A putative cap binding protein and the methyl phosphate capping enzyme Bin3/MePCE function in telomerase biogenesis

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Telomerase reverse transcriptase (TERT) and the noncoding telomerase RNA (TR) subunit constitute the core of telomerase. Additional subunits are required for ribonucleoprotein complex assembly and in some cases remain stably associated with the active holoenzyme. Pof8, a member of the LARP7 protein family is such a constitutive component of telomerase in fission yeast. Using affinity purification of Pof8, we have identified two previously uncharacterized proteins that form a complex with Pof8 and participate in telomerase biogenesis. Both proteins participate in ribonucleoprotein complex assembly and are required for wildtype telomerase activity and telomere length maintenance. One factor we named Thc1 (Telomerase Holoenzyme Component 1) shares structural similarity with the nuclear cap binding complex and the poly-adenosine ribonuclease (PARN), the other is the ortholog of the methyl phosphate capping enzyme (Bin3/MePCE) in metazoans and was named Bmc1 (Bin3/MePCE 1) to reflect its evolutionary roots. Thc1 and Bmc1 function together with Pof8 in recognizing correctly folded telomerase RNA and promoting the recruitment of the Lsm2-8 complex and the catalytic subunit to assemble functional telomerase.
Telomeres are protective caps at the ends of eukaryotic linear chromosomes that prevent natural DNA ends from being recognized as double-strand breaks. Deficiency in maintaining a functional telomere structure results in cellular senescence and chromosome instability that lead to premature aging and cancer. Central to telomere maintenance is the extension of shortened telomeres by telomerase, a specialized reverse transcriptase that utilizes part of a long non-coding RNA subunit as the template for the synthesis of telomeric DNA repeats. Telomerase is comprised of the catalytic protein Telomerase reverse transcriptase (TERT, Trt1 in Schizosaccharomyces pombe), the telomerase RNA subunit (TR, TER1 in S. pombe), and an unknown number of other protein subunits involved in the biogenesis, regulation, and recruitment of the enzyme to telomeres. Mutations in most known telomerase components have been implicated in premature aging diseases, such as dyskeratosis congenita, aplastic anemia, idiopathic pulmonary fibrosis, Hoyeraal–Hreidarsdottir syndrome, Coats Plus syndrome, and Revesz syndrome (reviewed in ref. 3). Reactivation or upregulation of telomerase activity is observed in over 85% of cancers. Given the critical role of telomerase in controlling cell proliferation, it is not surprising that telomerase ribonucleoprotein (RNP) biogenesis is a tightly regulated hierarchical assembly process involving numerous factors and stages.

In S. pombe, the telomerase RNA subunit TER1 is transcribed by RNA polymerase II and the primary transcript contains two exons and an intron. This precursor is processed by the spliceosome. Deviating from a normal splicing reaction, the intermediates generated by the first spliceosomal cleavage reaction are released with the 5′ exon being further processed into mature telomerase RNA and the intron lariat-exon 2 being degraded. This "discord" of splicing intermediates is promoted by RNA elements within TER1 that are unfavorable for a transition to the second step of splicing. Interestingly, the spliceosome also functions as a 3′ end processing machinery for telomerase RNA in other species, but the underlying mechanisms for releasing splicing intermediates as cleavage products are remarkably diverse.

Prior to spliceosomal cleavage, the hetero-heptameric Sm complex binds TER1 directly upstream of the 5′ splice site and recruits the methyl transferase Tgs1, which hypermethylates the 7-monomethylguanosine cap at the 5′ end of TER1. Following spliceosomal cleavage, the Sm complex is replaced by the Sm-like complex Lsm-2-8, which protects TER1 from degradation by exonucleases like the exosome. Importantly, the binding of Lsm-2-8 to telomerase is also a prerequisite for the binding of the catalytic subunit Trt1 to assemble an active enzyme.

Recent genetic and biochemical studies identified the Pof8 protein as a new factor involved in telomerase biogenesis. Originally described as a putative F-box protein, profile database searches revealed structural similarities between Pof8 and the La-related protein (LARP) superfamily. The La protein and La-related proteins bind to diverse noncoding RNAs and participate in RNA processing and RNP assembly. Genuine La protein recognizes the 3′ oligo-uridine tail present on most nascent RNA polymerase III transcripts to protect them and in many cases influences the folding of the RNA into functional conformations. Based on sequence conservation and domain organization, the LARP family has been divided into four subfamilies (LARP1, 4, 6, and 7). Pof8 shows the most similarity with LARP7 proteins. Members of this family have predominantly been studied in the context of the 7SK complex. However, recently additional roles in the context of other RNP proteins have been described, including a function for LARP7 as a molecular bridge between U6 snRNA and a subset of box C/D small nucleolar RNAs that guide U6 2′-O-methylation.

In the ciliated protozoa Tetrahymena thermophila and Euplotes aediculatus, the respective LARP family proteins Pof6 and Pof4 are bona fide telomerase subunits associated with the active enzymes. Further characterization of Tetrahymena pof6 revealed its function as RNA chaperone in the hierarchical assembly of telomerase, whereby pof6 induces a conformational change in the RNA that is favorable for the binding of TERT. The human La and Larp7 proteins have also been implicated in telomere maintenance. Larp7 loss-of-function mutations in patients with Alazami syndrome being associated with very short telomeres and Larp7 knockdown causing a reduction in telomerase activity in cancer cells. Whether human Larp7 is directly associated with telomerase RNA and functions in an analogous way to pof6 has remained unclear.

The fission yeast Pof8 protein binds to telomerase RNA and promotes the association of the Lsm2-8 complex with TERT, which in turn promotes the binding of the catalytic subunit Trt1. Loss of Pof8 reduces TERT stability, largely compromises telomerase assembly, and results in critically short telomeres. Structurally, Pof8 is comprised of a conserved N-terminal La module (La motif plus RNA recognition motif 1 (RRM1)) and a C-terminal RRM2. The conservation of the RNP3 motif (YW-X-D/Q) and the position of the α3 helix, as revealed by the crystal structure and NMR studies of the C-terminal domain, identifies RRM2 as an αRRM despite the absence of an extended α3 helix. This places Pof8 structurally as well as functionally in the same family as pof6 and LARP7. The loss of stability of the holoenzyme in the absence of Pof8 and the observation that Pof8 is associated with nearly 100% of telomerase enzyme support a role in stabilizing an active conformation of the holoenzyme, rather than functioning solely as an assembly chaperon.

The biochemical and structural basis for the constitutive presence of Pof8 on telomerase was elegantly explained by a recent study that revealed direct interactions between Pof8 and several subunits of the Lsm complex. The authors showed that Pof8 and Lsm bind telomerase RNA cooperatively, with Pof8 specifically recognizing a correctly folded pseudoknot and Lsm-2-8 interacting with the oligo-uridine tail at the 3′ end of the RNA. Extensive protein–RNA contacts and cooperative binding ensure that correctly folded TER1 is protected and then licensed for assembly with the catalytic subunit.

As LARP family proteins often function in complex with other factors, we wondered whether Pof8 has binding partners in addition to known telomerase components. Here we show that Thc1, a previously uncharacterized open reading frame, and Bmc1, the putative fission yeast ortholog of metazoan methyl phosphate capping enzymes, form a complex with Pof8, revealing an intriguing compositional similarity between telomerase and the 7SK RNP in metazoans. Deletion of either thc1 or bmc1 results in decreased TER1 steady-state levels, telomerase assembly defects, and shortened telomere length. In aggregate, our findings provide further insights into telomerase biogenesis as a hierarchical assembly process. The structural similarity of Thc1 with human PARN provides an evolutionary link in the biogenesis of telomerase in distant organisms, whereas Bmc1 functioning in telomerase biogenesis adds to the plethora of recently discovered interconnections between functionally diverse non-coding RNAs, including snRNAs, snoRNAs, RNaseP, and now 7SK RNA.

