REPORT

The interaction between the yeast telomerase RNA and the Est1 protein requires three structural elements

JOHNATHAN W. LUBIN,^{1,3} TIMOTHY M. TUCEY,^{1,2,3} and VICTORIA LUNDBLAD^{1,4}

¹Salk Institute for Biological Studies, La Jolla, California 92037-1099, USA

²Division of Biological Sciences, University of California San Diego, La Jolla, California 92093-0130, USA

ABSTRACT

In the budding yeast *Saccharomyces cerevisiae*, the telomerase enzyme is composed of a 1.3-kb TLC1 RNA that forms a complex with Est2 (the catalytic subunit) and two regulatory proteins, Est1 and Est3. Previous work has identified a conserved 5-nt bulge, present in a long helical arm of TLC1, which mediates binding of Est1 to TLC1. However, increased expression of Est1 can bypass the consequences of removal of this RNA bulge, indicating that there are additional binding site(s) for Est1 on TLC1. We report here that a conserved single-stranded internal loop immediately adjacent to the bulge is also required for the Est1–RNA interaction; furthermore, a TLC1 variant that lacks this internal loop but retains the bulge cannot be suppressed by Est1 overexpression, arguing that the internal loop may be a more critical element for Est1 binding. An additional structural feature consisting of a single-stranded region at the base of the helix containing the bulge and internal loop also contributes to recognition of TLC1 by Est1, potentially by providing flexibility to this helical arm. Association of Est1 with each of these TLC1 motifs was assessed using a highly sensitive biochemical assay that simultaneously monitors the relative levels of the Est1 and Est2 proteins in the telomerase complex. The identification of three elements of TLC1 that are required for Est1 association provides a detailed view of this particular protein–RNA interaction.

Keywords: telomerase; TLC1; Est1; RNA structure

INTRODUCTION

In most species, telomerase contributes to the maintenance of chromosome termini using a conserved mechanism for elongation of the G-rich strand of telomeres. The synthesis of telomeric DNA by telomerase relies on a conserved catalytic core composed of a reverse-transcriptase-like subunit and a template-containing RNA, although additional telomerase-associated proteins contribute to holoenzyme function. In humans, telomere erosion in cells that lack telomerase can be a substantial contributing factor to age-dependent limitations on tissue renewal in the lung, liver, pancreas, or bone marrow, resulting in impaired organ maintenance and consequent effects on life span (Savage and Alter 2008; Armanios 2009; Garcia 2011; Guo et al. 2011; von Figura et al. 2011).

The RNA subunit of telomerase provides more than simply a template which dictates the synthesis of telomere

repeats. It contributes to the fidelity of the catalytic reaction cycle and also performs crucial roles in localization and accumulation of the enzyme complex (Blackburn and Collins 2011). Derivation of separate secondary structure models for telomerase RNAs from ciliates, vertebrates, and budding yeasts have revealed several conserved motifs, which include a single-stranded region encompassing the template and a pseudoknot present in a central core domain as well as a 3' stem-terminus element (Theimer and Feigon 2006). However, telomerase RNAs display an exceptional degree of evolutionary diversity, with a striking degree of length variation (ranging from 147 nt to >2 kb) (Ye and Romero 2002; Chakrabarti et al. 2007; Kachouri-Lafond et al. 2009) which is accompanied by sequence divergence even within the conserved structural motifs. The tolerance to such a high degree of variation might be explained if the RNA subunit does not fold into a compact well-defined three-dimensional structure with fixed orientation of structural elements. Consistent with this premise, the budding yeast telomerase RNA has been proposed to form a flexible scaffold for protein assembly (Zappulla and Cech 2004), with independent binding domains for the regulatory Est1 protein and the catalytic Est2 subunit (Evans and Lundblad

³These authors contributed equally to this work.

⁴Corresponding author
E-mail lundblad@salk.edu

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2002; Livengood et al. 2002; Seto et al. 2002; Chappell and Lundblad 2004). This architecture appears to be maintained in the fission yeast telomerase complex as well, where the RNA subunit similarly mediates the indirect association of Est1 with the catalytic subunit (Leonardi et al. 2008; Webb and Zakian 2008).

