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21st Century Genetics: Mass Spectrometry of Yeast Telomerase

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Abstract

Telomerase is a specialized reverse transcriptase that maintains the ends of chromosomes in almost all eukaryotes. The core of telomerase consists of telomerase RNA and the reverse transcriptase that uses a short segment without the RNA to template the addition of telomeric repeats. In addition, one or more accessory proteins are required for telomerase action in vivo. The best-studied accessory protein is Est1, which is conserved from yeasts to humans. In budding yeast, Est1 has two critical in vivo functions: By interaction with Cdc13, a telomere-binding protein, it recruits telomerase to telomeres, and it also increases telomerase activity. Although budding yeast telomerase is highly regulated by the cell cycle, Est1 is the only telomerase subunit whose abundance is cell cycle–regulated. Close to 400 yeast genes are reported to affect telomere length, although the specific function of most of them is unknown. With the goal of identifying novel telomerase regulators by mass spectrometry, we developed methods for purifying yeast telomerase and its associated proteins. We summarize the methods we used and describe the experiments that show that four telomerase-associated proteins identified by mass spectrometry, none of which had been linked previously to telomeres, affect telomere length and cell cycle regulation of telomerase by controlling Est1 abundance.

Telomeres, the DNA–protein structures at the ends of eukaryotic chromosomes, are usually elongated by a telomere-dedicated reverse transcriptase called telomerase. The catalytic core of telomerase consists of an RNA component (called TLC1 in budding yeast) that serves as the template for elongating the G-rich strand of telomeric DNA and the catalytic reverse transcriptase subunit (Est2 in budding yeast; TERT in higher cells). In addition, the telomerase holoenzyme contains one or more species-specific accessory proteins. For example, budding yeast has two such subunits, Est1, which is found from yeasts to mammals, and Est3, which is not evolutionarily conserved (although in mammals its function may be performed by the structurally related TPP1 protein [Lee et al. 2008; Yu et al. 2008]). In budding yeast, all four of these subunits—TLC1, Est1, -2, and -3—are essential for telomerase action in vivo (for review, see Wellinger and Zakian 2012).

As with many cellular processes, yeast genes affecting telomeres were first identified in genetic screens, including all four of the essential telomerase subunits, Est1, -2, -3, and TLC1 RNA (Lundblad and Szostak 1989; Singer and Gottschling 1994; Lendvay et al. 1996). Genes affecting the telomere length were also identified by screening the deletion

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collection of nonessential genes and the DAmP collection of essential genes (Askree et al. 2004; Gatbonton et al. 2006; Ungar et al. 2009). As a result of these combined efforts, approximately 370 Sc genes are reported to affect the telomere length. However, the mechanism of action of most of these genes is not known, and many probably act indirectly. Moreover, the two groups that examined the deletion collection identified mostly nonoverlapping gene sets, raising concerns about the reliability of these screens.

Inspired by the success of mass spectrometry (MS) to identify novel components of telomerase in both the ciliate *Tetrahymena* and human tumor cells, we reasoned that MS would allow us to focus on the subset of yeast proteins that affect the telomere length by direct effects on telomerase. MS of purified *Tetrahymena* telomerase identified multiple previously unknown telomere proteins, such as p65, a telomerase RNA-binding protein required for wild-type levels of telomerase RNA and a trimeric complex, p75-p45-p19, that is related to the budding yeast CST complex (*C*TC1-*S*TN1-*T*EN1) and affects telomere structure and replication (Witkin and Collins 2004; Witkin et al. 2007; Min and Collins 2009; Jiang et al. 2015; Wan et al. 2015). MS of telomerase from human cells identified both telomerase RNA-binding proteins and ATPases such as pontin and reptin that affect the telomere length (Fu and Collins 2007; Venteicher et al. 2008). TCAB1, another telomerase-associated RNA-binding protein discovered by MS, controls intracellular trafficking of human telomerase (Venteicher et al. 2009).

