The methyl phosphate capping enzyme Bin3 is a stable component of the fission yeast telomerase holoenzyme

Jennifer Porat¹, Moaine El Baidouri^{2,3}, Jorg Grigull⁴, Jean-Marc Deragon^{2,3,5}, & Mark A. Bayfield^{1*}

1. Department of Biology, York University, Toronto, Canada

2. LGDP-UMR5096, Université de Perpignan Via Domitia, Perpignan, France

3. CNRS LGDP-UMR5096, Perpignan, France

4. Department of Mathematics and Statistics, York University, Toronto, Canada

5. Institut Universitaire de France, Paris, France

*Correspondence: bayfield@yorku.ca (M.A.B.)

Abstract:

The telomerase holoenzyme is critical for maintaining eukaryotic genome integrity. In addition to a reverse transcriptase and an RNA template, telomerase contains additional proteins that protect the telomerase RNA and promote holoenzyme assembly. Here we report that the methyl phosphate capping enzyme (MePCE) Bin3 is a stable component of the *S. pombe* telomerase holoenzyme. Bin3 associates with the telomerase and the U6 snRNA through an interaction with the recently described LARP7 family member Pof8, and we demonstrate that these two factors are evolutionarily linked in fungi. Our data suggest that the association of Bin3 with telomerase is independent of its methyltransferase activity, but rather that Bin3 negatively regulates TER1 levels and telomere length. Taken together, this work yields new

insight into the composition, assembly, and regulation of the telomerase holoenzyme in fission yeast as well as the breadth of its evolutionary conservation.

Introduction:

To ensure complete DNA replication, the termini of eukaryotic chromosomes contain tandem repeats, or telomeres, that can be continually extended as the ends of linear chromosomes are lost through DNA replication. Telomeres serve as protection from DNA degradation, end-to-end fusions, chromosomal rearrangements, and chromosome loss^{1,2}. With very few exceptions, eukaryotes extend telomeric DNA sequences through the telomerase holoenzyme, a complex containing the telomerase reverse transcriptase (Trt1 in the fission yeast *Schizosaccharomyces pombe*), an RNA template (TER1 in *S. pombe*), and accessory proteins that promote complex assembly and tethering to telomeric DNA¹⁻⁴. While the reverse transcriptase/RNA template core of telomerase is generally well-conserved among eukaryotes, major differences exist, including in the sequence and structure of the RNA template. In contrast to ciliate telomerase RNAs, which are small RNA Polymerase III transcripts⁵, yeast and metazoan telomerase RNAs are longer and transcribed by RNA Polymerase II as precursors. These precursors then undergo an extensive maturation process to yield the mature form that integrates into the telomerase holoenzyme^{3,4,6,7}.

In *S. pombe*, the mature form of TER1 is generated from an intron-containing precursor through a spliceosome-catalyzed reaction involving release of the 5' exon prior to exon ligation⁸. TER1 maturation then proceeds with the sequential binding of the Sm and Lsm complexes. Sm

proteins, well-characterized for their role in splicing, bind TER1 immediately upstream the 5' splice site and promote 3' maturation and the addition of a 5' trimethylguanosine (TMG) cap by the methyltransferase Tgs1⁹. The Sm complex is then replaced by the Lsm2-8 complex, which serves to protect the mature 3' end of TER1 from exonucleolytic degradation and promotes the interaction between TER1 and Trt1 in the active telomerase holoenzyme. The switch from the Sm to Lsm complexes correlates with distinct, non-overlapping substrates: TER1 precursors are exclusively bound by the Sm complex, while the mature form of TER1, ending in a polyuridylyate stretch upstream the spliceosomal cleavage site, is only bound by the Lsm2-8 complex⁹.

More recently, a role has been proposed for Pof8, a La-related protein 7 (LARP7) homolog, in telomerase assembly and telomere maintenance in *S. pombe*. Ciliate LARP7 family proteins, including p65 from *Tetrahymena thermophila*, have previously been characterized for their functions in telomerase assembly¹⁰. Binding of p65 to stem IV of the *T. thermophila* telomerase RNA TER results in a conformational change to the RNA and subsequent binding of the reverse transcriptase TERT, suggesting a p65-dependent hierarchical assembly of the telomerase holoenzyme¹¹. Pof8 is hypothesized to function similarly in *S. pombe*, with Pof8 binding to TER1 promoting recruitment of the Lsm2-8 complex and enhancing the interaction between TER1 and Trt1. Accordingly, Pof8 deletion strains possess shorter telomeres indicative of a defect in telomerase function^{12–14}. Pof8 also functions in telomerase RNA quality control through recognition of the correctly folded pseudoknot region and subsequent recruitment of the Lsm2-8 complex to protect the 3' end from degradation¹⁵. Telomerase-related functions in LARP7 family proteins, particularly Pof8, map to a conserved C-terminal domain characteristic of

LARP7 family members, the extended RNA recognition motif (xRRM). The Pof8 xRRM is a major determinant for Pof8-mediated telomerase activity, contributing to TER1 binding and complex assembly^{13,16}. As other fungal Pof8 homologs also possess an xRRM, it is anticipated that Pof8 may have a conserved role in telomere maintenance beyond fission yeast^{13,17}.

