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Telomere Maintenance in Fission Yeast Requires an Est1 Ortholog

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Summary

Telomerase regulation is critical to genome maintenance yet remains poorly understood. Without telomerase's ability to synthesize telomere repeats, chromosome ends shorten progressively, as conventional DNA polymerases cannot fully replicate the ends of linear molecules [1]. In *Saccharomyces cerevisiae*, telomerase activity in vivo absolutely depends on a set of telomerase accessory proteins [2–4] that includes Est1p [5], which appears to recruit or activate telomerase at the site of polymerization [6, 7]. Thus, *est1Δ* cells have the same cellular senescence phenotype as cells lacking either the catalytic protein subunit of telomerase or its template-containing RNA subunit [8, 9]. While the telomerase protein is highly conserved among eukaryotes, the apparent lack of Est1p homologs has frustrated efforts to describe a common mechanism of telomerase recruitment and activation. Here, we describe *Sp*Est1p, a homolog of Est1p from the evolutionarily distant *Schizosaccharomyces pombe*. Like *Sc*Est1p, *Sp*Est1p is required for telomerase activity in vivo. Coupled with the identification of an orthologous Est1 protein in humans [10], this suggests a much wider conservation of telomerase regulation than was previously known. Strikingly, in cells with compromised telomere function (*taz1Δ*), *Sp*Est1p loss confers a lethal germination phenotype, while telomerase loss does not, indicating that *Sp*Est1p plays an unexpected additional role in chromosome end protection.

Results and Discussion

We have identified the *Schizosaccharomyces pombe* Est1 protein (*Sp*Est1p, ORF SPBC2D10.13) by profile comparison to the Est1 protein of *S. cerevisiae* (see the Experimental Procedures in the Supplemental Data available with this article online, [10]). *S. pombe est1$^+$* encodes a 491 residue polypeptide of 57 kDa predicted molecular weight. *Sc*Est1p lacks detectable homology to *Sp*Est1p over large parts of its sequence, and only a small region near the amino terminus shows a significant degree of homology (Figure 1A). This region comprises the so-called EST1 domain [10] and a diverged version of the tetratricopeptide repeat, a motif implicated in protein-protein interactions (Figure 1A). Interestingly, these domains lie in a region of unknown function in the *Sc*Est1p sequence, outside the previously identified DNA binding and RNA recognition domains of *Sc*Est1p [11, 12] (Figure 1B). Localization of expressed *Sp*Est1p in live cells reveals that the protein is nuclear and accumulates in discrete foci, which are primarily associated with the nuclear periphery at the nucleolar boundary (not shown). This pattern of localization resembles that of the *S. pombe* telomere binding protein Taz1p, and this similarity suggests that expressed *Sp*Est1 may colocalize with telomeres.

Deletion of *est1$^+$* results in phenotypes expected for cells with telomere maintenance deficiencies. Tetrad dissection of a heterozygous diploid *est1Δ/est1$^+$* strain resulted in four evenly sized colonies, indicating that *est1$^+$* is not essential for viability and confers no apparent initial growth defect. However, null mutants of *est1$^+$* display a senescence phenotype virtually identical to that observed in telomerase null (*trt1Δ*) strains [13]. At approximately 60–70 generations of growth, *est1Δ* strains begin to exhibit a pronounced loss of viability (Figure 2A). Telomere length also decreases during successive passage, and this decrease correlates tightly with the loss of cell viability (Figure 2B). At the point of "crisis," when population viability is lowest and telomere length has diminished to the limits of detection, *est1Δ* cultures accumulate elongated cells and display a high degree of chromosome instability and missegregation (Figure 2C). The kinetics of the onset of senescence and subsequent crisis in *trt1Δ* and *est1Δ* strains are nearly indistinguishable. No additional phenotypes were evident in *trt1Δest1Δ* double mutant strains, and both single and double mutant strains enter senescence with identical kinetics (not shown).

In addition to *Sp*Est1p, a second *S. pombe* gene was identified that displayed similar levels of profile homology to *Sc*Est1p (ORF SPBC2F12.03c; Figures 1A and 1B). While this gene product is primarily associated with the nucleus [14], telomere lengths in cells lacking this gene were normal. Likewise, these cells displayed no obvious growth defects or other distinguishing phenotypes. Therefore, we concluded that this protein does not act in any nonredundant capacity to maintain telomeres under standard culture conditions.