Results

Pof8 forms a complex with Thc1 and Bmc1. To gain further insight into how Pof8 mediates the loading of the Lsm2-8 complex onto TERT, we immunoprecipitated epitope-tagged Pof8 expressed under the control of its endogenous promoter. Silver staining of the immunoprecipitated proteins revealed enrichment of four bands in
the tagged samples over untagged controls (marked with an asterisk in Supplementary Fig. 1a). The samples were then analyzed by multidimensional protein identification technology (MudPIT) and 19 proteins with an average distributed Normalized Spectral Abundance Factor (dNSAF) of >0.005 in the tagged samples were found to be enriched in the Pol8 immunoprecipitates (Supplementary Fig. 1b and Supplementary Table 1). Among them were Pol8 itself and Lsm4, a member of the Lsm2-8 complex, which had previously been shown to directly interact with Pol8 based on crosslinking experiments\(^3\). Four additional members of the Lsm2-8 complex were also enriched in the Pol8 pulldown but did not meet our stringent cutoff criteria. Based on their molecular weights, the two most prominently enriched proteins are Pol8 itself (49 kDa) and the heat-shock protein Ssa2 (70 kDa). Ssa2 is an abundant chaperon that frequently associates with epitope-tagged proteins and was also present in lower amounts in the untagged control immunoprecipitation (IP). We therefore considered its enrichment in the Pol8 IP a consequence of tagging Pol8 and did not further investigate it in this study. Two proteins caught our attention as they were only detected in the tagged Pol8 samples: sequence orphan SPCC18b5.09c and SPBC2A9.10, a predicted transcriptional and translational regulator of the Bin3 family. Their respective sizes are consistent with the two lower bands marked with asterisks in Supplementary Fig. 1a.

Sequence orphan SPCC18b5.09c encodes for a 13.5 kDa protein with no reported sequence homology to previously characterized proteins. However, a profile sequence search on the HHpred\(^3\) server revealed similarity with the nuclear cap-binding protein 3 (NCBP3) and the poly(A)-specific ribonuclease (PARN) (Fig. 1a, b). The nuclear cap-binding complex (CBP20 and 80) and PARN have previously been implicated in the biogenesis of human telomerase\(^34-38\), raising the possibility that SPCC18b5.09c may carry out an analogous function in fission yeast. Based on our results detailed below, we will subsequently refer to SPCC18b5.09c as Thc1 (Telomere holoenzyme component 1).

The open reading frame SPBC2A9.10 shares sequence similarity with proteins in the MePCE/Bin3 family (Fig. 1c). As the name Bin3 is ambiguous, referring to Bicoid interacting protein 3, first identified in Drosophila and characterized by the presence of an AdoMet binding domain, as well as the unrelated Bridging-Integrator 3 protein, members of the former family are now preferentially referred to as Methyl phosphate capping enzymes (MePCE). In accordance with the nomenclature conventions for fission yeast genes and to maintain the link to the founding members of the family, Drosophila Bin3 and MePCE, we will refer to SPBC2A9.10 as Bmc1 (Bin3/MePCE 1). The RNA methyltransferase activity of mammalian MePCE adds a methyl group to the ϕ-phosphate of the 5′ cap of RNA polymerase III transcribed 7SK RNA, a major transcriptional regulator, and U6 snRNA, a component of the spliceosome\(^39\). The presence of a MePCE ortholog in fission yeast is puzzling since the 7SK RNA has not been identified in any yeast species and an orthologous protein is absent from the Saccharomyces cerevisiae genome, indicating that U6 snRNA can be stabilized in ways not involving a MePCE ortholog. As high throughput knockout studies reported S. pombe bmc1\(^+\) (SPBC2A9.10) as essential for viability\(^40,41\), its target RNAs if any have remained uncharacterized thus far. Based on the known enzymatic activity, polIII transcripts such as U6 snRNA are prime candidates. However, we wondered whether the interactions of Bmc1 with Pol8 is indicative of involvement in the biogenesis of telomerase, despite the absence of a 5′ triphosphate cap structure on TER1.

Reduced TER1 level and short telomeres in thc1\(Δ\) and bmc1\(Δ\) cells. We next deleted thc1\(^+\) in haploid cells and one copy of bmc1\(^+\) in diploid cells. Strains deleted for thc1 were readily obtained. Surprisingly, sporulation of bmc1\(^+\)/bmc1\(Δ\) cells also gave rise to healthy haploid colonies. The gene deletions were verified by polymerase chain reactions (PCRs; Supplementary Fig. 2a–d) and further confirmed by the absence of reads mapping to the respective loci in RNA sequencing experiments (Supplementary Fig. 2e, f). It is not clear to us why prior attempts to delete bmc1 have not yielded viable knockout cells\(^40,41\). While we cannot rule out the possibility of a spontaneously arising suppressor mutation in our strains, backcrossing bmc1\(Δ\) strains to wild-type S. pombe obtained from the American Type Culture Collection (Lindner 972; ATCC\#38366) followed by tetrad dissections yielded four viable colonies for 10 out of 14 tetradas, as well as the expected two-to-two segregation of the selection marker (Supplementary Fig. 2g).

To confirm the physical interactions suggested by the mass spectrometry result, we introduced an N-terminal epitope tag on Pol8 and C-terminal epitope tags on Thc1 and Bmc1. Following IP of Pol8, Thc1 (Fig. 2a) and Bmc1 (Fig. 2b) were readily detected. Since Pol8 has been shown to directly bind TER1\(^35\) and structure-function predictions were consistent with Thc1 and Bmc1 also interacting with RNA, we tested whether the Pol8-Thc1 and Pol8-Bmc1 interactions are nucleic acid-dependent. The interactions were neither impaired by treatment with RNase A nor Benzonase, indicating that the interactions are not mediated by TER1 (Fig. 2a, b). Similarly, Thc1 coprecipitated with Bmc1 in a non-nucleic acid-dependent manner (Fig. 2c). In summary, these experiments confirmed the associations indicated by the mass spectrometry and suggested that Pol8, Bmc1, and Thc1 are part of a protein complex (Fig. 2d). As the IPs were carried out in S. pombe cell-free extract, it remains possible that interactions are mediated by additional proteins.

Based on the known function of Pol8, we next assessed whether Thc1 and Bmc1 also function in telomerase biogenesis. Examination of the steady-state levels of TER1 in cells deleted for Thc1 by reverse transcriptase–quantitative PCR (RT-qPCR) revealed a threefold reduction in TER1 levels (Fig. 3a), similar to the reduction we and others had previously observed in cells deleted for pof8\(^15–17\). Deletion of bmc1 also resulted in a reduction of total TER1 levels, albeit not quite to the same extent as the other single gene deletions (Fig. 3a). Detection of TER1 with primers annealing to the thc1 locus (SPBC2A9.10) as essential for TER1 (Fig. 2a, b). Thc1 and Pof8-Bmc1 interactions are nucleic acid-dependent. The interactions were neither impaired by treatment with RNase A nor Benzonase, indicating that the interactions are not mediated by TER1 (Fig. 2a, b). Similarly, Thc1 coprecipitated with Bmc1 in a non-nucleic acid-dependent manner (Fig. 2c). In summary, these experiments confirmed the associations indicated by the mass spectrometry and suggested that Pol8, Bmc1, and Thc1 are part of a protein complex (Fig. 2d). As the IPs were carried out in S. pombe cell-free extract, it remains possible that interactions are mediated by additional proteins.

