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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Defining the functional surface of the Est2 catalytic subunit of yeast telomerase

A thesis submitted in partial satisfaction of the requirements for the

degree Master of Science

in

Biology

by

Lisa Nguyen

Committee in charge:

Professor Vicki Lundblad, Chair
Professor Lorraine Pillus
Professor Ella Tour

2013

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Chair

University of California, San Diego

2013

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The biochemical experiments in chapter 2.1, 2.2, 2.3, and 2.4 was done in collaboration with Timothy Tucey.

ABSTRACT OF THE THESIS

Defining the functional surface of the Est2 catalytic subunit of yeast telomerase

by

Lisa Nguyen

Master of Science in Biology

University of California, San Diego, 2013

Professor Vicki Lundblad, Chair

Telomerase is a ribonucleoprotein complex important for elongating ends of chromosomes. The catalytic subunit of telomerase (called Est2 in budding yeast) is made up of multiple domains. Here, I used a genetic strategy for defining the functional surface of Est2. A reverse-mutagenesis strategy that targets specific amino acids was used to select for candidate mutations. These mutations were then screened for effects on telomere replication in an over-expression dominant negative (ODN) and loss-of-function (LOF) assay. This strategy has generated a collection of separation-of-function (*sof*) alleles in each domain that are proposed to be located on the surface of the Est2 protein. Subsequent biochemical analysis has shown that the amino-terminal (TEN) domain of Est2 plays a direct or indirect physical interaction with Est3 and that multiple regions (TEN, TRBD and RT domains) of Est2 have an RNA binding function.

INTRODUCTION

Telomerase is a ribonucleoprotein complex responsible for elongating ends of chromosomes in humans and most other eukaryotes. These chromosome ends are made up of thousands of DNA repetitive sequences called telomeres. The Hayflick Limit states that human fibroblasts can divide only a limited number of times before undergoing replicative senescence.[1] Telomeres shorten after each cell division due to the "end replication problem" and when telomeres reach a critical length, cells are no longer able to progress through the cell cycle and replicative senescence occurs. Telomerase solves the "end replication problem" by adding tandem G-rich DNA repeats to the 3' ends of linear chromosomes to help protect from loss of genetic information. Down-regulation of telomerase activity leads to telomere erosion, which triggers a DNA damage response that leads to replicative senescence, a process in which cell division is inhibited and apoptosis occurs. This process can contribute to age-related impairments that affect organ homeostasis and decrease lifespan.[2] Conversely, the up-regulation of telomerase activity is associated with uncontrolled cell proliferation in advanced human carcinomas.[2] As such, telomerase is important for developing anti-cancer therapeutics. In humans, telomerase deficiency is associated with several telomere-mediated syndromes, such as dyskeratosis congenita characterized by bone marrow failure and pre-mature aging.[2,3]

Telomere length homeostasis is achieved by an interconnected series of regulatory mechanisms that balance positive (i.e. telomerase) and negative (i.e. nuclease, incomplete semi-conservative DNA replication) activities. Telomerase is preferentially targeted to shorter telomeres, ensuring that telomere length does not fall below a critical level.[4] However, the number of nucleotide additions per cell cycle is independent of telomere length.[4] Moreover, telomere elongation is restricted to the phase, where telomerase acts on only ~10% of telomeres in each cell cycle.[4,5] Lastly, there are very low levels of telomerase in the cell; telomerase levels are lower than the number of chromosome ends.

Elimination of telomerase caused by either defects in the catalytic core or regulatory subunits will result in the EST (for Ever Shorter Telomeres) phenotype, characterized by the gradual decrease in proliferative capacity. Proliferation is inhibited by continuous telomere shortening and the eventual absence of cell division, also referred to as senescence.[6,7,8] Replicative senescence in yeast is typically measured by assessing the ability of a single colony to propagate and grow into a full-sized colony after successive generations. Telomeres shorten after each successive generation in telomerase-defective mutants, though visible growth defects are detected by ~50 generations (Fig. 1). After ~75 to 100 generations of growth, most cells cease to grow and can no longer divide (Fig. 1).

In the budding yeast *Saccharomyces cerevisiae*, telomerase is composed of three ever shorter telomere (EST) subunits, Est1, Est2 and Est3, in a complex with the 1.3-kb yeast telomerase template *TLC1* RNA that provides a scaffold for protein assembly.[9] Est1 and Est3 subunits serve as regulatory proteins, whereas the Est2 subunit and *TLC1* RNA make up the catalytic core of the enzyme.[10] Est2 is required for telomerase activity *in vivo* and in *vitro,* whereas Est1 and Est3 are only required for telomerase activity *in vivo*.[10]

Est1, Est2 and Est3 subunits are known to play certain roles in yeast telomerase. The Est1 subunit of telomerase functions to (1) mediate the recruitment of the telomerase enzyme to telomeres by electrostatically binding to Cdc13, a single-stranded telomere binding protein.[11,12,13,14] Est1 also (2) has a sequence specific affinity to single-stranded G-rich telomeric DNA and (3) interacts with the telomerase complex by directly binding to the bulged stem in *TLC1* RNA.[4,15] Telomerase RNA is encoded by the *TLC1* gene and is referred to as *TLC1* RNA in yeast.[16] *TLC1* RNA has been shown to have independent binding domains for Est1 and Est2, thereby bridging the indirect association between Est1 with Est2. Telomerase RNA (TR) is not well conserved among species and displays a wide variation in length and dramatic sequence variation. TR provides a template in which only a small region of RNA is used for telomeric DNA synthesis. The template domain is composed of a pseudoknot structure important for binding Est2 and two stem-loop structures that associate with Est1 and Ku.[6,17,18] Next, Est2, also known

as the TERT (telomerase reverse transcriptase) subunit, is the reverse transcriptase (RT) catalytic subunit (in complex with *TLC1* RNA) that makes up the catalytic core of telomerase. In addition, Est2 is the limiting subunit of telomerase. Lastly, the function of Est3 is largely unknown, though studies have shown that the association of Est3 with RNA relies on its association with Est2.[19] Although much is proposed about these telomerase subunits, many details of how these subunits interact with each other (and other regulatory proteins) and the mechanistic basis of telomerase elongation remain elusive.

In this study, I am closely examining Est2, the catalytic subunit of telomerase of yeast telomerase. Est2 is absolutely essential for normal chromosome telomere replication, as evidenced by telomere shortening and senescence phenotypes following deletion of Est2 and even single point mutations in certain motifs of Est2.[20] These essential mutations include the triad of aspartic acid residues (*est2-D530, est2-D670, est2-D671)* that are important in binding metal ions. Regions of the catalytic subunit in yeast are relatively well-conserved among distantly related species, including humans. Therefore, the information in Est2 studies may be applicable to the human telomerase protein.

The Est2 protein subunit of yeast telomerase is proposed to have four functionally distinct domains, which include the amino-terminal (TEN) domain, telomerase RNA binding domain (RBD), reverse transcriptase (RT) domain, and the C-terminal domain.[21] Sequence analysis indicates that four domains are relatively well conserved among distantly related species.[22] The TEN domain is the least conserved among phylogenetic groups (required in humans, yeast, and ciliated protozoa) and acts to localize telomerase to the nucleolus.[21] The RBD (RNA binding domain) has RNA binding properties; it is very well conserved among species and is required for telomerase function *in vivo* and *in vitro*.[23] The reverse transcriptase (RT) domain contains highly conserved aspartic acid residues that are necessary for the formation of the catalytic active site (containing the nucleotide-binding pocket), and thus essential for nucleotide addition.[20] Mutations in these conserved residues result in loss of all enzymatic activity both *in vivo* and in *vitro*.[20] The

C-terminal domain is the least conserved domain and is known to be essential for enzymatic activity in humans but not in yeast.[22,23]

Although much is proposed about the domains of Est2, many details pertaining to their function are left unanswered. As such, I aimed to define the functional surface of Est2 by using a reverse-mutagenesis strategy that targets specific amino acids to select for potential separation-of-function mutations. Subsequent biochemical and genetic analysis of these mutations will be performed in order to locate all of the regulatory sites on the surface of Est2 and gain insight on the mechanistic basis of telomerase regulation.

MATERIALS AND METHODS

Strains and plasmids. The strains used for the over-expression dominant negative in the synthetic lethality and telomere length assays were YVL3142 (a *yku80-Δ/pYKU80 URA3* strain) and YVL2967 (wild-type strain, MATa *ura3-52 lys2-801 trp1-* 1 *his3-* Δ200 *leu2-* Δ1), respectively, which was transformed with high-copy 2u *TRP1* plasmids expressing wild-type or mutant *Est2* genes expressed from the *ADH* promoter (derived from pVL4120). The *est2-Δ* strain used in the loss-of-function analysis was YVL3814 and transformed with single-copy *CEN LEU* plasmids expressing wild-type or mutant Est2 genes expressed from the native promoter. The strain used for anti-FLAG co-immunoprecipitation (IP) experiments was YVL3480 (Est1-G_6-myc_{12}), which was transformed with *CEN* plasmid *URA3* plasmids expressing wild-type or mutant *Est2* genes expressed from the native promoter (derived from pVL5681) and contains a $FLAG_3$-myc_{12}-G_6-Est2 epitope. Quikchange mutagenesis was used to introduce missense mutations into Est2. Standard methods were used to introduce plasmids into yeast. Strains used for the integration of Est2 were YVL2967 and YVL3528 for telomere length analysis and co-IP western analysis, respectively. The strains used for integrated *est2*$^{--}$ mutant strains were YVL 2967 and YVL 3528 which were transformed with integrating *CEN URA3* plasmids expressing wild-type or mutant *Est2* genes containing HpaI (pVL6323) or SwaI (pVL6322) restriction sites.