In budding yeast, the Est1 subunit of telomerase mediates a key regulatory event, which is recruitment of the telomerase enzyme to its site of action through interaction with the telomere-bound Cdc13 protein (Evans and Lundblad 1999; Pennock et al. 2001; Bianchi et al. 2004). Both chromatin immunoprecipitation and live-cell imaging of the telomerase RNA subunit have shown that telomerase associates with telomeres in late S phase, with association eliminated by the recruitment-defective *cdc13-2* mutation (Chan et al. 2008; Gallardo et al. 2011). Est1 therefore serves as a bridging protein, by binding both Cdc13 and the telomerase RNA to bring the catalytic core of the enzyme to its substrate. The Est1–TLC1 interaction is localized to a long helical arm (referred to as Helix IV in this study, based on the nomenclature used by Dandjinou et al. 2004), which can be relocated to ectopic locations within the telomerase RNA (Zappulla and Cech 2004). One determinant for Est1 has already been described, which is a conserved 5-nt bulge present in sub-helix IVc (Seto et al. 2002). We show here that a second conserved element—a single-stranded internal loop which is immediately adjacent to the pentanucleotide bulge—is also critical for recognition of TLC1 by Est1. These observations argue that the specificity of this interaction relies on two adjacent RNA binding motifs which together generate an interface for RNA recognition by Est1.

RESULTS AND DISCUSSION

A conserved internal loop in Helix IV of TLC1 is essential for in vivo function

Previous work has established that a 5-nt bulge (nucleotides 660–664) (Fig. 1A) mediates Est1 association with the telomerase complex (Seto et al. 2002). To address whether other features of Helix IV are required for TLC1 function, two mutations that deleted sub-helices IVb and IVc to generate *tlc1-201* ($\Delta 559$ –598) and *tlc1-101* ($\Delta 599$ –670), respectively (Fig. 1A), were assessed for effects on telomere length (Fig. 1B) and senescence (Fig. 1C and data not shown). Sub-helix IVb appeared to be dispensable for function, as telomere maintenance in the *tlc1-201* strain was indistinguishable from a wild-type *TLC1* strain, indicating that this sub-helix did not contribute to Est1 association with the RNA. In contrast, the *tlc1-101* strain, bearing a deletion which encompassed the previously characterized Est1-binding bulge, resulted in a severe telomere replication defect. The phenotype of the *tlc1-101* strain was comparable with that displayed by the *tlc1-47* strain, which expressed a variant of TLC1 which was only

deleted for the bulge (Fig. 1B,C). However, the telomere replication defects of *tlc1-101* and *tlc1-47* strains differed in one key aspect when examined in the presence of increased levels of the Est1 protein. In the presence of a high copy plasmid which expressed Est1 from the constitutive ADH promoter, the senescence defect of the *tlc1-47* strain was reversed (Fig. 1C) and telomeres were restored back to a length that approximated that of a wild-type strain (see below), as previously observed (Seto et al. 2002). In contrast, the severe growth defect and short telomeres displayed by the *tlc1-101* strain were unaffected by the presence of the ADH-Est1 plasmid (Fig. 1C and data not shown).

The fact that the telomere replication defect displayed by the *tlc1-47* mutant strain could be suppressed by enhanced Est1 expression argued that one or more binding sites for Est1 were still present in the TLC1 RNA bearing the $\Delta 660$ –664 deletion. Since Est1 overexpression failed to suppress the *tlc1-101* defect, we surmised that an additional binding site for Est1 might exist in sub-helix IVc. As a first step in testing this premise, we reexamined the predicted structure of this helix, based on a phylogenetic comparison of sequences from species from the *Saccharomyces* and *Kluyveromyces* clades (Fig. 1D). Comparison of this region of TLC1 revealed the presence of a conserved internal loop, adjacent to the 660–664 bulge, with two single-stranded sequences (5′-U₆₁₅UGCAA₆₂₁-3′ and 5′-A₆₄₈UG₆₅₀-3′; indicated in red in Fig. 1D) which were highly conserved. Previous work by Tomaska and colleagues analyzing conserved domains in the *Candida* clade telomerase RNAs proposed a similar structure, although with more relaxed pattern of sequence conservation, which may reflect the absence of Est1 homologs in several species in the *Candida* clade (Gunisova et al. 2009).