We next examined whether Thc1 and Bmc1 are stably associated with TER1 as previously reported for Pol8. TER1 was approximately 8000-fold enriched in immunoprecipitates of epitope-tagged Thc1 and Pol8 relative to untagged controls (Fig. 3c). A smaller, 64-fold-enrichment was observed with Bmc1, indicating a weaker interaction that is more easily disrupted under the experimental conditions. Alternatively, Bmc1 may not be a constitutive member of the telomerase complex and may only be associated with a fraction of the telomerase complex. Since telomere length regulation is exquisitely sensitive to changes in telomerase activity levels, we measured telomere length in the deletion strains. Telomeres were approximately 30% shorter than wild type in thc1\(Δ\) cells and 20% shorter in bmc1\(Δ\) cells (Fig. 3d). The reduction in telomere length in the mutants was observed across multiple isolates and was stable over the course of successive restreaks (Supplementary Fig. 3). Overall, these results confirm roles for Thc1 and Bmc1 in telomere maintenance and suggest functions in telomerase biogenesis or quality control.
Bmc1 function in telomerase biogenesis is independent of its catalytic activity. As vertebrate MePCE and Drosophila Bin3 bind the 5′ triphosphate caps on 7SK and U6 snRNA and function enzymatically by transferring a methyl group onto the γ-phosphate, it is not apparent how TER1 with its trimethylguanosine cap could be targeted by Bmc1. Nevertheless, sequence alignments and homology modeling showed that the amino acids involved in AdoMet binding and methyltransferase activity are largely conserved in fission yeast (boxed in red in Fig. 1c). Furthermore, an overlay of the AlphaFold predicted structure for Bmc1 with the human MePCE structure predicted striking structural similarities consistent with a methyltransferase activity.
for Bmc1 (Supplementary Fig. 4a). We therefore generated several mutants designed to abolish the putative capping activity based on structural and functional information on the orthologous proteins and introduced them on plasmids under the control of the endogenous promoter into S. pombe cells deleted for endogenous bmc1. Assessment of protein expression levels showed that F246A, GCN43ACA, and EPQ188APA were expressed at similar levels to wild-type Bmc1, whereas expression of VLD39AAA was approximately threefold reduced (Supplementary Fig. 4b). Using telomere length as the terminal readout of telomerase function in vivo, we found that each of the catalytic mutants rescued the telomere length phenotype, except for VLD39AAA (Supplementary Fig. 4c). As the expression of this mutant is reduced compared to wild type and the other mutants, it is likely that mutation of the VLD motif causes structural changes that compromise the stability of Bmc1. In summary, we surmise that the catalytic activity of Bmc1 is not required for its function in telomerase biogenesis. However, we cannot exclude the possibility that the mutants have residual catalytic activity, and a definitive conclusion will therefore require future experiments.

Fig. 1 Thc1 and Bmc1 share sequence and structural similarities with known RNA-processing factors. a HHPre<sub>d</sub> predictions for Thc1 reveal a putative cap-binding domain. Alignment and secondary structure elements of a putative cap-binding domain in Thc1 based on alignment with human (H.s.) polyadenosine-specific ribonuclease (PARN); b-strands are highlighted in blue and a-helices in red. The secondary structure elements predicted for Thc1 are labeled above the alignment. The conserved tryptophane that stacks against the guanosine base in the PARN structure is boxed in red. Symbols indicating the quality of the alignment match are depicted below the sequence: “|” very good, “+” good, “.” Neutral, “−” bad, and “=” very bad. b Alignment of the same region of Thc1 as in a with Histoplasma capsulatum (H.c.) Nuclear Cap Binding Protein 3 (NCBP3). c Multiple sequence alignment of human (H.s.) MePCE (amino acids 412–689), mouse (M.m.) MePCE (aa 395–677), S. pombe (S.p.) Bmc1, and putative Bmc1 orthologs from S. japonicus (S.j.) and S. octosporus (S.o.) using Clustal Omega. Secondary structure elements are annotated based on H.s. MePCE. Motifs characteristic of SAM-dependent methyltransferases are indicated in green, residues involved in SAH binding by human MePCE are boxed in pink. The following symbols are used below the alignment: “*” identical amino acid across all sequences, “.” amino acid groups of similar properties, “.” amino acid groups of weakly similar properties.

Fig. 2 Associations between Pof8, Thc1, and Bmc1. a Thc1 co-immunoprecipitates with Pof8. Co-IP of Thc1-2xV5 with 3xFLAG-tagged or untagged Pof8 by anti-FLAG. Western blot for input (10%, lanes 1 and 2) and IP (lanes 3–7) probed with anti-V5 to detect Thc1-2xV5 (top) and reprobed with anti-FLAG for 3xFLAG-Pof8 (bottom). Samples in lanes 5–7 were incubated at 37 °C for 15 min to assess whether the interactions are mediated by nucleic acid. An asterisk marks a protein of approximately 60 kDa that is recognized by the FLAG antibody even in cells that lack FLAG-tagged Pof8 (lane 3). This 60 kDa protein of unknown identity is enriched in the immunoprecipitate in an RNA-dependent, but FLAG-Pof8 independent manner. b Bmc1 co-immunoprecipitates with Pof8. IP with anti-FLAG in extracts from strains harboring Twinstrep-tagged Bmc1 and 3xFLAG-tagged or untagged Pof8. Plus indicates the presence of the tag. Western blot for input (10%, lanes 1 and 2) and IP (lanes 3–7) probed with anti-strep-tag II for Bmc1-Twinstrep (top) and reprobed with anti-FLAG for 3xFLAG-Pof8 (bottom). Samples in lanes 5–7 were treated as in a. Asterisk marks the same non-specific band as described in a. c Bmc1 co-immunoprecipitates with Thc1. Co-IP of Twinstrep-tagged Bmc1 with 2xV5-tagged or untagged Thc1. Western blot for input (10%, lanes 1 and 2) and immunoprecipitated (lanes 3–7) probed with anti-strep-tag II for Bmc1-Twinstrep (top) and anti-V5 for Thc1-2xV5 (bottom). Samples in lanes 5–7 were treated the same way as in a. d Schematic of interactions among the three proteins.
biochemical experiments and the identifications of those RNAs in fission yeast that are modified by Bmc1 and can serve as positive controls for loss of methyltransferase activity.

Pof8, Thc1, and Bmc1 function in the same pathway. The differences in the extent to which TER1 levels and telomere length were reduced following the deletion of pof8Δ, thc1Δ, or bmc1Δ, led us to investigate whether they have distinct roles during TER1 biogenesis. We constructed otherwise isogenic single, double, and triple mutants to compare TER1 steady-state levels by RT-qPCR and telomere length by Southern blotting. Neither TER1 levels (Fig. 4a) nor telomere length (Fig. 4b) were further reduced in any double or the triple deletion combination compared to the single pof8 deletion. It is noteworthy that telomeres in a pof8Δ deletion are already critically short as the terminal restriction fragment is almost entirely comprised of subtelomeric DNA and chromosome end fusions are readily detected in a pof8ΔΔ culture17. Any exacerbation of this phenotype would result in telomere loss and chromosome circularization as observed in trt1Δ cells48.