Conversely, LARP7 homologs in higher eukaryotes are best characterized in the transcriptional regulatory 7SK snRNP, which includes the RNA polymerase III transcribed 7SK snRNA, the methyl phosphate capping enzyme (MePCE), and hexamethylene bisacetamide-inducible protein (HEXIM1/2). The 7SK snRNP inhibits transcription by sequestering positive transcription elongation factor (P-TEFb) and preventing it from phosphorylating the C-terminal domain of the largest subunit of RNA Polymerase II, which is associated with the transition into productive transcription elongation^{18–20}. The La module and xRRM of LARP7 bind 7SK through its polyuridylyate trailer and the 3' hairpin, respectively, and promote MePCE recruitment to the complex^{21,22}. In addition to adding a γ -monomethyl phosphate cap (i.e. CH₃-pppN) to 7SK snRNA as a means of protecting it from 5' exonucleolytic degradation, MePCE remains stably bound to 7SK snRNA to stabilize the complex²¹. MePCE, a homolog of the *Drosophila melanogaster* Bin3/BCDIN3 protein, also catalyzes the addition of this atypical cap structure to U6 snRNA²³. Bin3/MePCE homologs are present in many eukaryotes, but to date little has been studied outside of humans and *Drosophila*²⁴. More recent work has identified BCIND3D, a related Bin3 family protein overexpressed in breast cancer cells, as the enzyme responsible for methylating the 5' monophosphate of histidine transfer RNA and pre-miR-145^{25,26}. Despite the

many insights into the role of Bin3/MePCE in RNA processing in higher eukaryotes, the function of the essential *S. pombe* Bin3 homolog has yet to be determined^{27,28}.

In this work, we have explored the RNA targets of the *S. pombe* Bin3 homolog in an effort to uncover its function. In addition to an expected association of Bin3 with the U6 snRNA, we present data showing that Bin3 is a stable component of the *S. pombe* telomerase RNP. Our results also provide evidence for an evolutionarily conserved interaction between Bin3 and LARP7 homologs. Importantly, we show that the Bin3-Pof8 interaction is required for the recruitment of Bin3 to both the U6 snRNA and the active telomerase holoenzyme. Additionally, we provide evidence that Bin3 does not catalyze the addition of a γ -monomethyl phosphate cap on TER1, suggesting a catalytic independent function. Rather, we present evidence that Bin3 may negatively regulate telomerase activity, as decreased Bin3 expression correlates with increases in steady-state levels of TER1 and longer telomeres. Taken together, our results identify Bin3 as a novel component of the *S. pombe* telomerase holoenzyme, thus adding a new layer of complexity to telomerase assembly and telomere maintenance.

Results:

***S. pombe* Bin3 is essential and interacts with the U6 snRNA and the telomerase RNA TER1**

While Bin3/MePCE is conserved in many eukaryotes, its presence in the fission yeast *S. pombe*, an organism lacking 7SK snRNA^{24,29}, is not well understood. Unlike the longer Bin3/MePCE homologs from higher eukaryotes, *S. pombe* Bin3 only contains a conserved methyltransferase/SAM-binding domain. Alignment of the methyltransferase domain of *S.*

pombe Bin3 to that of *H. sapiens* MePCE and *D. melanogaster* Bin3 reveals that previously identified residues critical for SAM and SAH binding and nucleotide binding³⁰ are highly conserved in *S. pombe* (Figure S1A,B). To validate genome-wide deletion screens identifying Bin3 as an essential fission yeast protein^{27,28}, we ectopically expressed GST-tagged Bin3 cDNA on the thiamine repressible pRep4x plasmid in a wild type strain and subsequently knocked out the chromosomal copy (Figure S1C). Counter-selection of the Bin3-expressing plasmid on 5'FOA resulted in lethality of the *bin3Δ* strain, with no phenotype for a wild type strain, confirming its essential nature. Furthermore, overexpression of GST-Bin3 in wild-type or *bin3Δ* cells (i.e. in the absence of thiamine) resulted in very slow growth, compared to normal growth when GST-Bin3 expression was repressed by addition of thiamine. Growth in the absence or presence of added thiamine had no effect on cells transformed with an empty vector (Figure S1D). These data thus suggest that in addition to Bin3 being essential, its overexpression is poorly tolerated in *S. pombe*.