To test whether this internal loop was required in vivo, a panel of five mutations was constructed (depicted in Fig. 2A). Two mutations changed the sequence of either strand of the two conserved blocks of sequence in the internal loop, without altering the proposed single-stranded secondary structure (*tlc1-202* and *tlc1-203*). Two additional mutations changed the sequence of either of the two single strands of the loop (*tlc1-204* and *tlc1-205*), creating a structure that would be predicted to form a duplex and thereby eliminate the internal loop. Finally, a mutation which deleted the predicted terminal hairpin was generated (*tlc1-206*). These five mutations were introduced into a *tlc1-Δ* strain and examined for effects on telomere length and senescence (Fig. 2B,C). Alteration of one of the two blocks of conserved nucleotides (5′-AUG-3′) resulted in a modest reduction in telomere length (*tlc1-202*), indicating that this region of the internal loop made a minimal contribution to TLC1 function. In contrast, introducing a sequence change into the larger block of conserved nucleotides on the other strand of the internal loop (5′-UUGCAA-3′) resulted in extremely short telomeres (*tlc1-203*). Furthermore, two mutations which closed the internal loop to generate a

FIGURE 1. Deletion of sub-helix IVc cannot be suppressed by overexpression of Est1. (A) Proposed secondary structure of Helix IV of TLC1, with the boundaries indicated for two mutations, *tlc1-101* and *tlc1-201*, which delete sub-helices IVc and IVb, respectively; the previously described Est1-binding bulge (nucleotides 660–664), which is deleted by the *tlc1-47* mutation, is indicated by the smaller box. (B) Telomere length of the indicated strains, examined after ~75 generations of growth; size markers (in kb) are indicated. (C) Growth characteristics of TLC1, *tlc1-47*, and *tlc1-101* strains in the presence (left) or absence (right) of a high copy plasmid expressing the Est1 telomerase subunit from the constitutive ADH promoter; strains were examined at a point when senescence was most pronounced (~100 generations). (D) Proposed secondary structures for sub-helix IVc from TLC1 RNAs from selected isolates from the *Saccharomyces* and *Kluyveromyces* clades; the two clusters of sequences indicated in red are invariant in the *Saccharomyces* clade, with partial conservation extending into the *Kluyveromyces* clade. Determination of structures was based on sequence alignments to identify conserved elements as well as comparison with previously published proposed secondary structures, aided by RNA Mfold.

predicted duplex (*tlc1-204* and *tlc1-205*) displayed a phenotype indistinguishable from that of a *tlc1-Δ* null strain. Removal of the nonconserved terminal hairpin (*tlc1-206*) also reduced telomere length, although the effect was less pronounced. The telomere replication defects displayed by these strains were not due to reduced levels of TLC1, as quantitation of the levels of the TLC1 RNA (normalized to levels of the U1 RNA) in extracts prepared from strains in which these *tlc1* mutations were integrated into the

genome did not reveal any significant differences, relative to wild-type TLC1 (normalized TLC1/U1 ratios for *tlc1-47*, *tlc1-202*, *tlc1-203*, *tlc1-204*, and *tlc1-206* were 1.0, 1.0, 1.0, 1.1, and 0.9, respectively; data not shown). Collectively, this analysis indicates that the conserved internal loop is a protein binding site which is essential for in vivo TLC1 function, with the 5′-UUGCAA-3′ and 5′-AUG-3′ sequence motifs on either single strand contributing to binding.