Deletion of pof8 affects stability and interaction between Thc1 and Bmc1. To further understand the difference in phenotype among the single gene deletions, we assessed the stability of the other subunits and integrity of the complex in the absence of each protein. Deletion of Pof8 resulted in an approximately fourfold reduction in Bmc1 (Fig. 4c) and no Bmc1 was detected in Thc1

Fig. 3 Thc1 and Bmc1 stabilize TER1 and are required for wild-type telomere length maintenance. a RT-qPCR quantification of TER1 by from DNase-treated total RNA extracted from wild type (WT), pof8Δ, thc1Δ, and bmc1Δ strains. TER1 was normalized to three endogenous control genes (act1, his1, and snR101) and is shown relative to expression in WT. The bars show the mean (±SD, n = 3). Significant values are marked above each bar (two-sided Dunnett’s test; ***p < 0.001, **0.001 ≤ p < 0.01, *0.01 ≤ p < 0.05). The schematic on the bottom indicates the position of the primers (red) used for TER1 amplification. b RT-qPCR quantification and statistical analysis as in a but using a primer pair in exon 2 of TER1, which is present in the precursor but not in the mature form of telomerase RNA. c RT-qPCR quantification of TER1 recovered from immunoprecipitations for 3xFLAG-Pof8, Thc1-2xV5, and Bmc1-Twinstrep normalized to untagged controls. The bars show the mean (±SD, n = 3) of enrichment of TER1 RNA relative to untagged control after normalization to RNA levels in the input using the same primer sets as in a. Statistical analysis as in a. d Telomeric Southern blot using genomic DNA from WT, pof8Δ, thc1Δ, and bmc1Δ strains. Genomic DNA was digested with EcoRI, separated by gel electrophoresis, transferred to a membrane, and probed for telomeric repeats and the rad16Δ locus as a loading control (LC).
Fig. 4 Functional interactions among Pof8, Thc1, and Bmc1. (a) Effect of single, double, and triple deletions of pof8, thc1, and bmc1 genes on TER1 levels measured by RT-qPCR. Mean (±SD, n = 3) fold-change values relative to wild type (WT). Significant differences from WT are marked above each bar (two-sided Dunnett’s test; ** p < 0.001, * p < 0.01, * p < 0.05). Telomeric Southern blot of the strains used in a. Two independent isolates of each deletion are shown. The rad16+ locus was used as a loading control (LC). c Pof8 stabilizes Bmc1. Western blot analysis using anti-Strep-tag II antibody for Bmc1-Twinstrep (top) and reprobed with anti-V5 antibody for Thc1-2xV5 (bottom). d Pof8 is required for the Thc1-Bmc1 interaction. Western blot analysis of co-immunoprecipitation using anti-V5 in strains containing Bmc1-Twinstrep and Thc1-2xV5 in pof8+ and pof8Δ backgrounds. The blot was probed with anti-Strep-tag II antibody for Bmc1-Twinstrep (top) and reprobed with anti-V5 antibody for Thc1-2xV5 (bottom). e Bmc1 stabilizes Thc1. Assessment of steady-state protein levels as in c for Thc1-2xV5 and 3xFLAG-Pof8 following the deletion of bmc1. f Bmc1 is not required for Thc1-Pof8 interaction. Western blot analysis of co-IP using anti-FLAG antibody in strains containing 3xFLAG-Pof8 and Thc1-2xV5 in bmc1+ or bmc1Δ backgrounds. The blot was probed with anti-V5 antibody for Thc1-2xV5 (top) and reprobed with anti-FLAG for 3xFLAG-Pof8 (bottom). g Thc1 stabilizes Bmc1 and Pof8 protein. Assessment of steady-state protein levels as in c for Bmc1-Twinstrep and 3xFLAG-Pof8 following the deletion of thc1. h Thc1 is required for the Pof8-Bmc1 interaction. Western blot analysis of co-IP using anti-FLAG in strains containing 3xFLAG-Pof8 and Bmc1-Twinstrep in thc1+ or thc1Δ backgrounds. The blot was probed with anti-Strep-tag II antibody for Bmc1-Twinstrep (top) and reprobed with anti-FLAG for 3xFLAG-Pof8 (bottom).
immunoprecipitates (Fig. 4d, compare lanes 3 and 4). When protein extract was prepared from bmcΔ1 cells, Thc1 levels were approximately twofold reduced, whereas Pof8 levels were close to wild type (Fig. 4e). Despite the reduction in Thc1, the protein still co-immunoprecipitated with Pof8 (Fig. 4f). In contrast, deletion of thc1 resulted in a threefold decrease of Bmc1 protein and a twofold reduction in Pof8 (Fig. 4g). Although Bmc1 was readily detected in the input, no Bmc1 was observed in the Pof8 immunoprecipitate (Fig. 4h). These results support physical and functional interactions within the complex, in which the stability of individual factors and the integrity of the complex depend on the presence of the other members. Most importantly, the association of Bmc1 with the complex depends on the presence of Pof8 and Thc1. In the absence of either Pof8 or Thc1, the interaction of Bmc1 with the remaining factor is compromised. The quantitative differences in the effects on TER1 levels and telomerase length between the single gene deletions can be explained by the loss of Pof8 or Thc1 causing the associated loss of Bmc1.

Further supporting the importance of stabilizing interactions between Thc1 and Pof8, recombinant 6xHis-Thc1 and untagged Pof8 co-expressed in Escherichia coli were readily purified as a complex (Fig. 5a). Whereas IP of the Lsm2-8 complex from extracts lacking endogenous Pof8 results in poor recovery of TER1 RNA and telomerase activity, the addition of purified Pof8-Thc1 complex to the cell extract was sufficient to increase the amount of TER1 recovered from an Lsm4-cMyc IP by fourfold and telomerase activity by up to ninefold (Fig. 5b).

**Effects on the Sm to Lsm2-8 switch.** Biochemical studies with purified proteins showed that Pof8 and Lsm2-8 interact directly and bind telomerase RNA cooperatively. To assess to what extent Thc1 and Bmc1 affect the association of Pof8 and Lsm2-8 with TER1 in vivo, we immunoprecipitated the Lsm complex via c-Myc-tagged Lsm4 from extract prepared from cells deleted for pof8, thc1 and bmc1. The deletions neither affected the level of Lsm4 protein in the extract nor the amount of immunoprecipitated Lsm4 (Fig. 5c). Nevertheless, the amounts of co-precipitating TER1 showed striking differences depending on the genetic background. Confirming previous results, deletion of pof8 causes a 100-fold reduction in the amount of TER1 associated with the Lsm complex (Fig. 5d). In stark contrast, deletion of thc1 only caused a twofold reduction in Lsm–TER1 association relative to the wild-type control. Deletion of bmc1 even enhanced the interaction, albeit slightly. Interestingly, despite the modest effect on TER1-Lsm association, loss of Thc1 resulted in a 15-fold reduction in telomerase activity associated with Lsm4 (Fig. 5e). Under the same conditions, the activity was undetectable in the absence of Pof8 and was two to fourfold higher than wild type in the absence of Bmc1. These findings suggest that Thc1 contributes to the assembly of active telomerase by enhancing the ability of Pof8 to recognize correctly folded TER1 or by providing an additional level of quality control, perhaps by assessing the processing state at the 5’ end of the RNA.

Prior to splicingosome cleavage, the Sm complex binds the AU6GG motif, which after cleavage becomes the oligo-uridine binding site for Lsm2-8. To assess whether members of the Pof8-Thc1-Bmc1 complex also affect the stability of the Sm complex on TER1, we assessed the amount of RNA precursor that is associated with Sm1. Although recovery of TER1 varied by up to 1.5-fold among strains, none of these differences were statistically significant (Fig. 5f). We conclude that Sm complex association with the TER1 precursor is unaffected by Pof8, Thc1, and Bmc1 consistent with this complex functioning downstream of splicingosome cleavage. Consistent with this notion, an association of Sm1 with the first exon of TER1 (mature form) was increased in each of the three deletions (Fig. 5g). These observations support a model in which the transition from Sm-bound to Lsm-bound form is an active process and that TER1 remains associated with the Sm complex for longer in the absence of Pof8, Thc1 or Bmc1.