Synthetic lethality assay. To assess the potential synthetic lethal effects of *Est2* mutants**,** plasmids expressing mutant alleles of Est2 (derived from pVL4120) were transformed into YVL3142. Single colonies were picked and grown to saturation in *TRP* medium for 2 days at 30°. 200ul from each culture was transferred into the microtiter dish using a multi-channel pipetter and plated using five-fold serial dilutions across each well onto *TRP 5FOA*-containing media to allow the growth of *5FOA* resistant colonies. Cells that retain *pYKU80* are *URA+* cells that can convert 5FOA to a toxic intermediate, however cells that lose *pYKU80* are *URA-* cells that are able to grow. Growth of the *yku80-Δ/pADH-est2-* strains was assessed at 30°, 34°, 36° after 3 days

.

Telomere length assay. Plasmids expressing mutant alleles of Est2 (derived from pVL4120) were transformed into YVL2967. Following ~100 generations of growth at "3x" streakouts, single colonies were picked and grown to saturation for 2 days at 30° in 5ml of *TRP* media. Genomic DNA was prepared using Wizard Genomic DNA Purification Kit (Promega) and digested with XhoI enzyme (New England Biolabs). Samples were separated on a 20-cm 0.7% gels and transferred on a nylon membrane then probed with poly d(GT/CA).

Co-IP Western. Co-immunoprecipitation of Est2 was examined in YVL3480 (a protease deficient strain containing $(myc)_{12}$ epitopes on Est1 and Est2 as well as $(FLAG)_3$ epitopes on Est2) with plasmids expressing mutant alleles of Est2 (introduced into pVL5681). An anti-myc western was performed to quantitatively measure proteins in the telomerase complex. After 2-3 days of growth at 30°, single colonies were picked and grown to saturation in *URA*$^-$ media for 2 days at 30°. Whole cell extracts were prepared from 250ml cultures were grown to O.D. 0.8 to 1.0. Extracts were incubated with anti-FLAG M2 agarose beads (Sigma). IPs containing $(myc)_{12}$ and $(FLAG)_3$ epitopes were separated on a 6% SDS-PAGE and probed with myc 2272 (Cell Signaling Technology) at dilution 1:1,000 and anti-rabbit IgG HRP conjugate (Promega) at dilution 1:10,000 for enhanced chemiluminesence (ECL) detection with pre-flashed film (FUJIFILM Science Lab Imagine Gauge)

Integration of Est2 alleles. *Est2* alleles were integrated into strain YVL2967 for telomere length and YVL3528 for co-IP western experiments. Restriction site marker was introduced (near the Est2 mutation site) into an *Est2* integrating plasmid pVL6323 and pVL6322 at HpaI or SwaI restriction sites, respectively, and subsequent *Est2* mutations were introduced. Integrating plasmids were linearized using BsrGI or ClaI to target the integration of *Est2* and subsequently introduced into yeast. After 2-3 days at 30°, single colonies were patched onto *URA*-containing plates and grown for 1 day at 30°. Grown patches were subsequently re-patched onto YPD-containing plates and grown for 1 day at 30°. Genomic DNA was isolated from YPD patches by

Wizard Genomic DNA Purification kit (Promega) and integration of the "pop-in" strain was diagnosed using two PCR reactions to detect the junction of endogenous *Est2* of yeast and that introduced by the integrating plasmid. PCR products were digested with either HpaI or SwaI to confirm that *Est2* is likely to be integrated into the genome. PCR products were sequenced to directly confirm the presence of the mutation for mutants with a positive diagnosis. Streakouts were made from YPD patches onto *5FOA*-containing plates and grown for 3 days at 30°. Single colonies were patched onto *5FOA*-containing plates to select for "pop-out" mutants, or cells that have lost the endogenous copy of *Est2* and the *URA*-containing vector backbone. Patches are grown for 1 days at 30° and diagnosed for the presence of "pop-outs" using the same methods described above.

Genetic Analysis. Est3 over-expression: A plasmid expressing *Est3* in high-copy (pVL1024, a 2u plasmid expressing Est3 protein under the control of the ADH promoter using a LEU marker) and empty vector (pVL1024) was transformed into the indicated integrated *Est2* mutant strains. After 2-3 days of growth at 30°, single colonies were picked and grown for ~75 generations at "2x" streakouts and the telomere length was assessed as described previously. *Rif2* over-expression: A plasmid expressing *Rif2* in high-copy (pVL 5690, a 2u plasmid expressing *Rif2* protein under the control of the *ADH* promoter using a *LEU* marker) and empty vector (pVL399, a 2u plasmid under the control of the *ADH* promoter using a *LEU* marker) was transformed into indicated integrated *Est2* mutant strains. After 2-3 days of growth at 30°, single colonies were picked and grown for ~100 generations at "3x" streakouts. Each streakout was grown for 2-3 days at 30° and *Rif2*-mediated suppression of senescence was assessed.

CHAPTER 1: A NOVEL STRATEGY FOR IDENTIFYING SEPARATION-OF-FUNCTION MUTATIONS IN *EST2*

Approaches to uncover protein function have often relied on the deletion of the gene of interest (at least for non-essential genes). Deletion of the gene will eliminate all the biological functions of that protein, and hence is unlikely to reveal the functional role of individual domains of the protein. In the standard loss of function approach, highly conserved residues are traditionally mutated to alanine with no assumptions as to whether these residues are located at the surface. The basis behind the change to alanine was that it would be less likely to disrupt protein structure. However, mutations to alanine could overlook functionally important residues. The standard loss of function approach also has a major caveat which is that it is difficult to differentiate whether the defect is caused by (1) structurally unstable protein or (2) a protein with a defect in a specific biochemical activity.

A technique that I have employed to resolve this caveat uses an approach specifically designed to identify separation-of-function mutations (i.e. missense mutations) that instead remove one biological role of that protein *in vivo*. One challenge is that the generation of missense mutations in a gene can create either a stably folded protein or an unfolded protein. A solution to this involves the use of an over-expression dominant negative assay, which allows us to distinguish between mutations that result in a fully stable protein or mutations that disrupt protein stability.[24] If a mutant protein confers an over-expression dominant negative (ODN) phenotype, then it is predicted to disrupt function when expressed in high copies in yeast strains.[24] This implies that the mutant protein is fully folded and functional enough to physically, and thus functionally displace endogenous, wild-type protein.[24] Since mutations on the interior of the protein are likely to cause the protein to unfold, mutated residues that produce an over-expression dominant negative phenotype are likely to be on the surface of the protein. Moreover, mutations in the inside of the protein that cause a structurally stable protein may not produce an ODN phenotype because internal residues are unlikely to define a functional surface. Lastly, residues may be present on the surface of the protein but may not have an ODN phenotype

because (1) the surface residue is not part of a functional surface or (2) is part of a functional surface but the single mutation is not severe enough to create a defect.[24]

Surface residues are of interest because they are more likely to be involved in regulating a biological function. However, it is a challenge to determine which residues are likely to produce a dominant negative phenotype when structural predictions are inadequate to predict surface residues. Several recent studies in the Lundblad lab have resolved this issue. Est3 was shown to associate with yeast telomerase through an OB-fold domain using a methodology that relies on an over-expression dominant negative assay to identify separation-of-function alleles (Fig. 2). It was concluded from this study that (1) the highest number of dominant negative alleles were found in charged residues when mutated to the opposite charge (i.e. charge swap mutagenesis), and as expected, (2) the mutants that displayed a severe dominant negative phenotype had a severe loss-of-function phenotype and (3) were separation-of-function alleles.[24] Subsequent work has shown that all of the mutations that confer an ODN phenotype reside on the surface of the experimentally determined Est3 structure, confirming that this strategy targets surface residues.[15] In a second study that uses the same methodology on *Est1*, data showed that the highest frequency of separation-of-function alleles was found on charged residues that were also highly conserved.[25] The reverse mutagenesis strategy (i.e. charge swap mutagenesis on conserved residues) in these papers is a fundamental tool for screening for separation-of-function alleles in the absence of any prior structural information, as is the case for the Est2 protein.

I have used the same strategy recently employed for *Est3* and *Est1* to select key residues on the Est2 protein. I employed mutagenesis on charged residues to the oppositely charged amino acid (ex. K → E) for highly conserved residues of Est2. ODN analysis was used to identify the separation-of-function mutations that encode for properly folded proteins that disrupt a single biological function on that protein.

CHAPTER 2: DOMAIN BY DOMAIN ANALYSIS OF *EST2*

I first selected candidate residues of Est2 for mutagenesis. My reverse genetics approach employs a protocol that selects for highly conserved residues based on protein alignments.[2,3] These highly conserved charged residues (D, E, H, K, R) of *Est2* were mutated to the opposite charge (D or E → R; R or K → E and H→ E), over-expressed in yeast and analyzed for their effects on telomere replication. In order to identify *est2*$^{-}$ mutations that interfere with wild type yeast telomere replication, I used two genetic yeast strategies. First, I conducted an initial ODN screen which monitored synthetic lethality in *yku80*-Δ strains. The *yku80*-Δ strain is highly sensitized to telomere replication defects and enhances the detection of phenotypes.[5] Then, selected mutations were examined in a secondary ODN screen for effects on telomere length when expressed in a wild type (*EST2*) strain. Mutagenesis on each of the four *Est2* domains was performed and mutations that had a dominant negative phenotype were found in three of the four domains. Mutations that gave a moderate to null phenotype in the ODN assay gave an equally strong phenotype when expressed under native conditions in the LOF assay. Figure 2 shows the initial success of my genetic strategy for generating mutations in predicted surface residues in *Est2*. The first part of my project focuses on an intensive mutagenesis of each domain of *Est2* using an ODN screen for isolating separation of function alleles. Wide ranges of phenotypes (from weak to severe) were observed. The second focus of my project focuses on biochemical analysis that assesses the enzymatic properties and binding partners of Est2.