We next sought to determine the RNA substrates of *S. pombe* Bin3 to better understand its role in the processing of fission yeast non-coding RNA(s). Following integration of a protein A (PrA) tag into the Bin3 genomic locus, we performed RIP-Seq and plotted Bin3 IP reads against the IP from an untagged strain, followed by validation of potential candidate substrates with northern blotting (Figure 1, S2, Table S1). Consistent with previous reports of MePCE interacting with U6 snRNA²³, U6 emerged as one of the most highly enriched Bin3-interacting RNA transcripts. *S. pombe* U6 is an unusual transcript in that it is transcribed by RNA Polymerase III yet contains an mRNA-type intron removed by the spliceosome^{31,32}. Alignment of RIP-Seq reads to the *S.*

pombe genome suggested that Bin3 interacts exclusively with the spliced form of U6. We confirmed this with northern blotting, demonstrating robust immunoprecipitation of mature U6 and no immunoprecipitation of the intron-containing precursor (Figure 1B). Chromatin immunoprecipitation studies suggest that 7SK and U6 snRNA may be co-transcriptionally modified by the human MePCE homolog³³, however our data are consistent with a model wherein *S. pombe* Bin3 interacts with U6 post-splicing.

Surprisingly, the *S. pombe* telomerase RNA TER1 was also highly enriched in Bin3 immunoprecipitates (Figure 1A, C). Given the previously established link between the human Bin3 homolog MePCE and human LARP7,²⁰ as well as the established link between the *S. pombe* LARP7 homolog Pof8 and the telomerase holoenzyme^{12–14}, we considered the possibility that Bin3 may also be part of the telomerase holoenzyme through an evolutionarily conserved interaction with LARP7 family members.

Bin3 interacts with the mature form of TER1

Since TER1 processing and maturation involves a spliceosome-catalyzed reaction⁸, we first set out to determine the processing state of Bin3-associated TER1 by sequencing the 5' and 3' ends through circularized rapid amplification of cDNA ends (Figure 2A). We also performed cRACE on TER1 immunoprecipitated by Pof8, the telomerase reverse transcriptase Trt1, and Lsm3, all known components of the mature TER1-containing active telomerase holoenzyme^{3,4,9,12–14}. All sequenced candidates had a discrete 5' end consistent with the reported 5' end^{3,4} and a similar

distribution of 3' ends ending immediately upstream the 5' splice site⁸, suggesting that Bin3 interacts with the full-length, 3' end-matured TER1 associated with the active telomerase holoenzyme. The heterogeneity observed at the 3' end of all candidates is likely the result of exonucleolytic nibbling prior to binding of the Lsm2-8 complex⁹. To investigate this further, we subjected TER1 RNA immunoprecipitated by Bin3, Trt1, Pof8 and Lsm3 to RNase H cleavage to generate shorter 5' and 3' fragments and compared fragment size by northern blot (Figure 2B). The sizes of the 5' and 3' ends of TER1 were similar across immunoprecipitates, further substantiating that Bin3 binds the mature form of TER1, rather than the intron-containing precursor.

Further TER1 processing in *S. pombe* involves the addition of a 5' trimethylguanosine (TMG) cap by Tgs1⁹. Since Bin3 homologs are normally associated with the formation of a γ -monomethyl phosphate cap, and to further understand when Bin3 interacts with TER1 with respect to TER1 processing, we examined the cap structure of Bin3-associated TER1 by immunoprecipitating RNA associated with Bin3 and subsequently re-immunoprecipitating this RNA with an anti-TMG antibody. The specificity of the anti-TMG antibody was confirmed by showing that it effectively enriched the TMG cap containing U5 snRNA over the TMG cap lacking U6 snRNA or an unrelated tRNA (Figure 2C). While Bin3-associated U6 snRNA was not immunoprecipitated by the anti-TMG antibody, in agreement with data demonstrating the presence of a γ -monomethyl phosphate cap³⁴, Bin3-associated TER1 RNA was effectively enriched by anti-TMG immunoprecipitation. These data are consistent with previous work demonstrating a TMG cap on TER1 in *S. pombe*, and also with Bin3 associating with TER1 following spliceosomal cleavage

and 5' TMG capping, similar to what has been posited for Pof8¹². This suggests that Bin3 interacts with the primary cohort of TER1 transcripts, and not a γ -monomethyl phosphate capped subset, and that TER1 is not a substrate for Bin3-catalyzed modification.

Bin3 interacts with components of the mature telomerase holoenzyme

Since our results indicate that Bin3 interacts with the mature form of TER1, we set out to confirm the presence of Bin3 in the mature *S. pombe* telomerase holoenzyme. We immunoprecipitated Bin3 and identified interacting proteins through mass spectrometry, followed by validation using co-immunoprecipitation (Figure 3). We identified Pof8, the Lsm2-8 complex, and Trt1 in Bin3 immunoprecipitates, all of which make up the catalytically active core telomerase holoenzyme^{9,12-14,35-37} (Table S2, S3). We also detected the RNase P and RNase MRP subunit Pop100 in Bin3 immunoprecipitations. Pop1, Pop6, and Pop7, subunits of RNase P and RNase MRP in budding yeast, have recently been identified as components of the budding yeast telomerase complex³⁸⁻⁴⁰, suggesting that the involvement of RNase P and RNase MRP subunits in telomere maintenance is evolutionarily conserved. The finding that Bin3 interacts with core components of the telomerase holoenzyme, and not the Sm complex nor the TMG capping enzyme Tgs1, suggests Bin3 does indeed associate with the active telomerase holoenzyme rather than a precursor. The involvement of Bin3 in the telomerase holoenzyme was further substantiated by gene ontology analysis to determine overrepresented biological processes and cellular components among the top 50 Bin3 interactors. Telomerase holoenzyme complex assembly and telomere maintenance via telomerase emerged as the top overrepresented biological processes (Figure 3A). Similarly, overrepresented cellular

components include the Lsm2-8 complex, the telomerase holoenzyme, and spliceosomal snRNPs (Table S4). We also repeated immunoprecipitations in the presence of benzonase to determine if these interactions are direct or mediated through a nucleic acid intermediate (Table S2). With the exception of Pof8, interactions between Bin3 and other components of the telomerase holoenzyme were lost, indicative of an interaction mediated by TER1.