**Thc1 and Bmc1 stabilize Pof8 on telomerase and are associated with telomerase activity.** With the deletion of pof8 affecting Lsm recruitment, telomerase activity, and telomere maintenance more strongly than thc1Δ and bmc1Δ, we next assessed the extent to which Thc1 and Bmc1 affect the association of Pof8 with TER1. Every single deletion caused a reduction in the amount of TER1 that co-immunoprecipitated with Pof8 (Fig. 6a). A 15-fold reduction was observed when both thc1 and bmc1 were absent, demonstrating that the stability of the Pof8-TER1 complex is clearly enhanced by the presence of Thc1 and Bmc1. Consistent with the co-IP data, telomerase activity associated with Pof8 was also reduced in the absence of either Thc1 or Bmc1 alone, or when both were absent (Fig. 6b). Although Lsm2-8 and Pof8 clearly still bind TER1 to some extent in the absence of Thc1 or Bmc1, the stability of the interaction between Pof8 and the core components of telomerase appears to be more transient in the absence of either protein.

To assess whether Thc1 and Bmc1 themselves are only transiently associated with the Lsm2-8 complex and Trt1, we precipitated Lsm4-cMyc from the extract in which either Thc1 or Bmc1 were also epitope-tagged. Thc1 and Bmc1 were readily detected in Lsm4 immunoprecipitates (Fig. 6c, d). Further supporting that Thc1 and Bmc1 are associated with active telomerase, we were able to detect telomerase activity following IP of epitope-tagged versions of Thc1 and Bmc1 (Supplementary Fig. 5). The activity associated with Thc1 was comparable to Pof8 IPs, while a reduced level of activity was recovered from Bmc1 IPs. However, this result must be interpreted with caution as different epitope tags are present on Pof8, Thc1, and Bmc1.

To gain insight into what fraction of telomerase is associated with Thc1 and Bmc1 respectively, we first asked whether either protein can be immunodepleted from the extract in a single step. Comparison of input and IP supernatant revealed that over 90% of Thc1 is removed from the soluble fraction in a single round of IP (Fig. 6d). Similarly, the supernatant is efficiently depleted of Bmc1 in a single round of IP (Fig. 6f). Using extracts from cells in which Thc1 and Lsm4 were both epitope tagged, we first immunoprecipitated Thc1 and then immunoprecipitated Lsm4-cMyc from the IP supernatant to assess how much telomerase activity remains in the supernatant following depletion of Thc1. To account for the non-specific reduction in telomerase activity associated with each round of IP, first-round mock IPs were carried out in parallel. Relative to mock depleted extract, 50% of telomerase activity was detected in Lsm4 IP after extracts had first been depleted of Thc1 (Fig. 6g). This is in stark contrast to the only 1% of activity detected when the extract was first depleted of Pof8 [17]. Even more strikingly, the analogous experiment performed with Bmc1 revealed that close to 90% of telomerase activity was recovered with Lsm4 from the supernatant of a Bmc1 immunodepletion (Fig. 6g). These results indicate that, unlike Pof8 and Lsm2-8, Thc1, and even more so, Bmc1, are only loosely associated with active telomerase.

**Deletion of thc1 affects the expression of several ncRNAs including TER1.** Considering the sequence similarity of Thc1 and Bmc1 with general RNA processing factors in other species, we asked whether the expression of RNAs other than TER1 is also affected by the deletion of either gene. For the deletion of thc1, 77
genes met the criteria of a twofold change in expression level with an associated adjusted \( p \) value of <0.05 (Supplementary Fig. 6a). Next to TER1 and thc1, 26 loci, including 11 non-coding RNAs were downregulated (Supplementary Table 2a). We also identified 49 loci that were upregulated, the majority of which were non-protein coding (Supplementary Table 2b). It therefore appears likely that Thc1 is involved in the biogenesis or regulation of additional RNAs than just TER1. Far fewer changes in gene expression were observed following the deletion of \( bmc1 \) with no loci meeting the criteria described above (Supplementary Fig. 6b). In light of U6 snRNA being a target of vertebrate MePCE, we specifically examined this locus but found no significant change in expression in either deletion. However, it must be noted that the knockdown of MePCE in human cells also did not reduce the
steady-state levels of U6 snRNA.\(^{39}\) While changes in expression levels are indicative of a specific RNA being a target for modification, RNA stability is not necessarily affected by the absence of the methyl group. It therefore will require further studies to assess whether Bmc1 functions specifically as a telomerase biogenesis factor or has additional functions in fission yeast.

**Discussion**

Previous work demonstrated that the *S. pombe* Pof8 protein is a LARP7 family member that functions in telomerase biogenesis by facilitating the loading of the Lsm2-8 complex onto TER1 through cooperative protein–protein and protein–RNA interactions.\(^{15–17,32}\) We now reveal that Pof8 carries out this function in complex with Thc1 and Bmc1, two previously uncharacterized proteins. Deletion of *thc1* or *bmc1* results in a decrease in Pof8 association with telomerase, decreased levels of TER1, and shorter telomeres. Although physical interactions support that Pof8, Thc1, and Bmc1 function together during telomerase assembly, they clearly have distinct roles in biogenesis with Pof8 remaining associated more tightly with active telomerase than Thc1 and Bmc1. It is further worth noting that the levels of telomerase activity associated with Pof8 in the extract from *bmc1Δ* cells were significantly lower compared to cell extracts from *bmc1Δ* cells (Fig. 6b). In contrast, the Lsm4 associated telomerase activity was slightly increased in the absence of Bmc1, suggesting that Bmc1 stabilizes the binding of Pof8 to telomerase but is not required for the stable association of the Lsm complex (Fig. 5e). These results support a model where the interaction of Pof8 with TER1 is stabilized by Thc1 and Bmc1, and both factors contribute to the assembly and stability of functional telomerase but do not necessarily remain associated with the active enzyme (Fig. 7).

To our knowledge, this is the first example of a member of the LARP7 family member functioning in a complex with MePCE/Bin3 outside the 7SK complex, indicating that the functional association of these two proteins predates the metazoan invention of the 7SK RNP as a transcriptional and translational regulator. Coincidentally, RIP-Seq experiments with epitope-tagged Bmc1 (Bin3) also identified TER1 as a target leading to an independent characterization of the Bmc1/Pof8 interaction by the Bayfield group.\(^{42}\)

It has been widely assumed that the 7SK complex emerged in metazoans to regulate transcription elongation, a process that gained in importance as intron size, and thereby primary transcript length, increased. This assertion is in part based on the inability to locate 7SK non-coding RNAs in the genomes of yeast and other unicellular organisms, but the high degree of divergence between vertebrate 7SK RNAs and those found in arthropods,\(^{49}\) justifies the old adage that absence of evidence is not evidence of absence. In light of well over 100 non-coding RNAs of unknown function in the fission yeast genome, it remains to be seen whether a 7SK-like complex exists here and in other unicellular organisms.