Chapter 2.1: TEN Domain

Out of the mutations screened from the TEN domain, 31 mutations were found to have a null, severe, or moderate ODN phenotype. Table 1 shows a number of mutants that conferred an ODN phenotype and the success of our methodology on the TEN domain. A subset of mutants (that had an ODN phenotype) were subsequently tested in a LOF assay and gave an equally strong telomere length defect phenotype (Table 1). I was therefore able to

generate comprehensive collection of separation-of-function alleles in the TEN domain that are proposed to regulate a biologically significant function on the surface of Est2. Furthermore, four mutations that conferred the ODN phenotype are conserved in the human TERT protein (the catalytic subunit of telomerase in humans).

From my initial mutagenesis strategy, I generated a panel of predicted surface residues that were further tested in a biochemical assay in collaboration with Tim Tucey, a graduate student in the lab. Our biochemical data has shown that a subset of these mutations has a role in RNA binding (Figure 4A and 4B). A number of studies have shown that *TLC1* bridges the association between Est1 and Est2, and that Est1 and Est2 both directly and independently bind to RNA at different sites. Therefore, *est2* mutations that have reduced binding to Est1 presumably have an RNA binding defect. To determine if any mutant Est2 proteins affect the Est1-*TLC1*-EST2 complex in the TEN domain, we performed an Est2 immunoprecipitation and looked for Est1 association in the telomerase complex. Our Est2 co-IP western data show that the Est1-*TLC1*-Est2 complex is affected by two (*est2-H61E and est2-N133D*) of the *est2* mutations we tested in the TEN domain. Mutations in the TEN domain are shown to have reduced binding to Est1, suggesting that amino acid residues from multiple domains of *Est2* contribute to *TLC1* association, not just the RNA binding domain (Fig. 4A and 4B).

Since it is unclear exactly how Est3 physically interacts with the telomerase complex, with conflicting reports implicating a direct interaction with either Est2 or Est1, we ask if Est3 interacts with Est2 in the telomerase complex. Using strains that I constructed, Tim performed an Est3 IP to see if the association of Est3 with telomerase is affected by *est2* mutations in the TEN domain. Our biochemical analysis has shown that a subset of these mutations (*est2-H61E, est2-N80K, est2-Y86K, est2-N91D, est2-N96D est2-N104D, est2-N104D, est2-N104D K111E, est2-N104D*) influence Est3 binding to the telomerase complex (Fig. 4C). We see that Est3 association is reduced in a number of *est2* mutants, presumably because the TEN domain of Est2 may have a direct or indirect physical interaction with Est3. Collectively, we show that mutations near the amino terminus of the Est2 protein result in loss of Est3 protein from the telomerase complex.

Chapter 2.2: RNA Binding Domain

The RBD domain makes extensive contacts with *TLC1* RNA component of telomerase. Out of the 26 mutations screened from the RBD domain, none of the mutations we tested showed a strong ODN phenotype. However, a subset of mutations showed a LOF phenotype (Table 2 and Supplemental Fig. 3). Unlike the data shown from the other domains, my findings suggest that the RNA binding determinants are resistant to ODN mutagenesis. ODN mutagenesis shows that mutations in this domain failed to confer an ODN phenotype (Table 2). Again in collaboration with Tim Tucey, we performed an Est2 IP and looked for Est1 association in the Est1-*TLC1*-Est2 complex to determine the effects of mutant Est2 proteins in the RNA binding domain. As expected, a subset of residues (*est2-N305K and est2-K306E*) showed reduced Est1 association in the telomerase complex. Here we showed that a subset of *est2* mutations in the RBD domain *(est2-N305K and est2-K306E*), isolated using LOF assay, is defective for *TLC1* RNA binding (Fig. 4A) but do not have ODN phenotypes (Table 2), presumably because RNA-binding-defective Est2 mutant proteins cannot associate with the telomerase complex and will fail to disrupt wild type telomere replication.

The RNA binding pocket of telomerase is formed by the three conserved residues (*est2-I260, est2-C261, and est2-P262*). These residues reside in the CP motif that is implicated in RNA binding in yeast. Studies have shown that mutated CP residues affect RNA binding and telomerase function in yeast as well as a wide range of phylogenetic groups. Therefore, we ask if the highly conserved ICP motif is required *in vivo*. Although implicated in RNA binding, the three mutations of this motif were assessed (*est2-I260E, est2-C261Y, and est2-P262R)* in an ODN and LOF assay and no telomere defect phenotype was detected in either case, suggesting that the highly conserved ICP motif is not required *in vivo*. This demonstrates that not all conserved residues (which are usually non-polar or hydrophobic) will result in an ODN phenotype, presumably because conserved residues tend to be involved in protein folding.

Chapter 2.3: Reverse Transcriptase Domain

The reverse transcriptase (RT) domain forms the catalytic active site with the nucleotide-binding pocket which is absolutely essential for telomeric DNA synthesis. Three invariant aspartic acid residues (*est2-D530A, est2-D670A, est2-D671A*) in this domain have been shown to be indispensable for enzymatic activity and are directly involved in catalysis.[20] Mutations in any of these three aspartate residues result in severely impaired telomerase activity—mutants display a senesce phenotype that is indistinguishable from null.[20] Furthermore, studies have shown these residues have dominant negative effects and were subsequently used as null mutants for comparisons in my assays. [20]

Out of the mutations screened in this domain, 11 mutations had an ODN phenotype. A subset of these mutations is also conserved in the human TERT protein (*est2-D530, est2-D670, est2-D671*, etc.) and *T. castaneum* (red flour beetle). A number of ODN mutations that I report (including the catalytic aspartic acid residues) in the RT domain of yeast line the catalytic cleft in the known structure of *T. castaneum*. Interestingly, I found 5 gain-of-function mutations that gave rise to longer telomeres (Table 3).

Our Est2 co-IP western analysis shows altered Est1 association in several mutations (*est2-D536K and est2-D646K*) that are adjacent to the catalytic pocket all affect, suggesting that the RT domain of Est2 is also responsible *TLC1* RNA binding. Collectively, we report that the TEN, RNA binding domain, and RT domain of Est2 contribute to *TLC1* association, not just the RNA binding domain as previously reported.

Chapter 2.4: C-terminal domain

Out of the mutations screened in this domain, 15 mutations had an ODN phenotype. Four of those mutations resulted in an ODN phenotype (Table 4). We did an Est2 IP and saw that there were no effects of *est2* mutations in the C-terminal domain on the Est1-*TLC1*-Est2 complex. Furthermore, when we performed an Est3 IP, we saw that association of Est3 with the complex was unaffected in the mutants we tested. Our data shows that the C-terminal domain of

Est2 is not involved in RNA binding or Est3 binding, suggesting that the C-terminal domain must be involved in some other unknown function. These data also support Est2 truncation experiments that show that the c-terminal domain is dispensable for telomere association.[26]

The biochemical experiments in chapter 2.1, 2.2, 2.3, and 2.4 was done in collaboration with Timothy Tucey.

CHAPTER 3: GENETIC ANALYSIS

A subset of est2^{--} mutations were integrated into the genome and genetic analyses were performed in order to probe for the possible functions of Est2. Again, I wanted to investigate how Est3 physically interacts with the telomerase function and asked whether Est3 associates with Est2. To do this, I overexpressed *Est3* in *Est2* mutant strains and looked for telomere replication defects after ~75 generations. If *Est3* over-expression suppresses a mutation in *Est2*, then this would suggest an Est2-Est3 interaction. If a telomere replication defect were due to telomerase association (more specifically, Est2-Est3 association), then we would expect *Est3* over-expression to suppress the telomere length defect. Although we see an Est2-Est3 interaction in the co-IP western experiments described previously, the mutants we tested here failed to suppress telomere replication defects. For instance, *est2-Y86K* mutant showed reduced Est1-*TLC1*-Est2 (Fig. 4C) association yet failed to respond to Est3 over-expression (Fig. 5).

To further investigate interacting factors of Est2, I asked whether mutations in the N- and C- terminal domains respond differently to factors (in this case, *Rif2*) that will influence replicative senescence in the absence of telomerase. *Rif2* (RAP-1 interacting factor) is a protein known to regulate telomere length in telomerase-proficient cells and influences growth in telomerase-defective cells. *Rif2* is known to attenuate senescence in null mutations in telomerase by acting as a negative regulator of the MRX complex, a complex that is involved in producing replicative senescence. To do this, we over-expressed *Rif2* into est2^{--} mutants strains with null phenotypes to see if *Rif2* will act to suppress senescence. If any of our est2^{--} mutants fail to respond to *Rif2* over-expression, this would suggest that the defect is a telomerase deletion-independent defect. My data show that *Rif2* over-expression in all the mutants I tested suppressed senescence and rescued the strain (Fig. 6).

FIGURES AND TABLES

A.

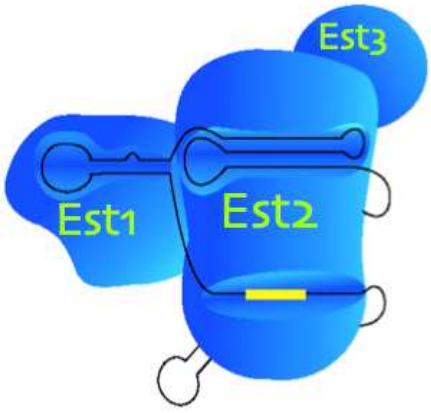


Figure 1. Schematic of Yeast Telomerase and the EST phenotype

(A) Yeast telomerase: The catalytic core of telomerase is made of the telomerase RNA *TLC1* (1.3 kb) and the reverse transcriptase Est2 (100 kDa). The in vivo regulatory factors are comprised of Est1 (80 kDa) and Est3 (18 kDa).