We confirmed the interactions between Bin3 and Pof8, as well as Bin3 and Trt1, through co-immunoprecipitation (Figures 3B, 3C, S3). We also performed co-immunoprecipitations with benzonase, demonstrating that the interaction between Bin3 and Pof8 persists with benzonase treatment, while the Bin3-Trt1 interaction is lost (Figures 3B and 3C). The apparent nucleic acid-dependence of the interaction between Bin3 and the majority of the telomerase holoenzymes is reminiscent both of the budding yeast telomerase RNA TLC1, which acts as a scaffold to nucleate telomerase holoenzyme assembly⁴¹, as well as the MePCE/Bin3-containing 7SK snRNP⁴². The finding that Bin3 and Pof8 interact directly is in agreement with previous reports demonstrating a protein-protein interaction between MePCE and LARP7 in the context of the vertebrate 7SK snRNP^{33,42}, suggesting the direct interaction may be evolutionarily conserved.

The apparent immunodepletion of Trt1 during Bin3 immunoprecipitation (Figure 3C, see flowthrough lanes) indicated an absence of detectable Trt1-containing telomerase holoenzymes lacking Bin3, substantiating the idea that Bin3 is a stoichiometric component of the *S. pombe* telomerase holoenzyme. Consistent with this, benzonase treatment prior to Bin3 immunoprecipitation resulted in reappearance of Trt1 in the immunoprecipitation flowthrough.

On the other hand, affinity purification of Bin3 did not completely deplete Pof8 from the Bin3 immunoprecipitation flow through, consistent with either Bin3-independent functions for Pof8 or with possible dissociation of Pof8 from some Bin3-containing complexes (see U6, below) (Figure 3B).

Bin3 is recruited to the active telomerase holoenzyme through the LARP7 family protein Pof8

We then tested whether Bin3 was recruited to the telomerase holoenzyme through its interaction with Pof8, much like the human LARP7 homolog promotes MePCE recruitment to 7SK snRNA²¹. Immunoprecipitation of Bin3 in the context of a *pof8Δ* strain resulted in a complete loss of TER1 binding, providing evidence that the interaction between Bin3 and TER1 is dependent on an interaction between Bin3 and Pof8 and Pof8 and TER1 (Figure 4A).

Unexpectedly, the interaction between Bin3 and U6 was also lost in the *pof8Δ*. Recent findings indicate that human LARP7 binds U6 to guide post-transcriptional modification^{43,44}, so it is tempting to speculate that Pof8 may also mediate Bin3 binding to U6 in fission yeast. To address this, we immunoprecipitated Pof8 alongside Bin3 and other components of the telomerase holoenzyme and looked for U6 enrichment. We observed slight enrichment of U6 in Pof8-myc immunoprecipitates relative to immunoprecipitation using an untagged strain or the Trt1-myc tagged strain (Figure 4B). We did not see enrichment for the intron-containing precursor, suggesting that like Bin3, Pof8 interacts with the spliced form of U6. The slight enrichment of U6 with a Pof8 immunoprecipitation, compared to the more robust immunoprecipitation seen with Bin3 and Lsm3, suggests that Pof8 may transiently bind U6, perhaps serving to load Bin3 on U6. Since LARP7 binding has been reported to disrupt the

catalytic activity of MePCE³³, a transient Pof8-U6 interaction may be consistent with U6 receiving a γ -monomethyl phosphate cap, compared to TER1, where stable Pof8 binding may prevent Bin3 catalytic activity.

Knowing that Bin3 interacts with TER1 and components of the telomerase holoenzyme, we next set out to confirm whether Bin3 is part of the catalytically active telomerase holoenzyme. We performed a previously described *in vitro* telomerase assay that relies on the presence of the TER1- and Trt1-containing telomerase holoenzyme to extend an oligonucleotide resembling telomeric DNA¹². Bin3 immunoprecipitates extended the oligonucleotide in a similar or greater manner to Trt1 and Pof8 immunoprecipitates, as well as showed the same loss of activity upon RNase A treatment, supporting the idea that Bin3, much like Pof8, is a component of the active telomerase holoenzyme (Figure 4C). Consistent with our previous results, we also observed a loss of activity for Bin3 immunoprecipitated from a *pof8Δ* strain, providing another line of evidence that Bin3 is recruited to the telomerase holoenzyme through Pof8. Since Trt1 is only recruited to the telomerase holoenzyme following Pof8 and Lsm2-8 binding, coupled with the idea that the assay relies on the presence of a reverse transcriptase, these results are consistent with a model in which Bin3 is recruited to TER1 through its interaction with Pof8 and remains bound through subsequent holoenzyme assembly and the catalytic cycle (Figure 4D).