FIGURE 2. A conserved internal loop is required for telomere length maintenance. (A) A depiction of mutations introduced into the conserved internal loop of sub-helix IVc, with blue boxes or triangles indicating sequence changes or deleted nucleotides, respectively. (B) Telomere length of strains bearing the indicated mutations (expressed from single-copy plasmids introduced into the *tlc1-Δ* strain YVL3554) examined after ~100 generations, at a point when the *tlc1-Δ* null strain (lane 11) has given rise to recombination-dependent survivors (Lundblad and Blackburn 1993). (C) Growth characteristics of the indicated mutant strains, which have been propagated for ~100 generations.

Mutations in the conserved internal loop are suppressed by Est1 overexpression

The obvious candidate for binding to this internal loop would be Est1, given the close proximity to the previously defined Est1 binding site at nucleotides 660–664. This predicts that increased expression of Est1 might similarly suppress mutations in this second proposed protein binding site. Consistent with this prediction, the severe telomere replication defect displayed by the *tlc1-203* strain, in which

the conserved 5′-UUGCAAA-3′ motif was mutated, was reversed by overexpression of Est1. Telomeres were restored to roughly wild-type telomere length, comparable with that previously observed for a *tlc1-47* mutant strain (Fig. 3A; Seto et al. 2002). Suppression of the *tlc1-47* and *tlc1-203* defects by Est1 overexpression was also specific, as increased expression of either Est2 or Est3 failed to suppress either mutation (data not shown). These observations indicate that in the presence of excess Est1, either of these two structures is sufficient to mediate association of Est1 with the complex. In contrast, a variant of TLC1 (*tlc1-207*), which combined the *tlc1-47* and *tlc1-203* mutations to eliminate both of the proposed binding sites, exhibited a different behavior in response to increased levels of Est1. Like the *tlc1-47* strain, the *tlc1-207* strain displayed a telomere replication defect that was indistinguishable from a *tlc1-Δ* null strain (Figs. 2C, 3A). However, the ADH–Est1 construct was unable to reverse the short telomere length and senescence phenotype of the *tlc1-207* strain (Fig. 3A and data not shown). Collectively, these overexpression data argue that the main determinants for association of Est1 with the telomerase complex are two adjacent structures on sub-helix IVc.

In addition to *tlc1-203*, the short telomere phenotypes of strains expressing either *tlc1-202* or *tlc1-206* were also restored to approximately wild-type length by enhanced expression of Est1 (data not shown). However, the *tlc1-204* and *tlc1-205* strains, which expressed mutant derivatives of TLC1 with the internal loop replaced by a duplex structure, could not be suppressed by the ADH–Est1 construct (Fig. 3B and data not shown). This indicates that, in the complete absence of the internal loop, the bulge at 660–664 nt is not sufficient to sustain association of Est1 with TLC1, arguing that the internal loop may make a more dominant contribution to the Est1–TLC1 interaction.

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The internal loop is required for association of Est1 with telomerase

The above genetic observations indicate that both the bulge and the internal loop of Helix IVc are required for Est1

FIGURE 3. Est1 association with telomerase is abolished by mutations in the conserved internal loop. (A) Telomere length of the indicated strains was examined after ~100 generations. (B) Growth characteristics determined as described in Figure 1C, although strains were examined at ~75 generations, when senescence was not as pronounced. (C,D). Anti-myc Western analysis of anti-FLAG immunoprecipitates following 6% PAGE of the indicated strains; the *tlc1* mutations were integrated into the genome of YVL3493 (with the exception of *tlc1*-207 and the accompanying *TLC1* control, which were present on single-copy plasmids in the YVL3701 *tlc1*-Δ strain). (E) Northern analysis monitoring levels of the *TLC1* RNA in the same immunoprecipitates shown in D.

binding to TLC1. To test this premise biochemically, association of a myc-tagged variant of Est1 with the telomerase complex was monitored using an assay that monitored the relative level of the Est1 and Est2 proteins following immunoprecipitation of Est2. To do so, we generated a strain in which both Est1 and Est2 could be detected by identical (myc)₁₂ epitopes present at the C terminus and N terminus, respectively, of the two Est proteins; in addition, the Est2 protein contained a triple FLAG epitope adjacent to the (myc)₁₂ epitope. These tagged versions were introduced into the genome in place of the untagged *EST1* and *EST2* genes, so that each tagged protein was expressed by its native promoter. The (myc)₁₂ and (FLAG)₃ sequences were also engineered in order to minimize any disruption of protein function, such that the resulting strain had wild-type telomere length (data not shown).