In *Tetrahymena* and immortalized human cultured cells, an MS strategy is facilitated by the high levels of telomerase in these cells. For example, *Tetrahymena* telomerase is isolated from a specific developmental stage, postmating cells, that undergo massive telomerase-mediated new telomere formation (Witkin and Collins 2004; Witkin et al. 2007). In contrast to these systems, telomerase is present in low amounts in most organisms, making it a challenge for MS approaches. Telomerase abundance has been best documented in budding yeast where there are about half the number of telomerase complexes as telomeres in post–S phase cells, when telomerase acts. The least abundant subunit is TLC1 RNA, estimated at approximately 30 copies per cell (Mozdy and Cech 2006). The protein subunits of budding yeast telomerase are also present in low amounts. Asynchronous yeast cells contain approximately 70, 40, and 80 molecules of, respectively, Est1, -2, and -3 (Tuzon et al. 2011; Wu and Zakian 2011). In yeast, where telomerase is expressed constitutively, its level is about what is needed to maintain wild-type telomere length. For example, when the amount of telomerase RNA is reduced, as in heterozygous *tlc1* /*TLC1* diploid cells, telomeres are shorter than in wild-type cells; that is, the *TLC1* gene is haplo-insufficient (Mozdy and Cech 2006). In humans, telomerase is not expressed in most somatic cells. Even in human stem cells, where it is expressed, telomerase expression is quite low as heterozygous alleles of both TERT and telomerase RNA lead to short telomeres and diseases characterized by stem cell failure and reduced life span (reviewed in Armanios and Blackburn 2013).

As in many organisms, the ability of budding yeast telomerase to lengthen telomeres is cell cycle–regulated. Telomerase-mediated telomere elongation is not detected in G_1 or early S phase yeast cells but rather is limited to late in the cell cycle (Diede and Gottschling 1999; Marcand et al. 2000). Of the four key telomerase subunits, Est1 is the only one whose abundance is cell cycle–regulated (Taggart et al. 2002). Est1 is present in approximately 20

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molecules per cell in G_1 phase, when telomerase is not active, and approximately 120 molecules per cell in G_2/M phase, when it is active (Wu and Zakian 2011). Est1 regulation is imposed in part by regulated degradation of its mRNA (Larose et al. 2007) and in part by proteosome-mediated Est1 degradation (Osterhage et al. 2006; Ferguson et al. 2013). Est1 interacts directly with both TLC1 RNA (Seto et al. 2002) and Est3 (Osterhage et al. 2006; Tuzon et al. 2011), and both interactions are critical for telomerase action. In addition, a direct interaction between Est1 and the Cdc13 component of the CST complex, which binds the single-strand G-tails that mark the very end of the chromosome, is critical for recruitment of telomerase to DNA ends in vivo and in vitro (Bianchi et al. 2004; Wu and Zakian 2011). The Est1–Est3 interaction is essential for Est3 incorporation into the holoenzyme and for its recruitment to telomeres (Osterhage et al. 2006; Tuzon et al. 2011).

Given that Est1 is required for telomerase recruitment and for an activation step, which is likely the recruitment of Est3, cell cycle–regulated abundance of Est1 is theoretically sufficient to explain why the telomerase holoenzyme acts only in late S phase. However, even if Est1 is expressed at high levels in G_1 phase, which results in formation of the holoenzyme, telomerase is still unable to lengthen telomeres (Osterhage et al. 2006). We speculate that this inactivity reflects the lack of an appropriate DNA substrate for the complex. Although yeast telomeres bear short (5–15-nt) TG_{1-3} extensions, often called G-tails, throughout most of the cell cycle, longer G-tails (20–25-nt) are generated late in the cell cycle (Wellinger et al. 1993; Larrivée and Wellinger 2004; Soudet and Teixeira 2014). Even though long G-tails are detected at the time when telomerase acts, their appearance does not require telomerase (Wellinger et al. 1996). Rather, long G-tails are generated by cell cycle–regulated C-strand degradation (Huertas et al. 2008; Bonetti et al. 2009).

The cell cycle–regulated appearance of long G-tails is cyclin dependent kinase (Cdk)-dependent (Frank et al. 2006; Vodenicharov and Wellinger 2006) and occurs mainly on the newly synthesized leading strand (Soudet and Teixeira 2014). The Mre11-Rad50-Xrs2 complex recruits the ATM-like kinase Tel1 to telomeres, which phosphorylates one or more substrates, and these modified proteins promote telomerase recruitment to telomeres (Sabourin et al. 2007; McGee et al. 2010). As the Mre11 complex binds preferentially to newly made leading strand telomeres (Faure et al. 2010), long G-tails are probably the preferred telomerase substrate. Thus, in addition to increased Est1, long G-tails and probably Cdk-mediated phosphorylation of one or more proteins, such as Cdc13 (Li et al. 2009), all contribute to telomerase being cell cycle–regulated.

Here we describe our success in purifying yeast telomerase for MS analysis. These efforts identified more than 100 telomerase-interacting proteins, of which ~70% had not been implicated previously in telomerase biology. So far, we have tested eight of the novel telomerase-associated proteins, and seven of these affect telomere length. We describe the methods that enabled us to obtain biochemically useful amounts of telomerase, identify a novel hetero-trimeric complex that regulates telomerase abundance, and speculate on why these studies did not identify many differences in telomerase-associated proteins in G_1 versus G_2 phase cells. (For more details of results and methods, see Lin et al. 2015.)