Evidence that Pof8 is required for telomerase holoenzyme assembly and maintenance of telomere length^{12–14}, as well as for Bin3 recruitment to telomerase, prompted the question of whether we could rescue the short telomere phenotype of a *pof8Δ* strain by overexpressing

Bin3. Consistent with a model in which Bin3 only assembles with the active telomerase holoenzyme through its interaction with Pof8, Bin3 overexpression in a *pof8Δ* strain was insufficient to rescue telomere shortening and the resultant chromosome fusions occurring after telomeres reach a critically short length¹² (Figure S4).

Bin3 negatively regulates TER1 accumulation and telomere lengthening

Following the finding that Bin3 is a component of the *S. pombe* telomerase holoenzyme, we wondered at the function of Bin3, and how it might interface with the stabilization and complex assembly roles previously described for Pof8^{12–15}. As Bin3 deletion is lethal and its overexpression results in a slow growth phenotype (Figure S1D), we varied Bin3 expression by expressing plasmid-encoded Bin3 under the *nmt1* promoter in *bin3Δ* cells grown in rich media containing thiamine. Under these conditions, we determined that thiamine-repressed, plasmid encoded Bin3 was expressed approximately two-fold lower than Bin3 endogenously expressed from the genomic locus (Figure 5A). Surprisingly, considering Pof8's role in TER1 stabilization, we found this lower expression of Bin3 relative to wild type cells resulted in increased steady-state levels of TER1 (Figure 5B). To determine the effects of Bin3 depletion on telomere length, we continually passaged *bin3Δ* strains in minimal media containing supplemented thiamine and visualized telomeres by southern blotting (Figure 5C). We observed a small but noticeable telomere lengthening relative to a wild type strain, as well as the disappearance of a lower band. Taken together, our results suggest that Bin3 may act as a negative regulator to limit TER1 levels and telomerase activity under wild type conditions, in contrast to the stabilizing roles of its binding partner Pof8.

Pof8-like proteins are associated with Bin3-like proteins in diverse fungal lineages

The interaction between Bin3 and Pof8, coupled with the conservation of such an interaction in other examined eukaryotes, led us to wonder at the extent of this interaction on a phylogenetic scale. While Bin3/MePCE and LARP7/Pof8 family members are ubiquitous in higher eukaryotes, their absence or presence is more varied in fungal lineages. We queried hundreds of representative fungal species for the presence of Bin3- and Pof8-like proteins and identified many species with a) both a Bin3 and Pof8 homolog, b) only a Bin3 homolog, or c) neither a Bin3 nor a Pof8 homolog (Figure 6, S5). Conversely, only four out of 472 examined species contained a Pof8-like protein but no Bin3-like protein, and in these species the Pof8 homolog was noted to have a shortened N-terminal domain that would lack the La module observed in other LARP7 family members, raising the possibility that members of this rare cohort may not be bona-fide LARP7 family members, at least as these are currently appreciated¹⁷. Based on the observed distribution, it seems likely that both genes were present in a fungi common ancestor, but Pof8 or both Bin3 and Pof8 were lost in certain lineages. Examination of the distribution of Bin3 and LARP7-family members in basal eukaryotic lineages reveals a similar pattern: Bin3 can be present without LARP7, but there are no instances where a LARP7-family member exists in a lineage lacking a Bin3 homolog (Table S9). Similarly, LARP7-family proteins are represented in Alveolates, Stramenopiles, Amoebozoa, Fungi, and Metazoans, while Bin3-family proteins are present in all eukaryotic lineages. Since the presence of Pof8/LARP7 is very highly linked to the presence of a Bin3 homolog, these data suggest that the function of LARP7

family members may be more intimately associated with the function of Bin3/MePCE than has been previously appreciated.

Discussion:

In this study, we have identified Bin3 as a novel component of the *S. pombe* telomerase holoenzyme. In addition to showing an interaction between Bin3 and U6 snRNA, we demonstrate that Bin3 interacts with the telomerase RNA TER1 and components of the active telomerase holoenzyme in a manner that is dependent on the presence of the LARP7 family protein Pof8. Together, our results indicating that Bin3 interacts with the mature, spliced form of TER1 and Bin3 immunoprecipitates possess telomerase activity *in vitro* strongly suggest that Bin3 is a constitutive component of the telomerase holoenzyme containing Trt1, Pof8, Lsm2-8, and Est1 scaffolded on TER1 (Figure 4D). The idea of Pof8 and Bin3 scaffolded on TER1 is reminiscent of LARP7 and MePCE in the 7SK snRNP, with Pof8/LARP7 recruiting Bin3/MePCE to the RNA substrate. Similar to our data showing Pof8 recruiting Bin3 to TER1 and the telomerase holoenzyme, LARP7 has also been demonstrated to recruit MePCE to the 7SK snRNP in human cells²¹. Thus, the protein-protein interaction and subsequent recruitment to RNA substrates by LARP7 appears evolutionarily conserved in fission yeast and higher eukaryotes. We also present data demonstrating that the presence of Pof8-like proteins is nearly universally correlated with the presence of Bin3-like proteins in fungi, suggesting that the Bin3-Pof8 interaction is highly conserved and more prevalent than previously anticipated. Since Pof8 is required to load Bin3 onto the telomerase RNA and U6 snRNA in fission yeast, this phylogenetic distribution raises

