACGAAG
Gal1-2 For (OP7753)	CGGTTTGTATTACTTCTTATTC
242 sphI/sacI rev	GCGCGAGCTCGCATGCTCAAGTAGGAGTATCTGGC
423 terminator for	GCATGCGAGCTCGAGAACAAGTAATGG
423 terminator rev	GCGCGAGCTCCTGCAGGTCG
Est1 sequencing forward 3 (M516)	TTCTTGCATTAACTTTGC
E1DB3RtoK rev	CTAATGCTCCTTTTACGAGCTCAAAAAAATACAG
E1DB2RtoK rev	CCAGGAAGCATTTAAACGTGATATATGC
M13F (-47)	CGCCAGGGTTTTCCCAGTCACGAC

Table A2. Plasmids

Plasmid	Genotype	Source
pRS416	*CEN URA3*	T. Graham
pRS423 *GAL-HA$_3$-EST1*	2µm *HIS3 GALp-HA$_3$-EST1*	A. Sathiyakumar
pRS425	2µm *LEU2*	T. Graham
pKF600	2µm *LEU2 GALp-HA$_3$-EST1*	This study
pVL242 R→A	2µm *LEU2 GALp-HA$_3$-EST1* (RxxLxxxN to AxxLxxxN in linker between *HA$_3$* and *EST1*)	J. Talley

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