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METHODS FOR ISOLATING YEAST TELOMERASE

We were unable to isolate telomerase until we overexpressed each of the four essential telomerase subunits (TLC1 RNA, Est1, Est2, and Est3) from the strong and inducible GAL1 promoter. Est1 and Est2 were expressed as GFP fusions, which is critical for the method we used to affinity purify telomerase. (Est3 was not expressed as a GFP fusion because doing so reduces its activity as inferred by short telomeres.) Based on levels of TLC1 RNA, this approach resulted in about a 10-fold increase in telomerase. Cells overexpressing telomerase have normal growth rates and cell cycle progression profiles, but their telomeres are hyperelongated, suggesting that the increase in telomerase increases telomerase action (Lin et al. 2015).

Isolation of telomerase was achieved by immunoprecipitation with a high-titer anti-GFP serum developed in the laboratory of Dr. Ileana Cristea (Princeton University) (Cristea et al. 2005). Another key to success was identifying conditions for efficient solubilization of the telomerase complex. We isolated telomerase from G_1 arrested cells (telomerase inactive) and G_2/M arrested cells (telomerase active). As a control, we fused the SV40 nuclear localization signal to GFP. We discarded proteins in the control immunoprecipitates from consideration.

PROTEINS ASSOCIATED WITH TELOMERASE

We determined the significance of the interactions of proteins that immunoprecipitated with telomerase using the SAINT algorithm (Significance Analysis of INTeractome), which considers the number of spectral counts in both experimental and control isolations, the frequency of detection across biological replicates, and protein length (Choi et al. 2011). Using a stringent SAINT score cutoff of 0.8, we identified 89 telomerase-associated proteins from G_1 phase and 72 from G_2/M phase cells. About 30% (37 proteins from both G_1 and G_2/M phases) of the proteins are still telomerase-associated after DNase I treatment of the extracts. Most proteins (58 of 74) were present with high SAINT scores in both G_1 and G_2/M phase isolations (Lin et al. 2015).

As expected, Est1 and -2 were among the most abundant proteins in the immunoprecipitate, as was Est3, even though Est3 was not GFP-tagged. All seven members of the Sm complex were also telomerase-associated. This result was expected as two members of the heptameric Sm complex, Smd1 and Smd3, have been shown previously to bind TLC1 in vivo (Seto et al. 1999). As noted above, previous genetic analyses link almost 400 genes to effects on telomere length, and 30% of the telomerase-associated proteins in our isolations fell into this group, almost fourfold higher than the fraction of yeast genes reported to affect telomere length. As all of the known telomerase subunits and many proteins linked previously to telomerase were present in our samples, we conclude that the multiprotein complex we identified is biologically relevant. We are even more encouraged that the majority of proteins in our samples have not been linked previously to telomerase and are candidates for novel telomerase regulators (Lin et al. 2015).

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THE TELOMERASE-ASSOCIATED Cdc48 COMPLEX IS A NOVEL REGULATOR OF TELOMERE LENGTH

Thirty-four of the proteins identified in our MS analyses are linked to proteolysis. These proteins include Cdc48, an AAA+ ATPase that is the catalytic subunit of several protein complexes. In addition, three proteins that associate with Cdc48—Npl4, Ufd1, and Shp1—were identified. All four of these proteins have the highest possible SAINT score (1.0) in both G_1 and G_2/M phase, indicating that their association with telomerase is authentic. Consistent with this interpretation, Est1 in present in immunoprecipitations of Cdc48 in cells that express wild-type levels of telomerase (Lin et al. 2015).

Cdc48 complexes, which are highly conserved in eukaryotes, were first identified because of their role in targeting misfolded proteins from the endoplasmic reticulum to the proteosome for degradation. Subsequent studies in diverse organisms revealed that these complexes function in a wide range of cytoplasmic and nuclear events. However, Cdc48 complexes had not been linked previously to telomerase in any organism. Whereas Cdc48 is the catalytic subunit, the other subunits in Cdc48 complexes recognize and bind ubiquinated proteins. The substrates of Cdc48 complexes are typically themselves subunits of multiprotein complexes. After the Ub-binding subunits in the Cdc48 complex bind the substrate, Cdc48 uses its ATPase activity to remove the substrate from the multiprotein complex. In most but not all cases, the removed subunit is then delivered to the proteosome for degradation, and the multiprotein complex from which the target protein was removed falls apart.