the question of whether Bin3 homologs in fungi lacking a LARP7 homolog have evolved a mechanism to bind RNA targets in a Pof8/LARP7 independent fashion.

Since TER1 undergoes several processing steps before assembly into the telomerase holoenzyme, we were able to assess TER1 processing state to determine when Bin3 interacts with TER1 with respect to the timing of TER1 maturation. We show that Bin3-associated TER1 has a mature 3' end, indicative of spliceosome-catalyzed end processing, and a 5' TMG cap. This is consistent with previously reported data on the timing of Pof8 binding; since our data indicates that the interaction between Bin3 and TER1 is dependent on Pof8, the most parsimonious explanation is that Pof8, and thus Bin3, bind TER1 following 3' maturation and TMG capping, preceding the binding of the Lsm2-8 complex¹². We show that the interaction between Bin3 and TER1 is dependent on Pof8, leading to the conclusion that Bin3 interacts with TER1 at the same stage as Pof8, resulting in the hierarchical assembly of the active telomerase holoenzyme.

The finding that Bin3-associated TER1 is immunoprecipitated by a TMG antibody argues against a catalytic role for Bin3 in TER1 processing. Our data, coupled with previous work characterizing the cap structure on the mature TER1 bound to Trt1^{3,4}, supports the idea that Bin3 has a capping-independent function in the telomerase holoenzyme. LARP7 binding to MePCE in the 7SK snRNP inhibits its capping activity; MePCE, in turn, promotes the interaction between 7SK and LARP7, thereby stabilizing the complex³³. In the *S. pombe* telomerase holoenzyme and U6 snRNP, we can hypothesize that Pof8 may also inhibit the methyltransferase activity of Bin3 by

binding to and obscuring the active site. This is consistent with our results suggesting a transient interaction between Pof8 and U6 that may function to load Bin3 on U6. In such a model, an interaction between Bin3 and Pof8 is necessary to recruit Bin3 to U6 and following dissociation of Pof8, the catalytic site of Bin3 would become available to cap U6.

Additionally, our work identifying Bin3 as a telomerase-associated protein brings to mind work identifying a box H/ACA motif at the 3' end of vertebrate telomerase RNA⁴⁵. Box H/ACA RNAs form an RNP complex with Dyskerin/Cbf5, Nop10, Nhp2, and Gar1 to guide noncoding RNA pseudouridylation^{46,47}. Although Dyskerin is a pseudouridine synthase, to date no evidence has pointed to a modification function for Dyskerin in the context of telomerase RNA. Rather, the Dyskerin-containing H/ACA complex has a structural role in telomerase biogenesis, with binding of the H/ACA RNP to telomerase RNA leading to a conformational change in the RNA that promotes 3' processing and maturation over exosome-mediated decay⁴⁸. This is reminiscent of the apparent catalytic independent role we observed for Bin3 in fission yeast telomerase, although our data point towards Bin3 having a role in limiting TER1 accumulation, perhaps as a negative regulator of telomere elongation.

Bin3 and Pof8 having seemingly opposing functions in TER1 maturation and the telomerase RNP may provide an explanation as to the viability of Pof8 knockout strains, while Bin3 knockouts are lethal. Assuming Bin3 functions in limiting telomerase activity by decreasing TER1 levels, a lack of Bin3 negative regulation may be detrimental, much like the shelterin component Tpz1 and the telomere cap complex subunit Stn1, both of which are negative regulators of

telomerase and essential proteins in *S. pombe*^{27,49}. In contrast, since Pof8 promotes telomerase activity, its deletion doesn't result in unchecked telomere lengthening. It remains to be found how exactly Bin3 limits TER1 accumulation but it is possible that Bin3 interfaces with decay pathways, such as the established exosome-mediated 3' decay pathway^{12,14} or an as yet undiscovered pathway analogous to the 5' decay of human telomerase RNA⁵⁰. Bin3 may therefore be important for maintaining telomere homeostasis by decreasing TER1 levels under conditions where cell cycle progression or telomere lengthening are unfavorable.

Another facet of this work builds on the idea that components of the telomerase holoenzyme are more conserved than previously appreciated. In addition to the ubiquitous telomerase reverse transcriptase, previous work has identified LARP7 family proteins in both ciliates and yeast^{10,12–14}, arguing a level of evolutionary conservation. Other recent findings implicate the human LARP7 homolog in telomere maintenance⁵¹, suggesting this conservation may extend to higher eukaryotes. We now also provide evidence indicating the presence of RNase P and RNase MRP subunits in fission yeast, much like the RNase P and RNase MRP subunits found in the budding yeast telomerase complex^{38–40}. The presence of RNase P and RNase MRP subunits in two divergent yeast species supports the importance of these factors in the function of a breadth of telomerase holoenzymes.