A hallmark of telomerase accessory proteins is their requirement for in vivo, but not in vitro, telomerase activity. As previously shown, immunoprecipitates of Trt1p from whole cell fission yeast extracts can extend a telomeric oligonucleotide in vitro in an RNA-dependent manner (Figure 3A, lanes 1 and 2, [15]). Interestingly,

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A

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scEst1          (78) VIPLVLKLLWLQIHEPTLQWFEHWFHDIMRLSNRRKFRVFRIFQKKMIQF
SPBC2D10.13     (58) ------DIIWSCCHYKIICHFRSRFREIHPRHVVEKKK----TKKVFFKF
SPBC2F12.03c   (128) MEKDVLTYLWMRVHYQVISFFKHRIYEASTQHDPELLS---SLVTMHIQY

scEst1               FKITHRYYYDIIEHLCAKYDMNSVISNALFAKLNLMQYTDGLS-------
SPBC2D10.13          LKTCAIFYQTCISELISKFQLDSYRPFFCKWTSSATVSSTISND------
SPBC2F12.03c         LNSTIQFYTTLIAIIGELYHLQCLSPLTSFFTSCVTPKTILESPLRKQGS

scEst1               --------------------------------------------------
SPBC2D10.13          --------------------------------------------------
SPBC2F12.03c         HNWKTSTNSQSRLAALFSSIFEDSCLEVDSVKRLLSGSPSSSSSPLKKDS

scEst1               --THEKIILNTSNPLTFSIVISLQRCVINLGSTHFYKTILNKESNKPKSV
SPBC2D10.13          --EMSSIPEASYSRNHMEALECVYNCFIYLGDMARYSSTCLKKRG-----
SPBC2F12.03c         SSNSLTYEPALTDHKPQYLVLCVYRSLIYIGDVHRYLAEVRSENV-----

scEst1               EGFEKSIRYLNIASLYLPAVGDTYFQRAKIYLITGKFSLYFFELVRGALV
SPBC2D10.13          -AYDRALGFYDLAHRTLPGNGMHRNQIAVVWASDECIVESIYWFSSALCS
SPBC2F12.03c         PDYQVSRRYYVMAANVAPDYGVHFHQLGLI--------------------

scEst1               RIPSKCALNNLKDFIL
SPBC2D10.13          EDPPKSALLNLLKQLI
SPBC2F12.03c         ----------------
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B

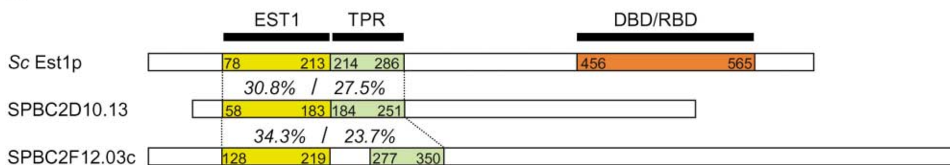


Figure 1. Sequence Comparison and Localization of *Sp*Est1p

(A) Sequence comparison of Est1 from *Saccharomyces cerevisiae* (*Sc*Est1p), *Schizosaccharomyces pombe* (SPBC2D10; hereafter referred to as *Sp*Est1p), and an Est1-like *S. pombe* ORF whose deletion led to no obvious telomere defect (SPBC2F12).

(B) Domain similarities between the sequences described in (A). The numbers between sequences indicate %similarity/%identity to *Sc*Est1p. Shaded regions include the EST1 domain (yellow), tetratricopeptide repeats (green), and the budding yeast DNA/RNA binding domain (red), and the corresponding amino acid numbering is specified. No DNA or RNA binding domain is apparent in either *S. pombe* sequence.

immunoprecipitates of *Sp*Est1p also yield telomerase activity, and *Sp*Est1p precipitates show a similar pattern of telomere synthesis to *Sp*Trt1p precipitates (Figure 3A, lanes 4–7). These results suggest that *Sp*Est1p is indeed part of the telomerase complex. As was shown for *Sc*Est1p, in vitro telomerase activity derived from immunoprecipitation of Trt1p was not impaired in strains that lacked *Sp*Est1p (Figure 3A, lanes 8 and 9). However, direct interactions between *Sp*Est1p and Trt1p in vivo are observed by coimmunoprecipitation of expressed *Sp*Est1p and Trt1p from *S. pombe* cellular extracts (Figure 3B). Taken together, our observations that *Sp*Est1p associates with and is required for telomerase activity in vivo, but not in vitro, suggest that *Sp*Est1p functions to activate or recruit telomerase. Therefore, *Sp*Est1p is a bona fide accessory component of the telomerase complex. This finding reveals a function that is evolutionarily conserved between *S. cerevisiae*, *S. pombe*, and, most likely, *H. sapiens* [10].