Shp1 and Npl4/Ufd1 are found in distinct Cdc48 complexes. Because Shp1 is implicated mainly in membrane fusion (Schuberth et al. 2004), we focused on the Cdc48-Npl4-Ufd1 complex. If this complex regulates telomerase, we anticipated that its depletion would impact the telomere length. As *CDC48*, *NPL4*, and *UFD1* are all essential genes, we used temperature-sensitive alleles. Even at permissive temperatures, *cdc48-3* cells have telomeres that are ~40 bp shorter than the ~300 bp telomeres in an otherwise isogenic wild-type control strain. This short telomere phenotype is rescued by introducing a plasmid-borne copy of *CDC48* into the strain, showing that short telomeres are due to limited Cdc48. In addition, *npl4-1*, but not *ufd1-2*, cells have short telomeres (Lin et al. 2015).

The effects of Cdc48 depletion on the telomere length suggest that the Cdc48 complex is a positive regulator of telomerase. Given that Cdc48 complexes usually target proteins for degradation, we used western blot analysis to identify telomere proteins whose abundance is Cdc48-dependent. We tested six proteins that affect telomerase activity: Est1 and Est2 (two of the three essential protein subunits of telomerase), Pif1 (a DNA helicase that removes telomerase from telomeres in vivo and in vitro (Boule et al. 2005), two telomere-binding proteins that function in telomerase recruitment (Cdc13 and Yku80) (Bianchi et al. 2004; Fisher et al. 2004; Wu and Zakian 2011), and Rap1 (a duplex telomere binding protein that recruits Rif1 and Rif2, two negative regulators of telomerase, to telomeres [Hardy et al. 1992; Wotton and Shore 1997; Teng et al. 2000]). We examined the abundance of each protein in wild-type and *cdc48-3* cells at both permissive and semipermissive temperatures.

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Est1 is the only one of the six proteins we tested whose level is affected by reduced Cdc48. Compared to wild cells, Est1 is 40 times more abundant in *cdc48-3* cells. Est1 levels are similarly elevated in *npl4-1* and *ufd1-2* cells. In addition, Est1 levels are no longer cell cycle–regulated in *cdc48-3* cells. Elevated levels of Est1 in Cdc48 depleted cells are the expected result if the Cdc48-Npl4-Ufd1 complex targets Est1 for degradation (Lin et al. 2015).

By several criteria, Est1 is an activator of telomerase (for review, see Wellinger and Zakian 2012). For example, overexpression of Est1 in wild-type cells leads to telomere lengthening (Zhou et al. 2000). Thus, we were surprised that *cdc48-3* cells have short telomeres despite containing a large excess of Est1. We showed that excess Est1 does not limit telomere length by titrating its interacting partner Est3 from telomeres, as overexpressing Est3 does not ameliorate the short telomere phenotype of *cdc48-3* cells. We reasoned that the Est1 that accumulates in *cdc48-3* cells must be defective in activation and hence different biochemically from the Est1 in wild-type cells. Given that the Cdc48 complex targets ubiquinated proteins for degradation, the obvious possibility is that Est1 is ubiquinated in Cdc48-depleted cells, and Est1-Ub is less able to activate telomerase (Lin et al. 2015).

To determine if Est1is ubiquinated, we expressed His6-tagged Ubiquitin (Ub) in *cdc48-3* and wild-type cells that also express Myc-tagged Est1. We isolated proteins bearing His6-Ub by passing cell extracts over a Ni column, which retains His6-labeled proteins. The retained proteins were eluted, separated on gels, and analyzed by both anti-His and anti-Myc westerns. Est1 is present in both extracts. Although Cdc48 complexes are often described as recognizing polyubiquinated proteins, only monoubiquinated Est1 is detected in these experiments. This finding is not unprecedented as Cdc48 complexes extract monoubiquinated transcription factors from both yeast and mammalian chromatin (Ndoja et al. 2014). Although Est1 ubiquination is detectable in *cdc48-3* and wild-type extracts, the fraction of Est1-Ub compared to total Est1 is approximately twofold higher in extracts from *cdc48-3* cells compared to wild-type cells (Lin et al. 2015).