(B) The EST phenotype: As cells divide, there is a progressive decrease in cell viability as shown by single colony streakouts (left) and a corresponding decrease in telomere length (right).

B.

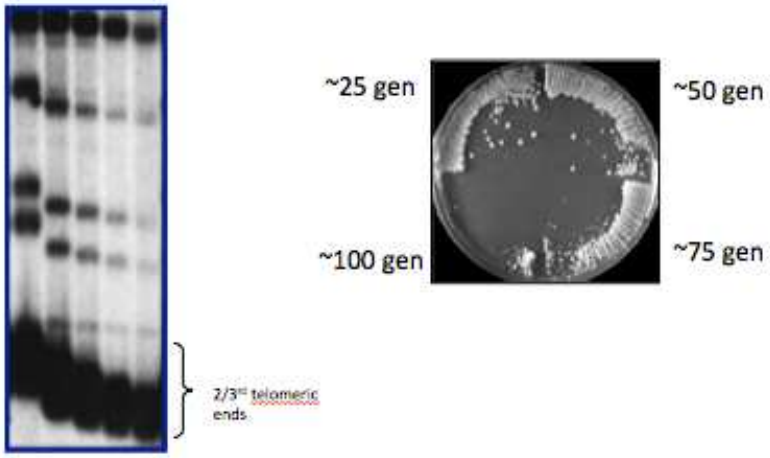


Figure 1. continued

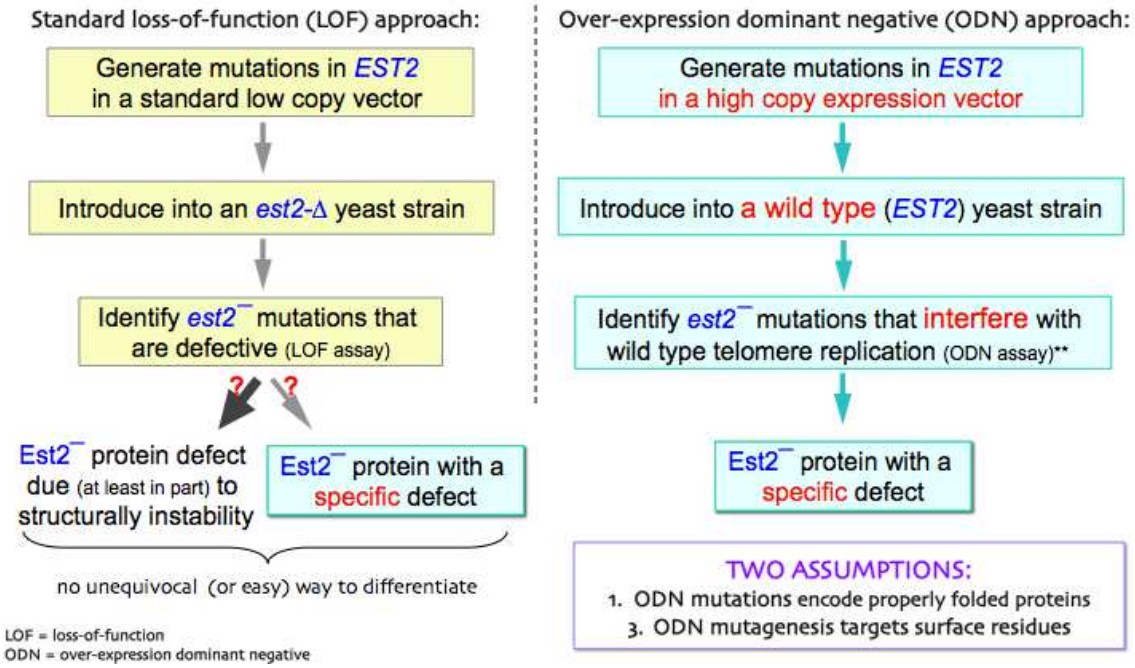


Figure 2. A genetic strategy for generating mutations in predicted surface residues

A.

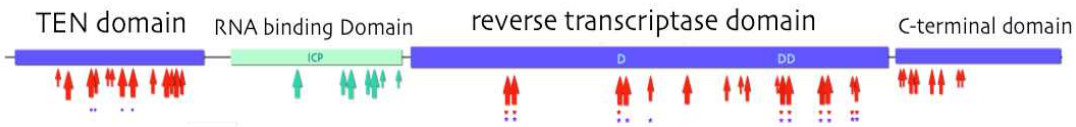


Figure 3. Over-expression dominant negative analysis of EST2

(A) Summary of ODN mutants in each domain of Est2
(B) Synthetic lethality analysis: a yku80-Δ/pYKU80 URA3 strain was transformed with a high copy plasmid (wild type or mutant alleles of EST2), under the control of the constitutive ADH promoter and examined for growth by plating 5-fold serial dilutions on 5FOA-containing media to select for the loss of pYKU80. Growth of the yku80-Δ/pADH-est2^{-} strains was examined at 30°, 34°, and 36°. Est2-D53 0A, Est2-D670A, and/or Est2-D671A null mutations were used as negative controls.
(C) Telomere length analysis: Southern blot of genomic yeast DNA, prepared after ~100 generations of growth from a wild type Est2 strain transformed with a high copy plasmid (wild type or mutant alleles of EST2), under the control of the constitutive ADH promoter and examined for telomere length. Telomeric bands were detected using a telomere-specific probe.

B.

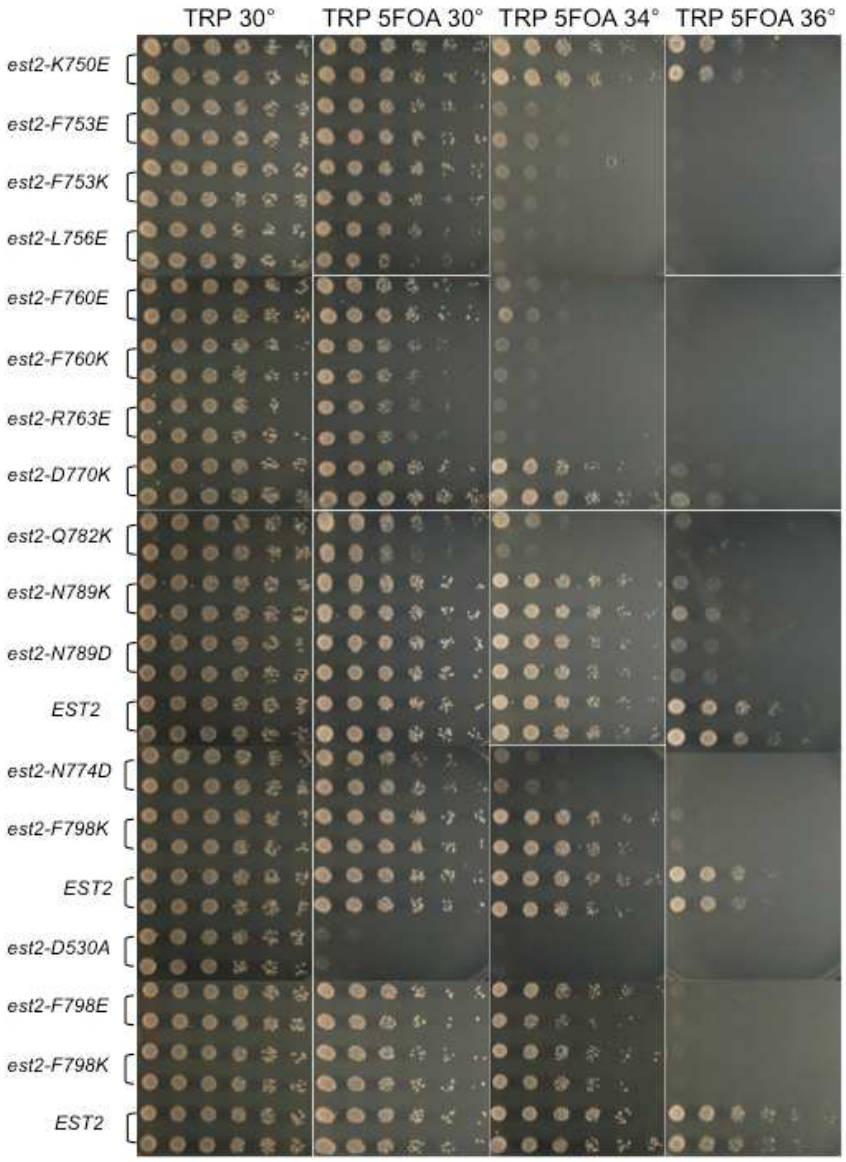


Figure 3. continued

C.