Should this not be the case, one will have to look elsewhere for the evolutionary origins of Larp7 and MePCE/Bin3 proteins functioning together in non-coding RNA processing or as stable components of a functional RNP. Prior to the discovery of a MePCE/Bin3 family member functioning in telomerase biogenesis, the presence of Larp7 family members as constitutive components of telomerase holoenzymes in evolutionarily distant unicellular species already suggested that the association of Larp7 with this RNP predates the existence of a 7SK complex. With Bmc1 and Pof8 functioning together in telomerase biogenesis, it is clear that the Larp7–MePCE/Bin3 partnership has ancient roots. Interestingly, the stability of Bmc1 is largely dependent on the presence of Thc1 and Pof8 indicating that most if not all Bmc1 in the cell is in complex with these two proteins and they are likely forming a functional unit on whichever RNAs they associate with.

It was recently reported that the 7SK complex interacts with the SMN complex in the cytoplasm to regulate the assembly of Sm complexes on snRNAs to connect spliceosome production with transcriptional output.\(^{30}\) It is thus noteworthy that telomerase RNAs in yeast share features with snRNAs, including the presence of Sm-binding sites and TMG cap structures.\(^{7,31}\) Whether this resemblance was critical in telomerase acquiring these biogenesis factors or whether telomerase is the more ancient target RNA remains to be investigated.

LARP7 family members share an atypical RNA recognition motif, named xRRM for the presence of an extended C-terminal RRM in Pof8 that lies across the C-terminus that lies across the C-terminus of the C-terminal 3-helix in Pof8 and MePCE/Bin3.\(^{52}\) While the C-terminal RRMs in Po8 shares most features with the xRRM domains in p65 and human Larp7, such as the presence of the conserved RNP3 sequence Y/W-X-D/Q on β2, a conserved arginine on β3, and charged/aromatic residues on the C-terminal end of helix α3. The C-terminal helix itself is considerably shorter in Po8 than in other family members. This may result in reduced RNA binding affinity as the extended α3 helix in Larp7 and p65 create a conserved binding pocket between the third and fourth turns of the α3-helix. Truncation of the α3-helix results in reduced binding affinity to substrate RNA, leading to the suggestion that the shorter α3-helix in Po8 will also result in a weaker binding affinity for RNA than seen with p65 and hLarp7.\(^{31}\) The α3 helix in Po8 is nevertheless important for RNA binding as mutations lower the affinity for TER1, reduce TER1
levels and cause telomere shortening similar to a deletion of the entire open reading frame. Recent structural and biochemical analysis revealed that TER1 interactions with RRM1 and xRRM2 of Pof8, as well as protein interactions with the Lsm2-8 complex, function cooperatively in binding to TER1. However, the addition of recombinant Pof8 to extracts from po8Δ cells was insufficient to restore telomerase activity. In contrast, we find that the addition of a preformed Pof8-Thc1 complex increased the association of TER1 with Lsm2-8 and strongly enhances in vitro telomerase activity (Fig. 5b). Together with our finding that thc1
Fig. 6 Thc1 and Bmc1 stabilize Pof8 on telomerase and are associated with Lsm proteins and active telomerase. a The absence of Thc1 and/or Bmc1 weakens the Pof8-TER1 interaction. RT-qPCR of TER1 following IP of FLAG-tagged Pof8 from thrclΔ, bmc1Δ, and combined deletion backgrounds relative to wild-type (WT) background. The bars represent the mean fold enrichment of TER1 relative to WT after normalization to TER1 levels in the input. Error bars represent standard deviation based on three biological replicates. b Telomerase activity assay from Pof8 IP samples of the same strains used in a. IP suspensions (10 and 20 μl) were used for telomerase activity assays; 20 μl of IP suspension was used for the RNase A treatment. c Thc1 co-immunoprecipitates with Lsm4. Extracts from strains containing V5 epitope-tagged Thc1 and untagged or cMyc-tagged Lsm4 were subjected to immunoprecipitation with anti-c-Myc followed by western blotting. The blot was probed with anti-V5 and reprobed with anti-cMyc antibodies. d Bmc1 co-immunoprecipitates with Lsm4. Extracts from strains containing TwinStrep tagged Bmc1 and untagged or Lsm4-cMyc were immunoprecipitated with anti-c-Myc followed by western blotting. The blot was probed with anti-strep-tag II and reprobed with anti-cMyc. e Depletion of Thc1-2xV5 from cell-free extract in a single round of immunoprecipitation with anti-V5 on Dynabeads; 20 μg of IP supernatant (S/N) was loaded next to a serial dilution of input to assess the extent of depletion; loading control (LC): α-tubulin. f Depletion of Bmc1-TwinStrep from cell-free extract in a single round of immunoprecipitation with anti-Strep-tag II on Dynabeads analyzed as in e, g Telomerase activity assay following sequential immunoprecipitations to assess the fraction of telomerase activity associated with Thc1 and Bmc1. The extract containing Thc1-2xV5 and Lsm4-cMyc was immunoprecipitated with anti-V5 and Lsm2-8 complex cannot load, leading to TER1 degradation. Whereas Pof8 and Lsm proteins are quantitatively associated with active telomerase, Thc1 and Bmc1 are only associated with a fraction of active enzyme.

Fig. 7 Proposed model of the role of Thc1 and Bmc1 during the stepwise assembly of telomerase. After spliceosomal cleavage, the Sm complex (purple) facilitates cap hypermethylation by Tgs1 and is subsequently replaced by the Lsm2-8 complex (brown). Pof8, Thc1, and Bmc1 function during the Sm to Lsm transition. Pof8 (pink) promotes the loading of Lsm2-8 onto TER1, while Bmc1 (green) and Thc1 (blue) stabilize Pof8 binding through protein–protein and likely through protein–RNA interactions. In the absence of Pof8 on TER1, the Lsm2-8 complex cannot load, leading to TER1 degradation. Whereas Pof8 and Lsm proteins are quantitatively associated with active telomerase, Thc1 and Bmc1 are only associated with a fraction of active enzyme.

Methods

Strains and constructs. S. pombe strains used in this study are listed in Supplementary Table 3. The bmc1 deletion was generated by replacing the entire open reading frame with the NatMX6 resistance cassette in a diploid strain generated fresh by crossing PP137 and PP139. The knockout fragment was generated by fusion PCR using primers listed in Supplementary Table 4 and contained the NatMX6 cassette and ~750 base pair (bp) of homology upstream and downstream of the bmc1 open reading frame. Cells were transformed as described23 and allowed to recover in a shaker at 25 °C overnight. Diploid transformants were then selected on yeast extract low adenine (YEA) plates supplemented with nourseothricin (NAT, 100 μg ml−1). The correct insertion of the NatMX6 cassette was confirmed by PCR and haploid cells were obtained by sporulation and selection on YEA plus NAT plates. The absence of the bmc1 ORF in the haploid cells was confirmed by PCR and sequencing.

The thc1 deletion was generated by replacing the open reading frame with the his3Δ gene using primers listed in Supplementary Table 4 and the same strategy as above except that haploid strain PP138 was used for transformation. Epitope tags were introduced as follows: 3xFLAG tag was introduced at the N-terminus of Pof8,
Liquid chromatography-tandem mass spectrometry. Peptides were loaded onto a 100-μm fused silica (Polymerix Technologies) capillary column packed with 3 cm of C18 (Phenomenex), 4 cm of strong cation exchange resin (Luna; Phenomenex), and 8 cm of reverse-phase C18 resin. Loaded microprecipitates were placed in line with an Agilent 1100 quaternary pump (Agilent Technologies, Palo Alto, CA). Overflow 50-μm fused silica tubing was used to decrease the flow rate from 0.1 ml min⁻¹ to about 200–300 ml min⁻¹. Three different elution buffers were used: 5% acetonitrile, 0.1% formic acid (Buffer A); 80% acetonitrile, 0.1% formic acid (Buffer B); and 0.5 M ammonium acetate, 5% acetonitrile, 0.1% formic acid (Buffer C). Eleven cycles with increasing salt concentrations followed by organic gradients slowly released peptides directly into the mass spectrometer.