The design of the Est1-(myc)₁₂ (FLAG)₃-(myc)₁₂-Est2 strain allows quantitative assessment of the degree of association of the Est1 protein with the Est2-TLC1 catalytic core of telomerase, by simultaneously monitoring Est1 and Est2 protein levels with the same antibody (anti-myc) following anti-FLAG immunoprecipitation of the Est2

subunit. Since numerous previous studies have established that Est1 and Est2 independently bind TLC1 (Evans and Lundblad 2002; Livengood et al. 2002; Seto et al. 2002; Chappell and Lundblad 2004), this assay provides an alternative means of quantitating the Est1–RNA interaction. This has several advantages relative to approaches used previously by our laboratory and others. For example, one commonly used assay monitored the relative ability of wild-type *TLC1* and a deletion derivative (*TLC1*– $\Delta 148$ –440) to be co-immunoprecipitated with a telomerase protein subunit (Chappell and Lundblad 2004; Lee et al. 2008). However, this strategy relies on a strain which expresses twice the normal level of TLC1; given that TLC1 is limiting for telomere length maintenance, altering the dosage of TLC1 may adversely affect the conclusions that stem from this method (Mozdy and Cech 2006). Furthermore, most strains that employ this assay express at least one of the two TLC1 variants from a plasmid; variations in plasmid copy levels (for example, see input lanes of Fig. 3D in Chappell and Lundblad 2004) can further limit the sensitivity of the *TLC1* vs. *TLC1*– $\Delta 148$ –440 assay. In contrast, in the assay used here, the epitope-tagged Est1 and Est2 subunits of the

telomerase complex were expressed from alleles integrated at their respective genomic loci, as were the mutant *tlc1* RNAs (with one exception).

Examination of anti-FLAG immunoprecipitates prepared from extracts of the Est1-myc₁₂ (FLAG)₃-(myc)₁₂-Est2 strain demonstrated that Est1 could be co-immunoprecipitated with Est2 with a highly reproducible 1:1 stoichiometry (Fig. 3C,D). Detection of both Est1 and Est2 in immunoprecipitates was dependent on the presence of the FLAG epitope, and as predicted by prior observations, association of Est1 could not be detected in a strain expressing the *tlc1*-47 mutation, which removed the 5-nt bulge (Fig. 3C). Similarly, mutations which removed the internal loop (*tlc1*-204 and *tlc1*-205) also eliminated interaction with the complex (Fig. 3D and data not shown), establishing that this conserved internal loop, like the 5-nt bulge, is required for Est1-TLC1 binding.