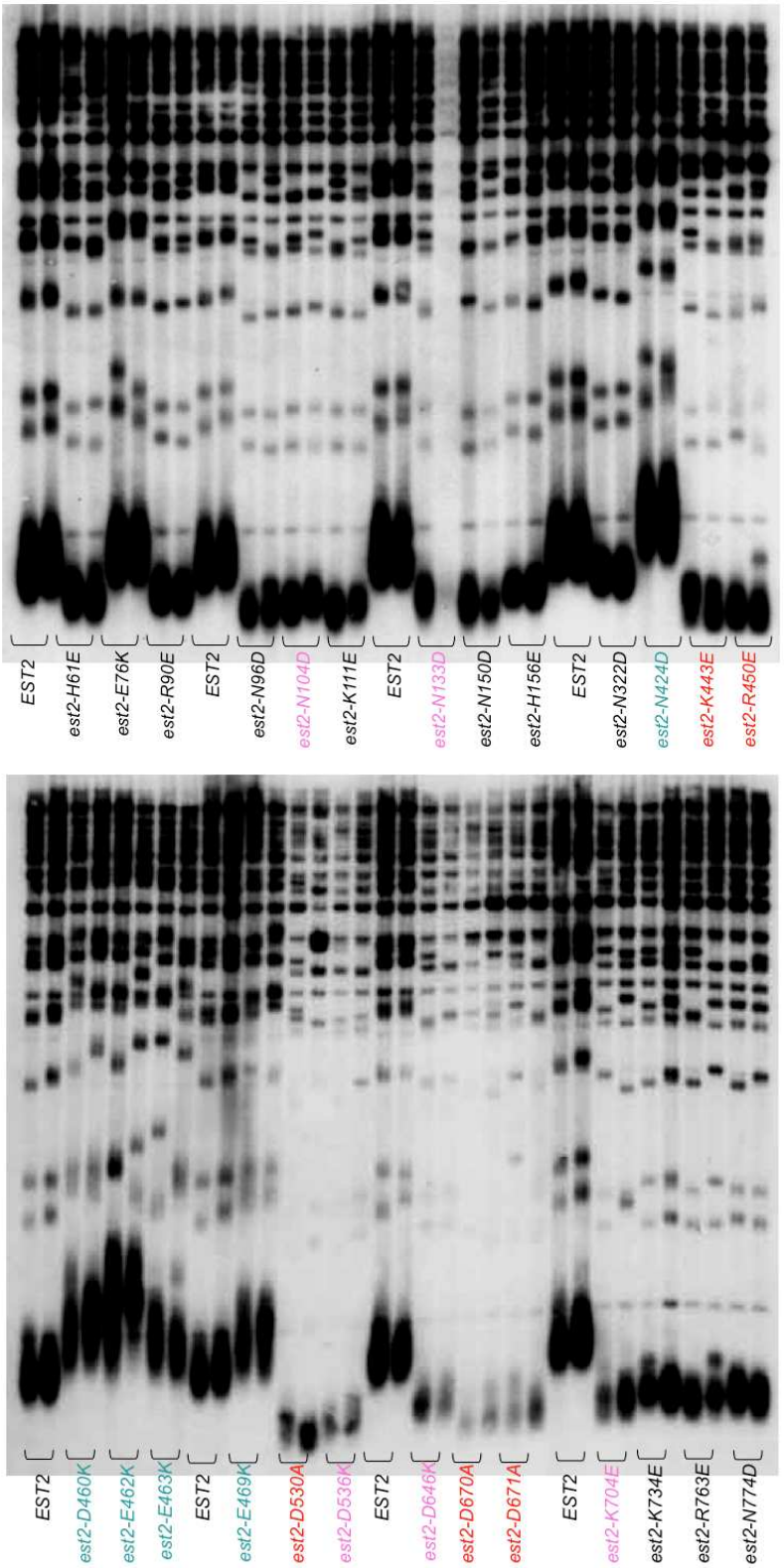


Figure 3. continued

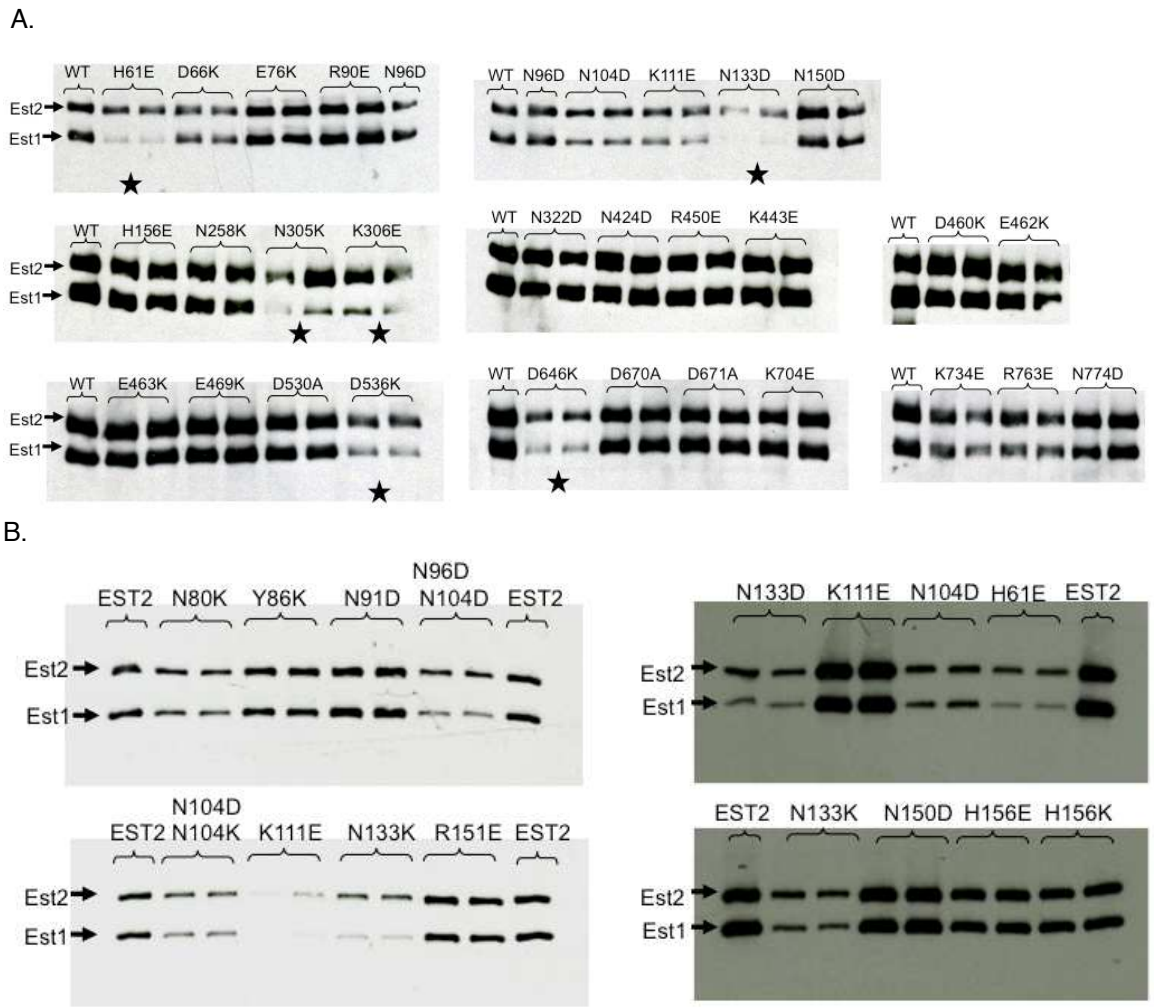


Figure 4. Biochemical analysis of Est2

(A) Est2-*TLC1* interaction is mediated in three domains of Est2. Anti-myc Western blots of anti-FLAG immunoprecipitations of Est2 were examined for Est1 association in the Est1-*TLC1*-EST2 complex. Single-copy plasmids (pCEN $FLAG_3$-myc_{12}-G_6-est2; wild type or mutant Est2) were transformed into Est1-G_6-myc_{12} strain. Mutants with an RNA binding defect are as indicated.

(B) Anti-myc Western blots of anti-FLAG immunoprecipitations of Est2 were examined for Est1 association in the Est2-*TLC1*-EST1 complex in integrated Est2 strains (Est1-G6-myc12 and FLAG3-myc12-G6-Est2, wild-type or mutant Est2).

(C) Est3 association in telomerase is affected by est2$^-$ mutations in the TEN domain. Anti-myc Western blots of anti-HA immunoprecipitations of Est3 to examine Est3 association in integrated Est2 strains. Single-copy plasmids (pCEN $Est3_3$-G_6-myc_6-HA_3) were transformed into $FLAG_3$-myc_{12}-G_6-Est2 (wild-type or mutant Est2)/Est1-G_6-myc_{12} strains.

C.