First chromatography step only implemented the reversed phase starting with a 10 min 100% A wash; from 0 to 40% B between 10 and 80 min; to 100% B from 80 to 90 min; to 100% A from 90 to 100 min. Steps 2–8 used a short salt bump of increasing buffer C concentration set at X = 15, 20, 30, 40, 60, 70, and 80%; starting with a 3-min 100% A wash; 3% C and 100% – X% A from 3 to 5 min; to 100% A from 5 to 10 min; to 15% B from 10 to 25 min; to 45% B from 25 to 17 min. Chromatography steps 9 and 10 comprised a long high salt wash starting with a 2 min 100% A wash; 100% Buffer C from 2 to 22 min; followed by a 5-min 100% A wash; followed by the acetonitrile gradient to 20% B from 27 to 37 min; to 70% B from 37 to 105 min; to 100% B from 105 to 115 min; back to 100% A from 115 to 117 min. The last step was a 2-h wash with buffer A.

The application of a 2.5 kV distal voltage electro-sprayed the eluting peptide directly into an LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA) equipped with a custom-made nano-LC electrospray ionization source (Florens and Washburn 2006). Full MS spectra were recorded over a 400 to 1600 m/z range, followed by five tandem mass (MS/MS) events sequentially generated in a data-dependent manner with the most intense precursor in all MS spectra (at 35% normalized collision energy). Data were acquired in positive ion and centroid mode with activation Q energy set at 0.250, activation time of 30 ms, and an isolation width of 1 m/z. Dynamic exclusion was enabled for 120 s with a repeat count of 2 and an exclusion list size of 500. peptide/spectrum files were extracted from m files in mz file format using RawDistiller v.1.099. MS/MS spectra were searched using ProLuCID v. 1.3.98 with a mass accuracy of 500 ppm for both precursor and fragment ions. Trypsin specificity was imposed on both ends of candidate peptides with up to 3 missed cleavages. Static modification was imposed for cysteine alkylation (+57 Da) and variable modification was allowed on methionine for oxidation (+16 Da). Tandem mass spectra were searched with these parameters against a protein sequence database containing 5084 S. pombe proteins downloaded from NCBI on 2017-03-07 and 193 sequences from common contaminants (human keratins, IgGs, proteolytic enzymes). To estimate false discovery rates (FDR), each protein sequence was randomized (keeping the same amino acid composition and length) and the resulting “shuffled” sequences were added to the database, for a total search space of 10554 amino acid sequences.

DTASelect and CONTRAST v.1.952 were used to select and sort peptide–spectrum–match spectra and to compare results from duplicate analyses of immunoprecipitated samples and 2 negative controls. Peptide/spectrum matches were retained only if they had a Delta CN score (DelTCN) of at least 0.8, and a minimum cross-correlation score of 1.8 for singly, 2.2 for doubly, and 2.6 for triply charged spectra. Additionally, the peptides had to be a minimum of seven amino acids in length, fully tryptic, and mapped to at least 2 MS/MS spectra. With these criteria, the FDRs were 0.7 ± 0.7, 1.1 ± 0.2, and 3.8 ± 1.2 at the spectral, peptide, and protein levels, respectively. Proteins that were subsets of others were removed using the parsimony option in DTASelect on the proteins detected after merging all runs. Proteins that were identified by the same set of peptides were grouped together, and one accession number was arbitrarily considered as representative of each protein group. An in-house software, NSAF7 v. 0.0.1 (https://github.com/tzw-wen/kite/tree/master/windowsapp/NSAF7x64), was used to create the final reports on all detected peptides and nonredundant proteins identified across the different runs and calculate label-free distributed normalized spectral abundance factor (dNSAF) quantitative values for all detected protein/ protein groups59–61.

Co-immunoprecipitation. For other IPs, cell-free extracts (5.5 mg) were diluted to 5 mg ml⁻¹ with TMG(300) buffer plus supplements. An aliquot (100 µl) was frozen as input control. Magnetic protein G Dynabeads (30 mg ml⁻¹; Invitrogen, 10004D) were coated with anti-c-Myc 9E10 (Sigma–Aldrich, M4439), anti-FLAG M2 (Sigma–Aldrich, F3165), anti-V5 (Thermo Fisher, R069 25), or anti-strep-tag II (Abcam, ab76949) (20 µg per 100 µl of bead suspension) by incubation for 30 min at room temperature in 200 µl of 1x PBS + 0.1% (v/v) Tween-20. The magnetic beads and StrepTactin Sepharose (GE Healthcare, 28935599) were washed three times with 1 ml of TMG(300). IPs were performed with 60 or 75 µl (sequential IP) of 500 µl elution buffer (50 mM Tris-HCl pH 7.5, 250 mM sodium chloride, 0.1% (v/v) NP40, and 10% (v/v) glycerol) at 4°C with gentle rotation. Recombinant Po0f/6xHis-Thc1 was added to 100 nM during the incubation for experiments in Fig. 5b. Beads were collected using a magnet or centrifugation at 300 x g for 2 min (StrepTactin Sepharose). The beads were then washed four times with 1 ml TMG(300) plus supplements and 0.1% (v/v) Tween-20, 250 µl TMG(300) buffer plus supplements. Finally, beads were resuspended in 100 µl TMG(50) plus supplements and 0.4 µl U1 RNasin (Promega) and frozen in liquid nitrogen.

Protein digestion. The immunoprecipitated protein eluates were treated with 0.1 U of benzonase (Sigma, E8263) for 30 min at 37°C and further diluted with the same volume of 100 mM Tris-HCl, pH 8.5. The samples were then split into three 400 µl aliquots, mixed with 100 µl of 100% trichloroacetic acid (TCA), and incubated at 4°C overnight. After centrifugation, 20,000 x g for 30 min at 4°C, the pellets were washed twice with 500 µl cold acetone and centrifuged at 20,000 x g for 10 min after each wash. Air-dried pellets were resuspended in 30 µl of 100 mM Tris-HCl, pH 8.5, 8 M Urea, and pooled. 4.5 µl of 0.1 M Tris(2-carboxyethyl)-phosphine (Sigma–Aldrich, TCEP) was added, and the samples were incubated at 37°C overnight. After the incubation, 1.8 µl of 0.5 M CAM was added and incubated for 30 min at rt in the dark. Proteins were then digested with 1 µl of 0.1 µg µl⁻¹ Lys-C at 37°C overnight. The samples were then diluted to 2.1 µl Urea with 100 mM Tris-HCl, pH 8.5, 8 M Urea, and 100 µl of 100 µg ml⁻¹ trypsin at 37°C overnight. Finally, 90% formic acid was added to a final concentration of 5% and samples were analyzed by MudPIT mass spectrometry as described39.
Western blot analysis. Denatured protein extracts or immunoprecipitates were loaded onto 4–12% NuPAGE Bis-Tris gel (Life Technologies, NP0321BOX) for electrophoresis in 1× MOPS buffer (Life technologies, NP0001) at 160 V for 60 min. Proteins were transferred to Protran nitrocellulose membranes (Whatman) in western transfer buffer (3.03 g 1−1 Tris, 14.4 g 1−1 glycerol, 20% (v/v) methanol) at 100 V for 1 h with a cold pad stored at −20 °C prior to use. Blots were processed using an iBlind Western Device (Life Technologies, SLE20002). Primary antibodies were mouse anti-FLAG M2 (Sigma Aldrich, F3165) at 1:4000 dilution, rabbit anti-Flag (Sigma, F3165) at 1:4000 dilution, rabbit anti-c-Myc (Santa Cruz Biotechnology, sc-7989) at 1:1000, and mouse anti-tubulin (Sigma, T5168) at 1:10,000 dilution. Primary antibodies for V5 epitope tag were rabbit anti-V5 (Abcam, ab9116) at 1:2000 dilution (Figures 2 and 4d, f), mouse anti-V5 (Thermo fisher, R060-25) at 1:5000 dilution (Figures 4c, e and 6e), or mouse anti-V5-HP (Thermo fisher, R961-25) at 1:10000 dilution (Figure 6c). Secondary antibodies were visualized with ECL plus substrate (Pierce, 32132) on a Typhoon FLA9500 (GE Healthcare) for co-ip blots and ChemiDoc MP (ImageAmp) for denatured protein extract blots. Image Lab (6.0.1) was used to quantify the western blots.