These three mutations (*tlc1*-47, *tlc1*-204, and *tlc1*-205), which conferred a telomere replication defect comparable with that of a *tlc1*-Δ null strain, reduced the amount of the Est1 protein in the anti-FLAG immunoprecipitates to below detection levels. Thus, although the genetic data implied that the internal loop was more important for binding of Est1 to TLC1, we were unable to detect a difference between mutations in the 5-nt bulge vs. the internal loop in this biochemical assay. Nevertheless, for mutations that did not confer a null phenotype, this biochemical assay was sensitive enough to detect a reduction in Est1 levels in anti-Est2 immunoprecipitates which closely correlated with the severity of the in vivo phenotype. For example, the *tlc1*-203 mutation, which altered the highly conserved 5'-UUGCAA-3' motif of the internal loop, conferred a pronounced telomere replication defect which was slightly less severe than the null phenotype exhibited by the *tlc1*-47, *tlc1*-204, and *tlc1*-205 strains. This slight retention of function was reflected in the ability to detect a very low level of Est1 in the *tlc1*-203 immunoprecipitates (Fig. 3D, left panel), which was abolished by in the *tlc1*-207 mutant strain which combined the *tlc1*-47 and *tlc1*-203 mutations (Fig. 3D, middle panel). The relative association of Est1 with telomerase in the *tlc1*-202 and *tlc1*-206 strains similarly correlated with telomere length. The *tlc1*-202 strain, in which the smaller conserved region of the internal loop (5'-AUG-3') was mutated, exhibited a moderate reduction in the ability of Est1 to associate with telomerase, with Est1 association further reduced in the *tlc1*-206 mutant strain which lacked the terminal hairpin (Fig. 3D, right hand panel). Since the hairpin deleted by the *tlc1*-206 mutation does not exhibit any conserved sequence elements (Fig. 1D), it might simply provide structural stability for the conserved loop; however, we cannot rule out the possibility of an additional interaction between Est1 and this region of the RNA. For all of these mutations, the interaction between the catalytic Est2 protein and the RNA was unchanged (Fig. 3D,E). This demonstrates

that the altered association of Est1 with the complex was not an indirect consequence of reduced TLC1 levels and also indicates that the in vivo defects displayed by this set of mutant RNAs was not simply due to global misfolding.

An additional single-stranded region of TLC1 is required for Est1 binding

The analysis shown in Figure 1 indicated that sub-helix IVb was dispensable for Est1 function, in contrast to the two conserved binding sites for Est1 in sub-helix IVc. However, the deletion of sub-helix IVb to generate the *tlc1*-201 mutation still retained an internal single-stranded loop at the base of sub-helix IVc (indicated by the gray box in Fig. 4A). Furthermore, a prior phylogenetic analysis of Helix IV supported the idea that a discontinuous duplex at the base of sub-helix IVc is conserved (Dandjinou et al. 2004; Zappulla and Cech 2004). To investigate this further, mutations which eliminated several adjacent secondary structures were constructed (Fig. 4A) and examined for effects on telomere length maintenance (Fig. 4B). These results showed that conversion of the *tlc1*-201 RNA to a version with a continuous duplex stem (*tlc1*-209) resulted in short telomeres (Fig. 4B) and reduced association of Est1 with the telomerase complex (Fig. 3D), which was not due to reduced levels of the *Tlc1*-209 RNA in either extracts or immunoprecipitates (Fig. 3E and data not shown). This was also not the consequence of the particular nucleotide changes used to convert *tlc1*-201 to *tlc1*-209, as the same mutation had no effect when introduced into the wild-type TLC1 RNA to create *tlc1*-208 (Fig. 4B). Furthermore, increased expression of Est1 reversed the telomere length defect of the *tlc1*-209 mutant strain (Fig. 4C), similar to effects observed with mutations in the 5-nt bulge and the conserved internal loop. Figure 4 further shows that one additional internal loop (at nucleotides 532–535), which was eliminated by *tlc1*-210 and *tlc1*-211, was not required for function.

The experiments reported here provide a comprehensive analysis of all of the proposed secondary structural elements in Helix IV of TLC1. These results lead us to propose that three discrete features of sub-helix IVc are required for Est1 binding: the previously characterized pentanucleotide bulge, an immediately adjacent internal loop, and a single-stranded region at the base of sub-helix IVc. Both the bulge and internal loop structures exhibit sequence conservation (Fig. 1D) which presumably mediates specificity for binding by Est1; whether these two motifs collaborate or instead represent two discrete RNA–protein interactions will most likely await a structural determination of the Est1:RNA complex. In contrast, there does not appear to be any sequence specificity at the base of sub-helix IVc, suggesting that this third structural feature simply provides a flexible hinge which may help ensure accessibility of the two Est1 binding motifs. These results also demonstrate that sub-