Our results indicating that Bin3 is part of the telomerase holoenzyme were quite unexpected, as Bin3 has been well-characterized in higher eukaryotes for its role in the processing, maturation, and protection of RNA Polymerase III transcripts^{23,33}. An intriguing idea involves

polymerase switching through telomerase RNA evolution. Ciliate telomerase RNA is transcribed by RNA Polymerase III and subsequently binds the LARP7 homolog p65 through the terminal polyuridylate stretch common to RNA Polymerase III transcripts^{10,52}. Since MePCE and LARP7 also associate with RNA Polymerase III transcripts, it is possible that the Pof8-bound RNA Polymerase II transcribed TER1, and the *S. pombe* telomerase RNP as a whole, represent an intermediate step in the evolution of telomerase RNA between ciliates and the RNA Polymerase II transcribed telomerase RNA of higher eukaryotes. Future work should investigate the presence of Bin3/MePCE homologs in the telomerase holoenzymes of other species, particularly in ciliates, which possess both an RNA Polymerase III transcribed telomerase RNA and a LARP7 homolog. Such findings will continue to develop emerging ideas regarding conservation in telomerase RNA processing and the composition of telomerase RNPs.

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419 **Author contributions:**

420 J.P. conceived of the study, designed, performed, and analyzed all experiments, and wrote the
421 first draft of the manuscript with input from all authors. M.E.B. and J-M.D. performed
422 phylogenetic analysis. J.G. and J.P. analyzed RNA-Seq data. M.A.B. conceived of the study,
423 designed experiments, supervised the project, and edited the manuscript.

424

425 **Materials and Methods:**

426 Strains, constructs, and growth media

427 Strains were grown in yeast extract with supplements (YES) or Edinburgh Minimal Media
428 (EMM), as indicated. Expression of genes under the control of the *nmt1* promoter was
429 repressed with the addition of 15 μ M thiamine. Tag integration and knockouts were generated
430 with a previously described PCR-based strategy and verified by PCR and western blotting⁵³
431 (primer sequences in Table S6). Protein A-tagged strains were generated according to⁵⁴.
432 Correct genotypes were selected on YES plates with the corresponding antibiotic (200 μ g/mL
433 G418, Sigma; 100 μ g/mL Nourseothricin, GoldBio; 100 μ g/mL phleomycin, Invivogen). Other
434 strains were created by mating and antibiotic selection. A list of strains is provided in Table S5.

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436 Native protein extract and immunoprecipitation

437 *S. pombe* cells were grown in YES at 30°C to mid-log phase, harvested, and subject to cryogenic
438 disruption using a mortar and pestle. Cell powder was lysed in 50 mM NaCl, 20 mM Hepes pH

7.4, 55 mM KOAc, 0.5% Triton X, 0.1% Tween-20, 0.2 mM PMSF, 1:100 Protease Inhibitor Cocktail (Thermo, 78430), and 0.004 U/ μ L RNase inhibitor (Invitrogen, AM2694). For Protein A-tagged strains, immunoprecipitation was carried out with Rabbit IgG-conjugated (MP-Biomedicals, SKU 085594) Dynabeads (Invitrogen, 14301) as described⁵⁵. Myc-tagged proteins were immunoprecipitated with Protein G Dynabeads (Invitrogen, 10003D) coated with anti-myc antibody (Sigma, M4439). Beads were washed 4 times with 400 μ L lysis buffer. For RNA immunoprecipitations, bound RNA was isolated by treatment of beads with 0.1% SDS and 0.2 mg/mL Proteinase K (Sigma, P2308) at 37°C for 30 minutes, followed by extraction with phenol: chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation.

RNA preparation and Northern blotting

Total RNA was isolated from native protein extracts by incubation with 0.5% SDS, 0.2 mg/mL proteinase K (Sigma, P2308), 20 mM Tris HCl pH 7.4, and 10 mM EDTA pH 8.0 for 15 minutes at 50°C, followed by phenol: chloroform extraction and ethanol precipitation. Northern blot analysis was performed as described using 8% TBE-urea polyacrylamide gels⁵⁶. Briefly, electrophoresed RNA was transferred to positively charged nylon membranes (Perkin Elmer, NEF988001) with the iBlot 2 system (Thermo, IB21001) and probed with ³²P γ -ATP-labeled DNA probes (probe sequences provided in Table S7). For RNase H digestion, RNA was incubated with 25 pmol RNase H probe (provided in Table S7) for 5 minutes at 65°C and slow cooled to 37°C, followed by digestion with 5 U RNase H (NEB, M02975) for 30 minutes at 37°C. The reaction was stopped with the addition of 25 μ M EDTA pH 8.0 and phenol: chloroform extraction and

ethanol precipitation. TMG-capped RNAs were isolated from RNase H-treated immunoprecipitates with an α -TMG agarose conjugate (Sigma, NA02A), according to ⁴.