*Sc*Est1p has been shown to bind single-stranded telomeric oligonucleotides in vitro [11], and it has been suggested that this binding activity is an important feature of *Sc*Est1p function. However, using both *E. coli*-produced and in vitro-translated *Sp*Est1p, we were unable to detect either single- or double-stranded DNA binding activity under a wide range of conditions. This might reflect a very low binding affinity or unsuitable binding conditions in our assays. Alternatively, DNA binding by Est1p may not be a conserved feature of telomerase regulation. Instead, *Sp*Est1p may be recruited to telomeres by interactions with other proteins, a prominent candidate being *Sp*Pot1p, a conserved protein that does appear to bind single-stranded telomeric DNA and is necessary for protecting telomeres from degradation in vivo [16].

Human cells lacking telomerase activity can maintain telomeres through an alternative, homologous recombination-based process [17]. Fission yeast strains lacking telomerase can also escape from senescence to generate "survivor" strains [18]. Like *trt1*Δ populations, *est1*Δ strains generate survivors with similar rates of recovery and growth. Most fission yeast survivors have lost all telomeric DNA and have undergone intramolecular end joining of each of the three fission yeast chromosomes to yield circular chromosomes that are stably maintained [18]. Thus, survivors arise when chromosome ends are sufficiently "uncapped" to act as substrates

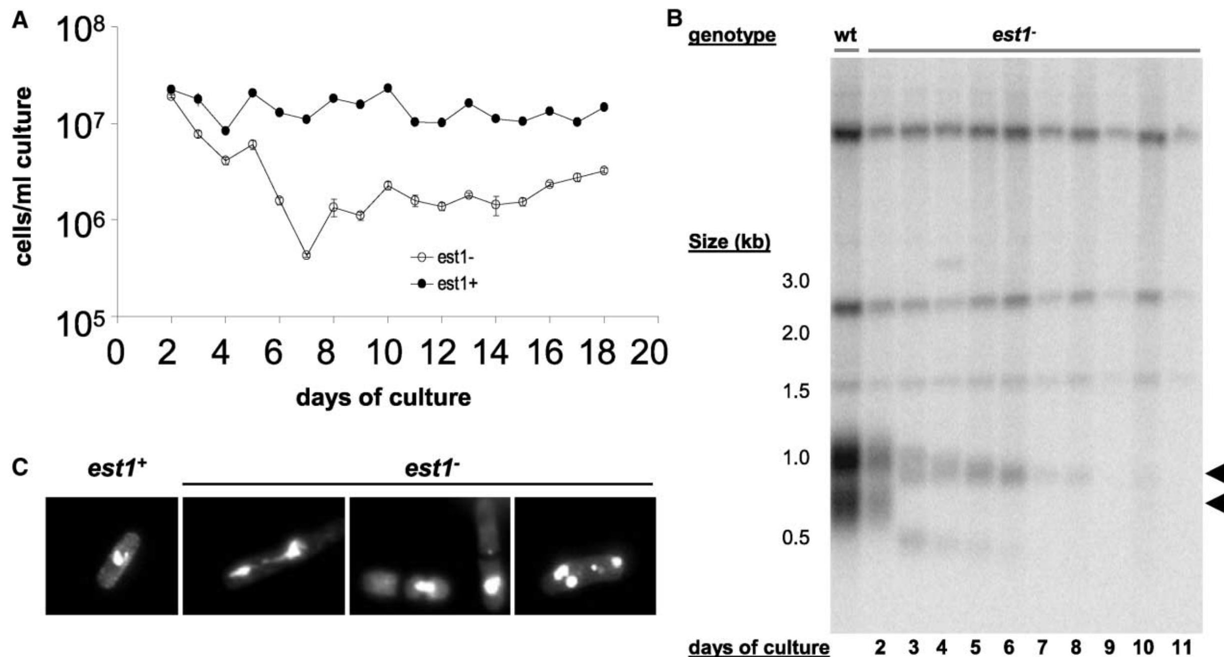


Figure 2. Growth and Senescence of *est1*Δ Strains

(A) *est1*$^{+}$ (closed circle) and *est1*Δ (open circle) offspring of a heterozygous *est1*$^{+/\Delta}$ diploid were cultured immediately following tetrad dissection. The error bars represent the standard deviation of three replicate measurements. Crisis occurred only in the *est1*Δ strains on day 7 of continuous culture.