FIGURE 4. A single-stranded region at the base of sub-helix IVc contributes to TLC1-Est1 association. (A) Proposed secondary structure of Helix IV, with various mutations indicated as in Figure 1D; the *tlc1-209* mutation is a combination of the mutations shown in *tlc1-201* and *tlc1-208*. (B) Telomere length of strains expressing the mutations shown in A, after 75 generations of growth. (C) Telomere length of the *tlc1-209* strain in the presence or absence of a high copy ADH-Est1 plasmid, compared with a *TLC1* strain with the same plasmid.

helix IVb is dispensable for in vivo function, at least in a laboratory setting; however, the fact that this sub-helix is conserved at least among the *Saccharomyces* clade (Dandjinou et al. 2004; Zappulla and Cech 2004) suggests a role for this sub-helix in TLC1 function, which has not yet been elucidated.

MATERIALS AND METHODS

Strains and plasmids

All in vivo observations were performed with derivatives of YVL3554 (*MAT α tlc1- Δ ::HIS ura3-52 lys2-801 trp- Δ 1 his3- Δ 200 leu2- Δ 1/p CEN URA3 TLC1*), which was transformed with single-copy *CEN TRP1* plasmids expressing wild-type or mutant *TLC1* genes expressed from the native *TLC1* promoter (derived from pVL795, as described below). The *TLC1 URA3* plasmid was subsequently evicted by propagation on 5-FOA-containing media, and strains were grown for ~75–100 generations prior to examination of senescence and/or telomere length, which were monitored as described previously (Lendvay et al. 1996; Chappell and Lundblad 2004). The parental strains used for the biochemical experiments shown in Figure 3E and 3D, respectively, were YVL3493 (*MAT α leu2 trp1 ura3-52 prb⁻ prc⁻ pep4-3 Est1-Gly₆-myc₁₂ FLAG₃-myc₁₂-Gly₆-Est2*) or YVL3701 (a *tlc1- Δ* derivative of YVL3493 containing a *TLC1 URA3* plasmid). *TLC1* mutations were introduced by Quikchange mutagenesis into pVL795 (*CEN TRP1 TLC1*) (Chappell and Lundblad 2004) to generate pVL2128 (*tlc1-47*; Δ 660–664), pVL2272 (*tlc1-101*; Δ 599–670), pVL5385 (*tlc1-201*; Δ 559–598), pVL5377 (*tlc1-202*; A₆₄₈TG₆₅₀ → CCA), pVL5379 (*tlc1-203*; T₆₁₅TGCAAA₆₂₁ → TCTTACC), pVL5378 (*tlc1-204*; T₆₁₅TGC AAACA₆₂₃ → ATAAAGT), pVL5602 (*tlc1-205*; A₆₄₃CTTTAT₆₄₉ → TGTGTTGCAA), pVL5386 (*tlc1-206*; Δ 625–641), pVL5640 (*tlc1-207*; Δ 660–664, T₆₁₅TGCAAA₆₂₁ → TCTTACC), pVL5689 (*tlc1-208*; A₆₇₃ → T, A₆₇₅ → T, A₆₈₃ → CG), pVL5599 (*tlc1-209*; Δ 559–598, A₆₇₃ → T, A₆₇₅ → T, A₆₈₃ → CG), pVL5600 (*tlc1-210*; Δ 559–598, A₆₉₃CGT₆₉₆ → TAAA), and pVL5601 (*tlc1-211*; Δ 559–598, T₅₃₂TTA₅₃₅ → ACGT).

Biochemical methods

Whole-cell extracts were prepared from 250-mL cultures grown to O.D. 0.8–1.0, and extract preparation and immunoprecipitations with anti-FLAG M2 beads (Sigma) were performed as described previously (Evans and Lundblad 2002). The Est1-(myc)₁₂ and (FLAG)₃-(myc)₁₂-Est2 proteins were detected on 6% SDS-PAGE gels with anti-myc 2272 (Cell Signaling Technology) at 1:1000 dilution and anti-rabbit IgG HRP conjugate (Promega) at 1:10,000. Northern analysis to monitor TLC1 RNA levels was conducted as described in Lee et al. (2008).

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Johnathan W. Lubin, Timothy M. Tucey and Victoria Lundblad

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