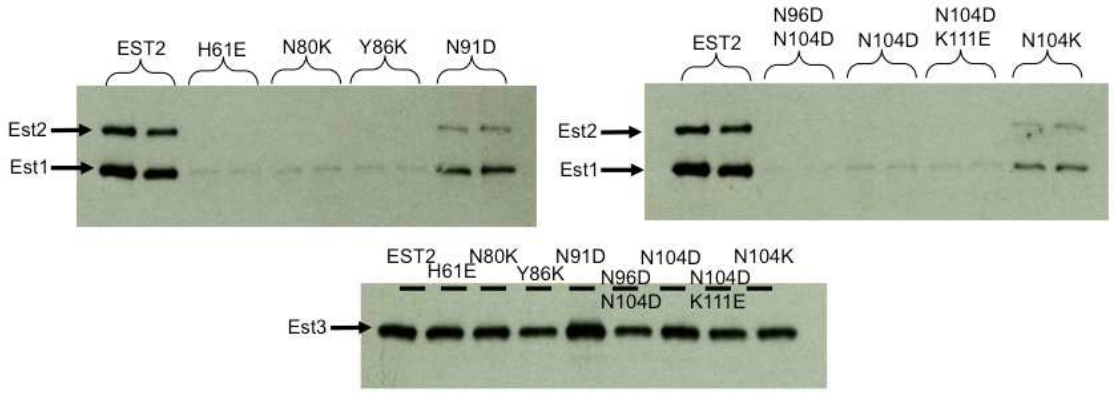


Figure 4. continued

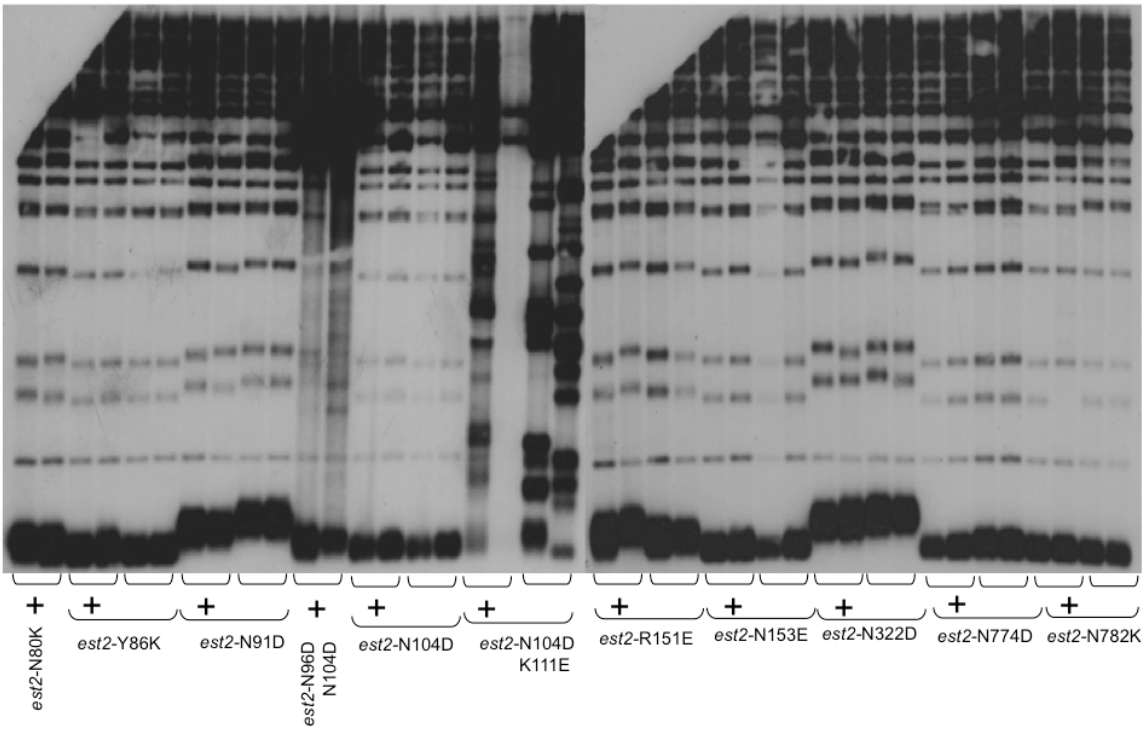


Figure 5. Over-expression of Est3 in integrated Est2 (mutant or wild type) strains does not suppress telomere length defects. Telomere length analysis: Southern blot of genomic yeast DNA, prepared after ~100 generations of growth from a integrated Est2 strain (mutant or wild type) transformed with a high copy plasmid expressing wild type Est3, under the control of the constitutive ADH promoter and examined for telomere length.

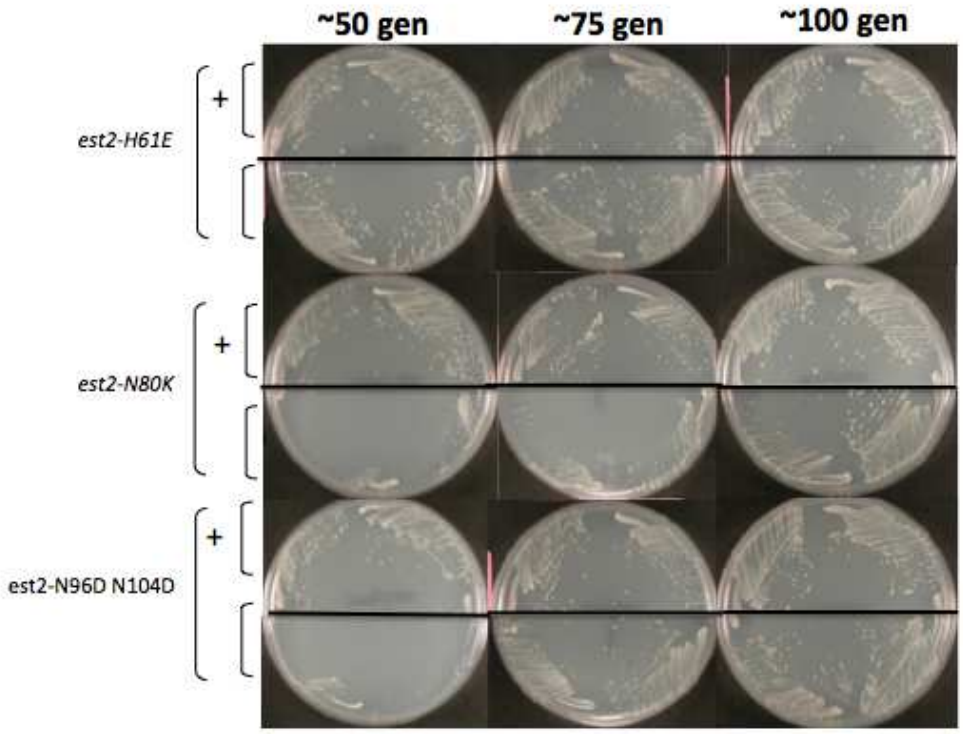


Figure 6. Over-expression of Rif2 in integrated Est2 alleles does not suppress senescence. Senescence analysis: Successive streakouts at ~50, ~75, and ~100 generations of an integrated Est2 strain (mutant or wild type) transformed with a high copy plasmid expressing wild type Rif2, under the control of the constitutive ADH promoter and examined for suppression of senescence.

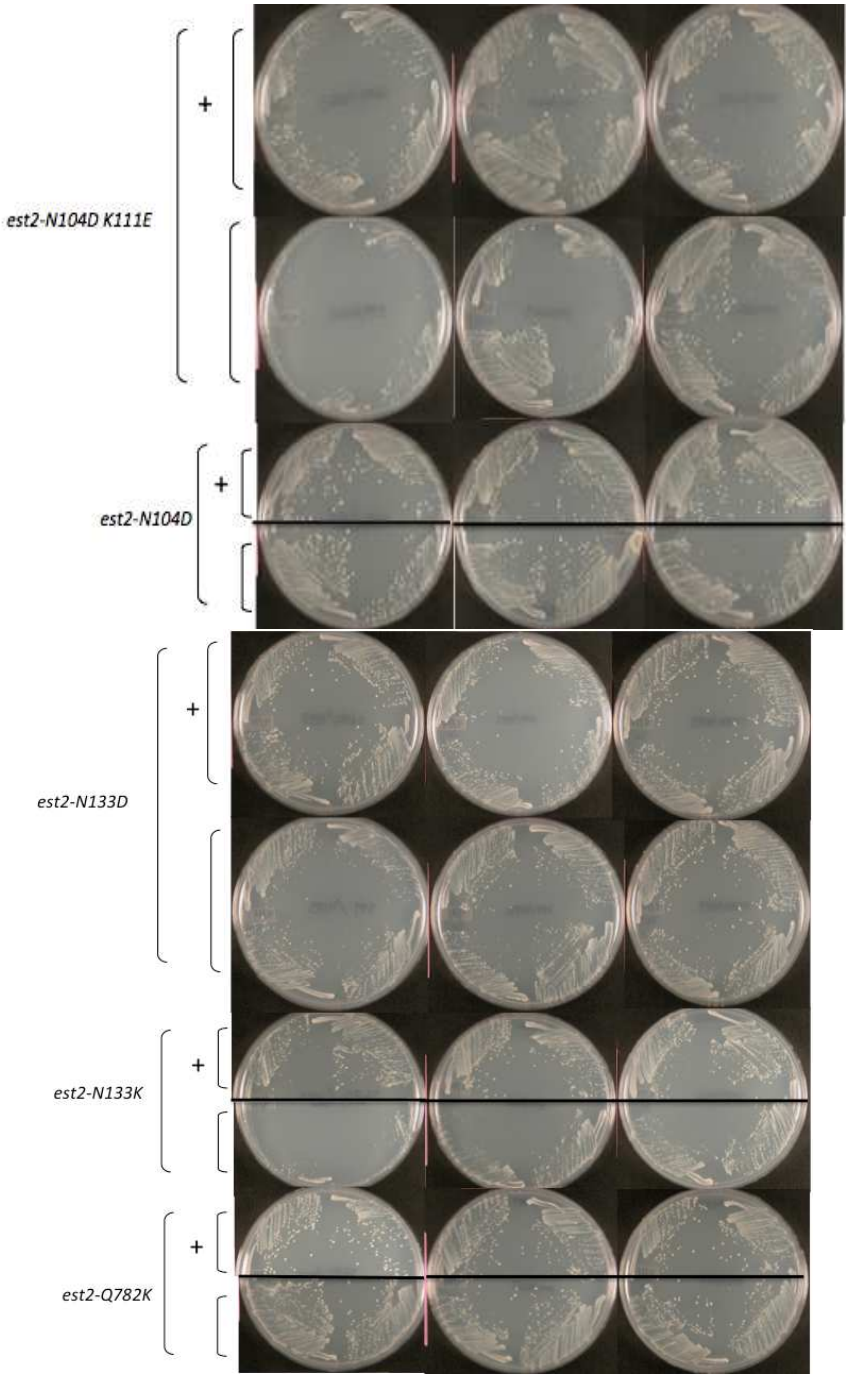


Figure 6. continued

TABLE 1. Complete list of missense mutation in the TEN domain (aa 1-180)