RNA extraction. For total RNA extraction, cell cultures (250 ml) were grown to a density of 6–8.5 × 10^6 cells ml−1 and collected by centrifugation, washed twice with H2O (500 ml), resuspended in 5 ml H2O and quick-frozen by dripping the cell suspension into liquid nitrogen. Cells were lysed in a 6850 Freezer mill (SPEX SamplePrep) using seven cycles (2 min) at a rate of 10 per second with 2 min cooling time between cycles. Lysed cells were transferred into 50 ml tubes containing 5 ml phenolchloroform:isosaliclyl alcohol (25:24:1, equilibrated with 50 mM sodium acetate, pH 5.2) and 5 ml 50 mM sodium acetate and 1% (w/v) SDS pre-treated (5 ml phenol:chloroform:isoamyl alcohol (25:24:1, equilibrated with 50 mM sodium acetate, pH 5.2) and 5 ml 50 mM sodium acetate and 1% (w/v) SDS). RNA was precipitated and resuspended in H2O. In the context of IP experiments, RNA was isolated from input and beads by incubation with proteinase K (2 µg ml−1) in 0.5 ml H20, 1 µl 10X TE, 10 µl 20% (v/v) polyacrylamide (29:1) gel containing 8 M urea and transferred to Biodyne B filters (Pall Corporation) at 400 mA for 1 h in 0.5× TBE buffer. RNA used for RT-PCR was treated with 8 U of DNase I (RNase-free, NEB, M0297S) was added followed by incubation at 37 °C for 20 min and 65 °C for 20 min. Reactions were then diluted with water 10-fold for total or input RNA and 20-fold for RNA isolated from IPs. Reactions in 384-well microplates contained 2 µl of the diluted cDNA, 5 µl of 2× PerfeCTa SYBR Fast Green Fast Mix (Quantabio, 760001) and 2 mM forward and reverse primers (Supplementary Table 5), and 1 µl of H2O. The qPCR reactions were performed in technical and biological triplicates in a Viia 7 Real-Time PCR system (Thermo Fisher Scientific). Microsoft Excel (v16.54) was used to compile the data from the qPCR reactions. The qPCR data for total RNA was normalized to three reference genes (act1, hist, and snR101) by the QuantiStudio Software V1.2.4 (Thermo Fisher Scientific) while RNA IP was normalized to the input (TER1 all, TER1 precursor). Finally, the relative fold change was calculated by comparing either to an untagged control or to wild type as stated in the figure legends.

Statistics and reproducibility. Statistical analysis for all qPCR data was performed on the log2-transformed values of biological triplicates. Each sample was compared with the wild type using t-test from the R package PMCMRplus. Homoscedasticity was tested with Levene’s and Bartlett’s tests and equal variances for the samples were assumed. In addition, the data were plotted as bar graphs in the original scale along with mean and standard deviation. Significant values in the figures are marked above each bar (**<p < 0.01, ***<p < 0.001, ****<p < 0.0001). Experiments for Figs. 2a, 3a, 4a, 5a, and 6a were independently repeated twice. Experiments for Figs. 2b, 4d, f, h, and 6e and Supplementary Figs. 2b–d, 3, 4, and 5 were repeated independently twice. Experiments for Figs. 2c, 4c, e, g, 5e, and 6c, d, f, and Supplementary Fig. 4b were performed once. In Fig. 4b, wild type, single deletion, and double deletions were repeated independently four times, and the triple deletion was repeated independently twice.

Telomerase activity assay. Telomerase was enriched by IP as described in the section “Immunoprecipitations.” Bead suspension aliquots (10 and 20 µl) were used in telomerase activity assays. Control samples were incubated in 20 µl of TMG(50) plus 20 ng of RNase A (Invitrogen) for 30 min at 30 °C prior to the telomerase reaction. Supernatants were removed and beads resuspended in 10 µl of 50 mM Tris-acetate at pH 8.0, 1.00 mM potassium acetate, 1.0 mM magnesium acetate, 5% (v/v) glycerol, 1 mM spermidine, 1.0 mM DTT, 0.2 mM dATP, dCTP, dGTP, 2 mM [α-32P]-dGTP (500 Ci/mmol), and 5 µl of oligo PBol871 (5’-GTACGGTTAACGGTACGTTACG-3’). Reactions were incubated for 90 min at 30 °C and stopped by the addition of 2 µl of proteinase K (1 µg ml−1 in 0.5% (w/v) SDS, 10 mM EDTA pH 8.3, 20 µM Tris–HCl pH 7.5). Following the addition of 1000 counts per minute of a 100-mer oligonucleotide labeled with [γ-32P]-ATP as recovery and loading control reactions were incubated at 42 °C for 15 min. DNA was extracted with phenol-chloroform:isosaliclyl alcohol (25:24:1, equilibrated with 5x TE) and ethanol precipitated overnight at –20 °C. Products were resuspended in RNA loading dye (5µl), heated in a thermocycler at 90 °C for 15 min, and electrophoresed through a 1.2% (w/v) pectin acrylamide (19:1) sequencing gel containing 8 M urea for 1.5 h at 80 W. Gels were dried and exposed to PhosphorImager screens and analyzed with a Typhoon scanner. Telomerase activity was quantified in ImageQuant TL (v7.0) except for Fig. 6g which was quantified by Fiji/ImageJ v5.3e.
proteinase K (Sigma-Aldrich, P2308) was added to a final concentration of 30 µg/ml. After incubation for 1 h at 50°C, the samples were precipitated with 3 ml of 5 M potassium acetate for 30 min on ice. The precipitates were removed with two rounds of centrifugation at 3200 × g for 10 min at 4°C. The supernatant was collected and mixed with 1 volume of 100% isopropanol for 1 h on ice followed by centrifugation at 10,500 × g for 10 min at 4°C. Genomic DNA was resuspended in 5 × TE with 50 µg ml⁻¹ RNase A. Resuspended DNA was then incubated for 1 h at 37°C followed by two rounds of extraction with phenol:chloroform:isoamyl alcohol (25:24:1, equilibrated with 5× TE) and one round of chloroform:isoamyl alcohol (24:1, equilibrated with 5× TE). DNA was ethanol precipitated and resuspended in 1× TE. DNA concentrations were determined on a Qubit 3.0 instrument using the dsDNA HS Assay Kit and Qubit 3.0 dsDNA HS assay protocol (Invitrogen, Thermo Fisher Scientific) with a Qubit 3.0 spectrophotometer (Invitrogen, Thermo Fisher Scientific). DNA concentrations equal to or greater than 30 µg/ml were used.

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