(B) Telomere length progressively shortens in *est1*Δ strains. Cultures from (A) were harvested, and genomic DNA was digested with EcoRI for Southern blotting with a telomeric oligonucleotide probe. Wild-type telomere signals are indicated (arrows), and they decrease in both length and intensity with successive passage in senescing strains.

(C) *est1*Δ strains display chromosome segregation defects during crisis. *est1*Δ and *est1*$^{+}$ strains were grown in liquid culture until they reached crisis (approximately 7 days). Cells were then harvested, fixed in methanol, and visualized by staining with 4′,6′-diamidino-2-phenylindole (DAPI). While missegregation phenotypes were not apparent in wild-type populations, approximately 20% of *est1*Δ cells examined after 60–70 generations of growth displayed such defects.

for end-joining reactions that are normally prohibited by telomeres. This uncapping presumably occurs through the progressive removal of telomere capping proteins, as their binding sites are lost from the eroding telomeres. A key telomere capping protein in fission yeast is Taz1p, which binds the double-stranded telomeric repeats and regulates the extent of telomerase activity such that loss of Taz1p leads to a 10-fold increase in telomere length [19]. In addition, *taz1*Δ telomeres are susceptible to DNA double strand break repair reactions that are prohibited at wild-type telomeres; thus, telomere fusions appear in *taz1*Δ cells grown under conditions that favor high levels of nonhomologous end joining [20].

As *taz1*Δ telomeres are highly elongated, *taz1*Δ strains might be expected to exhibit postponed telomere loss and senescence upon telomerase inactivation. Intriguingly, dissection of heterozygous diploid *trt1*$^{\Delta/+}$ *taz1*$^{\Delta/+}$ cells (whose telomere lengths are normal due to the presence of one *taz1*$^{+}$ allele in each diploid) results in *trt1*Δ*taz1*Δ daughters that show an initial synthetic growth defect [18] (Figure 4A). We observe a similar growth defect in *est1*Δ*taz1*Δ spores obtained from *est1*$^{\Delta/+}$ *taz1*$^{\Delta/+}$ diploids (Figure 4A). This similarity suggests either that functional telomerase and Taz1p serve some redundant, essential function, or that the absence of telomerase-mediated synthesis subjects *taz1*Δ telomeres to rampant degradation. In an attempt to explicitly address the prediction that elongated telomeres would suppress senescence, we deleted either *est1* or *trt1* at a single allele in a homozygous *taz1*$^{\Delta/\Delta}$ diploid strain. The resulting diploids maintain extremely long telomeres, as expected for *taz1*Δ cells (data not shown). Sporulation of these diploids should yield double mutant haploids that, while isogenic to *est1*Δ*taz1*Δ and *trt1*Δ*taz1*Δ daughters of heterozygous *taz1*$^{+/\Delta}$ diploids, inherit long and aberrantly structured telomeres from the parental strain.

Sporulation of “long telomere” *trt1*$^{+/\Delta}$ *taz1*$^{\Delta/\Delta}$ diploids results in few viable *trt1*Δ*taz1*Δ spores, again indicating that these genes have synergistic effects on viability (Figure 4B), although the few viable *trt1*Δ*taz1*Δ daughters appear visually normal when grown in liquid culture. Southern blotting reveals that these strains have long telomeres that do not shorten with successive passage, as shown previously for *trt1*Δ*taz1*Δ daughters of heterozygous *trt1*$^{+/\Delta}$ *taz1*$^{+/\Delta}$ diploids [18]. This finding reflects the fact that Taz1p loss allows telomeres to be maintained by recombination-based mechanisms, thus averting the complete telomere attrition and chromosome circularization seen in *taz1*$^{+}$*trt1*$^{\Delta}$ survivors [18]. In marked contrast, not a single *est1*Δ*taz1*Δ daughter could be isolated by sporulating *est1*$^{+/\Delta}$ diploids homozygous for *taz1*Δ (i.e., carrying long, aberrantly structured telomeres), despite screening over 50,000 spores.