est2 mutation	ODN phenotype	LOF phenotype	Tested in Est2 co-IP assay?	Integrated into YVL3528?	Integrated into YVL2967?
est2-H61E	strong	null	reduced		
est2-H61K	moderate	n.t.			
est2-D66K	n.t.	n.t.	wild type		
est2-E76K	long	n.t.	wild type		
est2-N79D	wild type	n.t.			
est2-N79E	wild type	n.t.			
est2-N79K	wild type	n.t.			
est2-N80K	very strong	null	wild type	Yes	Yes
est2-N80E	strong	n.t.			
est2-N80D	wild type	n.t.			
est2-V81E	moderate	?			
est2-L82E	wild type	n.t.			
est2-G85A	wild type	n.t.			
est2-G85K	wild type	n.t.			
est2-G85E	wild type	n.t.			
est2-Y86E	strong	n.t.			
est2-Y86K	strong	short	wild type	Yes	Yes
est2-K87E	wild type	n.t.			
est2-R90E	moderate	med short	wild type		
est2-N91D	moderate	med short	wild type		
est2-N96D	strong	short	wild type		
est2-N104D	Moderate/ Strong	very short	wild type	Yes	Yes
est2-N104K	Moderate/ strong	short	wild type	Yes	Yes
est2-N104E	moderate	n.t.			
est2-N96D N104D	very strong	null	slightly reduced	Yes	Yes
est2-N104D K111E	n.t.	null	reduced		
est2-K111E	strong	short	wild type	Yes	Yes
est2-H119E	wild type	n.t.			
est2-F127E	moderate	n.t.			
est2-F127K	moderate	n.t.			
est2-N133D	strong	short	reduced	Yes	Yes
est2-N133E	moderate	n.t.			
est2-N133K	moderate	null	reduced	Yes	Yes
est2-N140D	wild type	n.t.			
est2-N140E	wild type	n.t.			
est2-N140K	wild type	n.t.			
est2-Q142E	wild type	n.t.			
est2-Q146E	weak	n.t.			
est2-Q146A	moderate	n.t.			
est2-G149A	moderate	n.t.			
est2-N150D	strong	short	wild type	Yes	Yes

TABLE 1. continued

est2 mutation	ODN phenotype	LOF phenotype	Tested in Est2 co-IP assay?	Integrated into YVL3528?	Integrated into YVL2967?
est2-Q146E N150D	strong	n.t.			
est2-R151E	moderate	med short	wild type	Yes	Yes
est2-R151A	wild type	n.t.	wild type	Yes	Yes
est2-N153E	very strong	very strong	wild type	Yes	Yes
est2-N153K	strong	n.t.			
est2-N153D	strong	n.t.			
est2-E154K	moderate	n.t.			
est2-H156E	strong	med short	wild type	Yes	Yes
est2-H156K	very strong	med short	wild type	Yes	Yes
est2-K160E	wild type	n.t.			

TABLE 2. Complete list of missense mutation in the RBD-terminal domain (aa ~344-757)

est2 mutation	ODN phenotype	LOF phenotype	Tested in Est2 co-IP assay?	Integrated into YVL3528?	Integrated into YVL2967?
est2-K190E	wild type	n.t.			
est2-R218E	wild type	n.t.			
est2-E219K	wild type	n.t.			
est2-K243E	wild type	n.t.			
est2-H248E	wild type	n.t.			
est2-R250E	wild type	n.t.			
est2-Y253E	wild type	n.t.			
est2-Y253A	wild type	n.t.			
est2-Y253R	wild type	n.t.			
est2-N258D	wild type	n.t.	wild type		
est2-N258K	wild type	n.t.			
est2-I260E	wild type	n.t.			
est2-C261Y	wild type	n.t.			
est2-P262R	wild type	n.t.			
est2-H273E	wild type	n.t.			
est2-K285E	wild type	wild type			
est2-V283E	wild type	n.t.			
est2-F286E	n.t.	n.t.			
est2-F286A	wild type	n.t.			
est2-F286R	wild type	n.t.			
est2-K293E	wild type	wild type			
est2-E298K	wild type	wild type			
est2-K303E K304E	wild type	short			
est2-K303E	wild type	n.t.			
est2-K304E	wild type	n.t.			
est2-N305D	wild type	wild type			
est2-N305K	wild type	short	reduced		
est2-K306E	wild type	slightly short	reduced		
est2-N314D	wild type	n.t.			
est2-W321A	wild type	n.t.			
est2-N322D	moderate	slightly short	wild type		
est2-N322K	wild type	slightly short	wild type		

TABLE 3. Complete list of missense mutation in the RT-terminal domain (aa ~757-884)

est2 mutation	ODN phenotype	LOF phenotype	Tested in Est2 co-IP assay?	Integrated into YVL3528?	Integrated into YVL2967?
est2-W321A	wild type	n.t.			
est2-D338K	wild type	wild type			
est2-W341E	wild type	n.t.			
est2-W341R	wild type	n.t.			
est2-W341A	n.t.	n.t.			
est2-E385K	wild type	n.t.			
est2-R396E	slightly long	n.t.			
est2-D398K	slightly long	n.t.			
est2-E418K	wild type	wild type			
est2-N419D	slightly long	slightly long			
est2-N424D	long	n.t.	wild type		
est2-H425E	wild type	wild type			
est2-N426D	slightly long	slightly long			
est2-N432D	slightly long	n.t.			
est2-N434D	wild type	wild type			
est2-H435E	wild type	short			
est2-R439E	wild type	n.t.			
est2-E448K	n.t.	n.t.			
est2-R450E	very strong	n.t.	wild type		
est2-K443E	very strong	n.t.	wild type		
est2-D460K	very long	n.t.			
est2-E463K	n.t.	n.t.	wild type		
est2-E469K	n.t.	slightly long	wild type		
est2-N470D	n.t.	n.t.			
est2-E483K	wild type	med short/short			
est2-R486E	wild type	short			
est2-K488E	wild type	wild type			
est2-R489E	wild type	wild type			
est2-D530A	severe	n.t.	wild type		
est2-D530K	severe	n.t.			
est2-D536K	n.t.	n.t.	reduced		
est2-E554K	wild type	short			
est2-R560E	moderate	med short			
est2-D592K	severe	short			
est2-R626E	moderate	short	reduced		
est2-D628K	wild type	wild type			
est2-D642K	weak	med short	reduced		
est2-D662K	wild type	wild type			

TABLE 3. continued

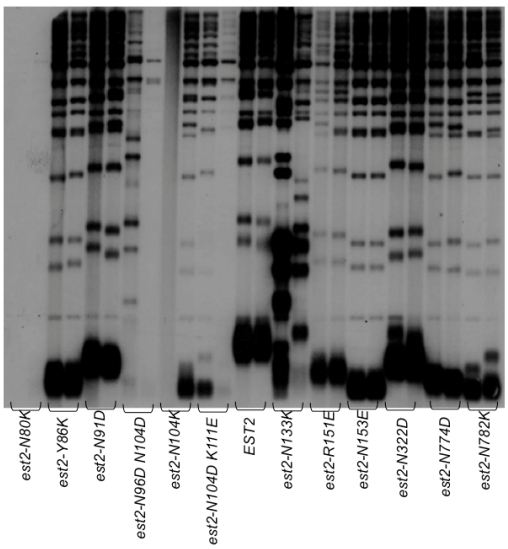
est2 mutation	ODN phenotype	LOF phenotype	Tested in Est2 co-IP assay?	Integrated into YVL3528?	Integrated into YVL2967?
est2-K667E	very strong	short	reduced		
est2-N701D	severe	null			
est2-D670A	severe	n.t.	wild type		
est2-D671K	severe	n.t.	wild type		
est2-D646K	n.t.	n.t.	reduced		
est2-D647K	n.t.	n.t.			
est2-K686E	wild type	n.t.			
est2-N697D	wild type	n.t.			

TABLE 4. Complete list of missense mutation in the C-terminal domain (aa 757-884)

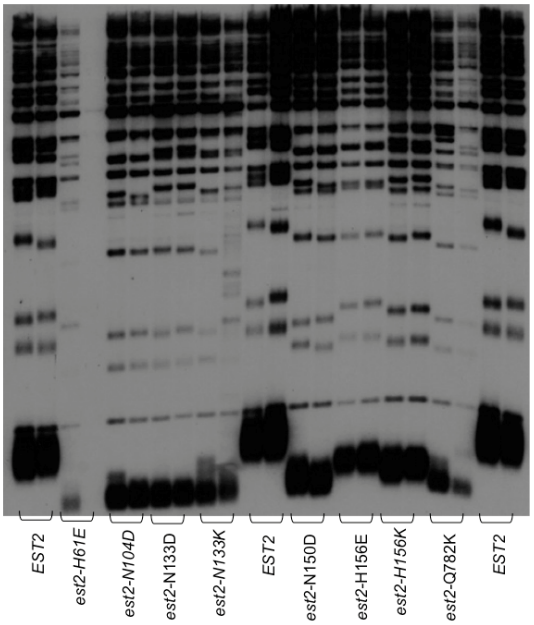
est2 mutation	ODN phenotype	LOF phenotype	Tested in Est2 co-IP assay?	Integrated into YVL3528?	Integrated into YVL2967?
est2-K704E	very strong	n.t.	wild type		
est2-E731K	moderate	n.t.			
est2-K734E	strong	very strong	wild type		
est2-N740D	moderate	n.t.			
est2-K750E	wild type	n.t.			
est2-F753E	strong	n.t.			
est2-F753K	strong	n.t.			
est2-L756E	strong	n.t.			
est2-F760E	strong	n.t.			
est2-F760K	very strong	n.t.		yes	
est2-R763E	very strong	very strong	wild type		
est2-D770K	wild type	n.t.			
est2-N774D	strong	very strong	wild type	yes	yes
est2-K788E	wild type	n.t.			
est2-N789D	weak	n.t.			
est2-Q782E	n.t.	n.t.			
est2-Q782K	very strong	n.t.	wild type	yes	yes
est2-N789D	moderate	n.t.			
est2-N789K	weak	n.t.			
est2-E792K	wild type	n.t.			
est2-F798E	strong	n.t.			
est2-F798K	strong	n.t.			
est2-D832K	wild type	n.t.			
est2-R840E	n.t.	n.t.			
est2-R849E	n.t.	n.t.			