THE TELOMERASE-ASSOCIATED UFD4 AFFECTS TELOMERE LENGTH AND Est1 ABUNDANCE

The demonstration that Est1 is cell cycle–regulated by a proteosome-dependent process (Taggart et al. 2002; Osterhage et al. 2006) led us and others to look for Est1 ubiquination. However, Est1-Ub is difficult to detect, probably because Est1 is not abundant in wild-type cells. Indeed, even when 10 times as much extract is used for wild-type versus *cdc48-3* cells, Est1-Ub is barely detectable in wild-type extracts, although it is easily seen in the mutant extract (Lin et al. 2015). There are hints from earlier studies that the E3 Ub ligase, Cdh1, affects telomere length (Ferguson et al. 2013), but because the authors were unable to detect Est1-Ub, it was unclear whether Cdh1 affects telomeres by modifying Est1.

There are more than 80 E3 ligases in yeast. To identify the E3 Ub ligase that modifies Est1, we tested the three E3 Ub ligases that we telomerase-associated by MS analysis: Ufd4, Tom1, and Ubr1. We reasoned that mutations in the E3 ligase that modifies Est1 should result in more Est1 and longer telomeres in both wild-type and *cdc48-3* cells. Deletion of

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UFD4 but not that of *CDH1* nor either of the two other telomerase-associated E3 ligases has these effects. The fact that telomeres increase in length in both wild-type and *cdc48-3* cells provides further support for the activation role of Est1 and for the hypothesis that Est1-Ub is less able to carry out this function (Lin et al. 2015).

THE COMPOSITION OF TELOMERASE IS NOT DRAMATICALLY DIFFERENT IN G_1 VERSUS G_2/M PHASE CELLS

MS identified a handful of proteins that are telomerase-associated only in G_1 phase (telomerase inactive) or only in G_2/M phase (telomerase active). One of the more interesting (or at least unexpected) of these is Sme1, one of the seven Sm proteins that bind not only telomerase RNA but also pre-mRNAs. The seven Sm proteins form a ring that encircles the RNA to which they bind. Thus, it is surprising that Sme1 is telomerase-associated in G_1 but not G_2/M phase, whereas the other six Sm proteins are telomerase-associated in both G_1 and G_2 phase. Other proteins are associated with both G_1 and G_2/M phase telomerase but are more abundant in one of the two phases. For example, proteins with roles in proteolysis tend to be more abundant in G_1 phase telomerase, whereas chromatin proteins are enriched to a greater extent in G_2/M telomerase (Lin et al. 2015).

Despite these examples, most telomerase-associated proteins are present in both G_1 and G_2/M phase cells. Perhaps, overexpression of telomerase masks some interactions that occur normally in only one of the two cell cycle phases. Alternatively (or in addition), the events that limit telomerase action to late S/G_2 phase may not impact the composition of the holoenzyme. For example, if, as we suggest, long G-tails are the in vivo substrate for telomerase, their cell cycle occurrence (in combination with Est1 regulation) can explain why telomerase action is cell cycle–dependent (Wellinger et al. 1993). This view is supported by the inability of telomerase to lengthen telomeres when the holoenzyme is assembled in G_1 phase by forced expression of Est1 (Osterhage et al. 2006). Another possibility is that posttranslational modification of telomerase subunits or telomerase regulators contributes to cell cycle regulation. For example, Cdc13, the G-tail binding subunit of CST, is phosphorylated by Cdk1 late in the cell cycle, and this modification increases its ability to interact with Est1 to recruit telomerase to telomeres (Li et al. 2009). Other telomerase regulators or subunits may also be modified in a cell cycle–dependent manner. One such example comes from our MS analysis that detected five sites of Est1 phosphorylation. Of these sites, three are phosphorylated in both G_1 and G_2/M phase cells, whereas the other two sites show cell cycle–restricted phosphorylation with one being phosphorylated only in G_1 and one only in G_2/M phase. It remains to be seen whether these cell cycle–restricted phosphorylation events affect Est1 stability and/or telomerase activity.

FUTURE PERSPECTIVES

We are in the process of determining whether other previously unidentified proteins that copurify with telomerase affect its activity. We start by asking whether mutations in the associated protein affect telomere length. In addition to the studies reported here, we have identified three copurifying proteins that affect both telomere length and TLC1 abundance. We also see telomere length effects for several other genes with roles in proteolysis. Thus,

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the MS approach is clearly very useful for identifying telomerase regulators. Our methods are applicable to other organisms with low levels of telomerase. For example, overexpression of telomerase RNA, Est1, and Trt1 (the catalytic subunit; homolog of Est2) results in lengthening of *Schizosaccharomyces pombe* telomeres. By expressing both Est1 and Trt1 as fusions to GFP, we are able to use the same strategy described here to identify multiple proteins associated with fission yeast telomerase (K McDonald, K-W Lin, C Webb, I Cristea, and VA Zakian, unpubl.).

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