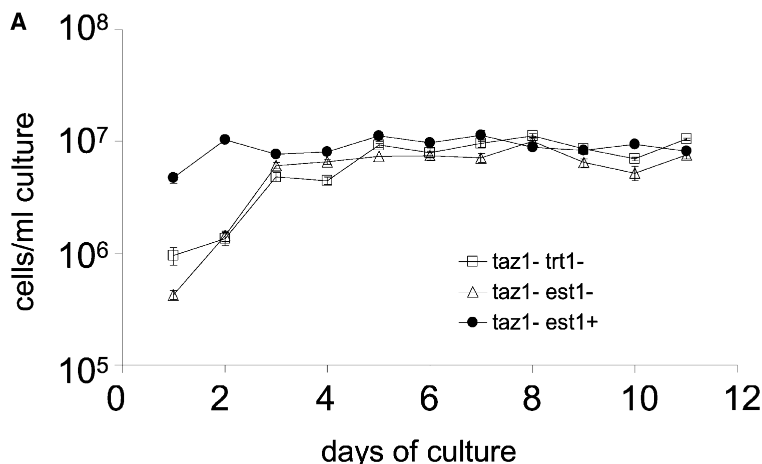
Figure 3. *Sp*Est1p and Trt1p Physically Interact, but *Sp*Est1p Is Not Required for Telomerase Catalytic Activity

(A) In vitro telomerase assay. Extension of the telomere sequence primer is only observed when Trt1p is present (lanes 1, 4, and 8) and is abolished in the presence of RNase (lanes 2, 5, and 9). Telomerase activity partitions with *Sp*Est1p (lane 4), although *Sp*Est1p is not required for in vitro telomerase-dependent primer extension (lane 8). The GFP antibodies used for immunoprecipitation show no cross-reactivity to Trt1-myc (lane 3).

(B) *S. pombe* cell lysates from strains expressing *Sp*Est1-GFP (lane 4, right panel, "GFP-IP control"), myc-tagged Trt1p (lane 4, left panel, "Myc IP control"), or both (lanes 1–3, both panels) were prepared for immunoprecipitation. GFP immunoblotting (left panel) shows that anti-GFP antibodies (lane 2, both panels) precipitate *Sp*Est1-GFP. Myc immunoblots (right panel) show that anti-GFP coprecipitates Trt1-myc in the presence (lane 2, right panel), but not in the absence (lane 4, right panel), of *Sp*Est1-GFP (lane 4, right panel). Similarly, anti-myc precipitated *Sp*Est1-GFP in the presence (lane 3, left panel), but not in the absence (lane 4, left panel), of Trt1-myc.

Thus, *Sp*Est1 has a critical telomere function that is distinct from that of telomerase itself. In principle, this result could indicate that growth is impossible in the absence of both *taz1*$^{+}$ and *est1*$^{+}$. However, the observation that viable *taz1*Δ*est1*Δ colonies arise, although infrequently, from spores of diploids that are heterozygous for *taz1* and thus contain normal telomeres (Figure 4A) rules out the possibility that *taz1*Δ and *est1*Δ are synthetic lethal per se. In addition, plasmid loss experiments show that an extrachromosomal copy of *est1*$^{+}$ can be lost from a haploid *est1*Δ*taz1*Δ cell (not shown), indicating that *est1*$^{+}$ is not essential in cells containing *taz1*Δ telomeres.

The complete inviability of *taz1*Δ*est1*Δ spores therefore indicates that germination of such spores is impossible if they have inherited aberrant (*taz1*Δ) telomeres from their parent. While meiosis in the long telomere *est1*$^{+/\Delta}$ *taz1*$^{\Delta/\Delta}$ diploids did yield daughters and was therefore successful, all of the viable progeny were *est1*$^{+}$, demonstrating that *est1*$^{+}$ becomes critical specifically during the germination period in strains harboring *taz1*Δ telomeres. Germination entails a particularly ex-