SUPPLEMENTAL FIGURES

A.

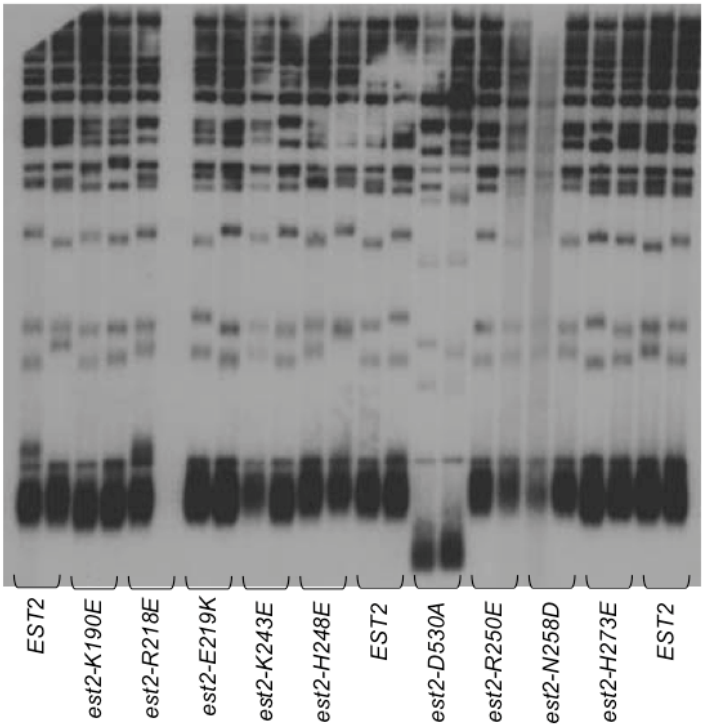


B.

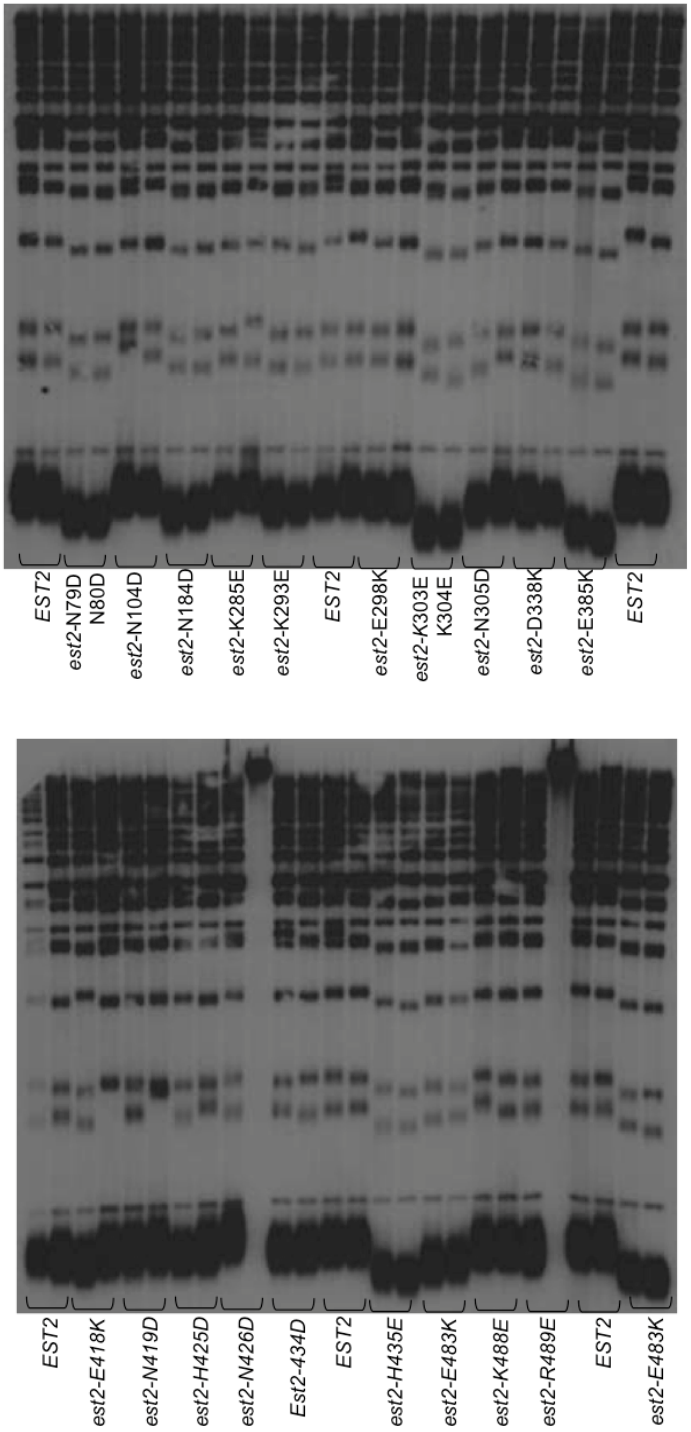


Supplemental Figure 1. Loss-of-function analysis of EST2

(A) Telomere length analysis: Southern blot of genomic yeast DNA, prepared after ~75 generations of growth of integrated Est2 strains (wild type or mutant) and examined for telomere length and
(B) after ~100 generations of growth for indicated strains.



Supplemental Figure 2. Over-expression dominant negative analysis of RBD domain of EST2. Telomere length analysis: Southern blot of genomic yeast DNA, prepared after ~100 generations of growth from a wild type Est2 strain transformed with a high copy plasmid (wild type or mutant alleles of EST2), under the control of the constitutive ADH promoter and examined for telomere length.



Supplemental Figure 3. Loss-of-function analysis of TEN and RBD domain of EST2. Telomere length analysis: Southern blot of genomic yeast DNA, prepared after ~100 generations of growth from an est2-Δ strain transformed with a single-copy plasmid (wild type or mutant alleles of EST2), under the control of the native promoter and examined for telomere length.

DISCUSSION

These results allow us to conclude that our ODN strategy is a powerful tool for finding separation-of-function mutations. The success of my ODN mutagenesis of Est2 as well as our lab's initial mutagenesis of Est3 (and subsequent mutagenesis of DNA replication proteins) has shown that this ODN strategy for locating separation-of-function mutations could potentially be widely applicable to finding functionally important residues where no structural information is available. Information from our initial screen with Est3 led me to mutagenize conserved charged residues of Est2 to the opposite charge. From my mutagenesis, I report numerous ODN mutations in each domain that affect telomere replication. Furthermore, our co-IP and genetic analysis of Est2 has uncovered specific biochemical functions for subset of these mutations. We determined that three domains of Est2 affect Est2-*TLC1* interactions and that the TEN domain is involved in mediating Est2-Est3 interactions. Moreover, a subset of the ODN mutations failed to show an interaction in these assays, suggesting that these residues may play some other specific biochemical function.

One important biochemical function of telomerase is telomerase activity or the rate at which telomerase adds nucleotides to chromosome ends. To assess Est2 mutant effects on telomerase activity, we should perform an enzymatic activity assay, which will assess the nucleotide additions of mutant Est2 proteins compared to wild-type. Telomere length defects can be due to multiple biochemical properties which cannot be assessed in a Southern blot assay. Therefore, performing a telomerase activity assay will allow us to specifically examine whether the telomere replication defected is caused by the rate of nucleotide addition or some other biochemical property (i.e. recruitment-defective telomerase to telomeres).

A subset of Est2 mutants was shown to disrupt RNA binding in the Est1-*TLC1*-Est2 complex. Northern analysis of *TLC1* RNA levels in the telomerase complex should be performed (from the same Est2 immunoprecipitation extracts) to rule out the possibility that reduced Est1 association with the complex was not a consequence of changes in *TLC1* levels. If levels of *TLC1* are unchanged in the Est2 IP extracts, then Est2-*TLC1* interaction is not perturbed and altered

association of Est1 is likely due to defects in Est1-*TLC1* association. Conversely, decreased *TLC1* levels suggest that altered association of Est1 in the complex is likely due to a defect in the Est2-*TLC1* interaction.

Interestingly, I found a number of gain-of-function residues that when mutated, gave rise to longer telomeres. Since these amino acids are in close proximity to each other, it is possible that they cluster together at the surface of the protein and that this surface may have a telomerase inhibiting function. One possibility may be that Est2 has an unknown binding partner that transiently associates to negatively regulate telomerase activity. Another possibility is that these amino acid regions in the RT domain may play a role in binding telomeric DNA. Since these are negatively charged amino acids mutated to positively charged amino acids, it may also be possible that the mutated residues have a higher binding affinity to DNA in the catalytic pocket. This would suggest that these residues are implicated in telomeric DNA binding. Est2 binding affinity to telomeric DNA can be measured using electrophoresis mobility shift assays (EMSA).

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