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Parental genotype	% Total viability	% Distribution of genotype (among viable daughters)				
		***taz1*Δ**	***est1*Δ**	***trt1*Δ**	**double**	**wild-type**
wild-type	97.6 (7.3)					100 (7.6) **(100%)**
est1 $^{+/\Delta}$	97.0 (19)		54.4 (14.0) **(50%)**			45.6 (8.2) **(50%)**
taz1 $^{\Delta/\Delta}$	9.8 (0.9)	100.0 (0.9) **(100%)**				
trt1 $^{+/\Delta}$ ***taz1*** $^{+/\Delta}$	79.0 (9.2)	23.9 (4.3) **(25%)**		36.6 (2.7) **(25%)**	17.8 (5.7) **(25%)**	21.7 (5.3) **(25%)**
est1 $^{+/\Delta}$ ***taz1*** $^{+/\Delta}$	71.7 (1.6)	23.2 (2.3) **(25%)**	30.8 (5.1) **(25%)**		22.8 (4.4) **(25%)**	23.2 (1.8) **(25%)**
trt1 $^{+/\Delta}$ ***taz1*** $^{\Delta/\Delta}$ **(long telomere)**	10.8 (1.0)	70.1 (0.9) **(50%)**			29.9 (0.38) **(50%)**	
est1 $^{+/\Delta}$ ***taz1*** $^{\Delta/\Delta}$ **(long telomere)**	7.3 (0.4)	100.0 (0.4) **(50%)**			0 **(50%)**	

Figure 4. *taz1*$^{+}$ Is Crucial for Viability in *est1*Δ Spores

(A) *est1*Δ*taz1*Δ (open triangle) and *trt1*Δ*taz1*Δ (open square) strains show accelerated senescence relative to *est1*Δ or *trt1*Δ alone (Figure 2A; *est1*$^{+}$ *taz1*$^{+}$ [closed circle]). Cell growth was measured as in Figure 2A.

(B) Distribution of genotypes among viable daughters arising from *taz1*Δ, *est1*Δ, and *trt1*Δ parental diploids. Diploids of the indicated genotype were sporulated, and allelic segregation and percent viability were assessed. All *taz1*Δ/Δ diploids yield low total spore viability due to the requirement of Taz1p for successful meiosis. The values in parenthesis indicate the standard deviation of three independent experiments, while the numbers in bold indicate the expected frequency of randomly segregating alleles. Diploids heterozygous for *est1* but homozygous for *taz1*Δ carry long, aberrantly structured telomeres and are referred to as "long," while diploids heterozygous for both *est1*$^{+}$ and *taz1*$^{+}$ carry normal telomeres that may be transiently inherited by *est1*Δ*taz1*Δ daughters. "Long" telomere diploids were unable to produce *est1*Δ*taz1*Δ daughters. In contrast, *trt1*Δ*taz1*Δ daughters were obtained from *trt1*$^{\Delta/+}$ *taz1*$^{\Delta/\Delta}$ diploids, albeit with a lower frequency than expected.

tended G_1 period as well as a "resetting" of the cell cycle, both of which distinguish it from other cellular processes. Cellular survival under these circumstances clearly requires *est1*$^{+}$, but does not require *trt1*$^{+}$, indicating that *Sp*Est1p plays a role that is independent of telomerase. Perhaps *Sp*Est1p, like its human counterpart [10], is required for regulating some critical aspect of telomeric chromatin accessibility.

The identification of Est1p in organisms other than budding yeast reveals a common requirement for telomerase regulation among eukaryotes. Electron microscopy has revealed that telomeres in mammals and protozoa can form a looped structure, termed a T loop [21–23], that could provide a means for telomerase regulation, as the chromosome terminus tucked into the T loop might be inaccessible to telomerase-dependent elongation. Dismantling of the T loop might follow chromosomal replication, exposing a free telomeric end to telomerase. While T loops have yet to be detected in yeasts, the conservation of Est1 in both mammals and yeasts suggests that telomerase accessory proteins are required even in the context of T loops and prompts further interest in the precise structural features that are conserved between yeast and mammalian telomeres.

Like its counterpart in budding yeast, *Sp*Est1p appears to recruit or activate telomerase, serving as a molecular bridge between telomerase and other telomere-associated factors and/or regulating telomeric chromatin structure. In *S. cerevisiae*, telomerase appears to be recruited via bridged protein contacts with Cdc13p [24, 25]. Although no Cdc13p homolog has been identified outside of budding yeast, the recently de-

scribed Pot1 protein of *S. pombe* and human appears to fulfill similar requirements for end protection [16], and shared structural features suggest that Pot1p is the ortholog of Cdc13p [26]. The conservation of regulatory strategies between highly diverse eukaryotes augments our tools for understanding the mechanisms that control telomerase activation, a key step in tumorigenesis.

Supplemental Data

Supplemental Data including the Experimental Procedures are available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

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