qRT-PCR

1 μ g Turbo DNase-treated RNA was reverse transcribed with the iScript cDNA synthesis kit (BioRad, 1708890), treated with 0.5 μ L RNase cocktail (Invitrogen, AM2286), and diluted 1:10. cDNA was quantified using the SensiFAST SYBR No-Rox kit (Bioline, BIO-98005) and 1 μ M of each primer (primer sequences provided in Table S7). qPCR settings were as follows: 95°C for 10 minutes and 40 cycles consisting of 10 seconds at 95°C, 20 seconds at 60°C, and 20 seconds at 72°C, followed by melting curve analysis. *Bin3 mRNA* levels were normalized to *his1 mRNA* levels and subsequently subject to paired two-tailed student's *t*-tests (n=3 biological replicates, each with 6 technical replicates).

RNA Seq and RIP Seq

Libraries were constructed from immunoprecipitated RNA samples and rRNA-depleted total RNA samples by the TCAC Sequencing Facility at the Hospital for Sick Children in Toronto. Libraries were sequenced on an Illumina Hi-Seq 2500 for 50-bp single-end reads. Reads were mapped to the *S. pombe* ASM294v2 genome using kallisto ⁵⁷ using parameters –single – fragment-length=50 –sd=5 and subsequently visualized on IGV ⁵⁸. Reads were filtered to include transcripts containing 4 or more reads in at least one of the Bin3 immunoprecipitated replicates and TPM values were subject to median-normalization. Gaussian mixture modeling (GMM) was applied to calculate the probability of an interaction using log of odds ⁵⁹. Reads

were further filtered to exclude transcripts with a log of odds score less than 3.17. The log of the average of median-normalized TPM values from the Bin3 immunoprecipitates was plotted against that of the control immunoprecipitations.

Circularized Rapid Amplification of cDNA Ends (cRACE)

Immunoprecipitated RNA was treated with 2 U TURBO DNase (Invitrogen, AM2239) and dephosphorylated with 1 U calf intestinal alkaline phosphatase (NEB, M0290), followed by decapping and circularization with RNA 5' Pyrophosphohydrolase (NEB, M0356S) and T4 RNA Ligase 1 (NEB, M0204S). TER1 was amplified from circularized RNA with the OneStep RT-PCR Kit (Qiagen, 210210) (primer sequences in Table S7). cRACE products were cloned into the pGEM-T vector (Promega, A1360) and sequenced by the TCAG DNA Sequencing Facility at the Hospital for Sick Children in Toronto.

Mass spectrometry and GO analysis

Half of the native protein extract was pre-treated with 0.625 U/ μ L Benzonase (Millipore, E1014) at 37°C for 30 minutes. The reaction was stopped with 5 mM EDTA pH 8.0 and Protein A immunoprecipitation was performed in the same manner as for RNA immunoprecipitations. After washes with lysis buffer, beads were washed with 0.1 M NH_4OAc and 0.1 mM MgCl_2 and eluted with 0.5 M NH_4OH for 20 minutes at room temperature. Eluates were lyophilized and subject to in-solution tryptic digestion. LC-MS/MS analysis was performed by the IRCM Proteomics Discovery Platform on a Q Exactive HF.

Raw data was processed and analyzed using the MaxQuant software package 1.5.1.2⁶⁰ and the fission yeast UP000002485 reference proteome (24/11/2019). Settings used for MaxQuant analysis were as reported⁶¹. Proteins present in immunoprecipitations from both biological replicates and absent from control immunoprecipitations were considered genuine Bin3 interacting partners and ranked by peak intensity. PANTHER overrepresentation tests (GO Ontology database released 2019-12-09) were conducted on the top 50 Bin3 interacting partners. Fisher's exact tests with FDR correction were performed using the GO biological process complete and GO cellular component complete data sets.

Co-immunoprecipitation and western blotting

Cells were harvested at mid-log phase, resuspended in lysis buffer (10 mM Tris HCl pH 7.5, 150 mM NaCl, 0.5% NP40, 1:100 Protease Inhibitor Cocktail), and lysed with acid-washed glass beads (Sigma, G8772). Immunoprecipitations were carried out as described and proteins were eluted by resuspending beads in 2X SDS loading buffer (100 mM Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue) and boiling at 95°C for 5 minutes. Western blot analyses were performed using monoclonal anti-myc (Cell Signaling, 2276S) at 1:5000 and monoclonal anti-beta actin (abcam, ab8226) at 1:2500 for primary antibodies and HRP-conjugated anti-mouse at 1:5000 for secondary antibodies. Protein A-tagged proteins were detected with HRP-conjugated polyclonal anti-Protein A (Invitrogen, PA1-26853) at 1:5000.

Telomerase activity assay

The telomerase activity assay was performed as described¹². Briefly, bead slurries (see immunoprecipitation) were incubated at 30°C for 90 minutes in a 10 µL reaction containing 50 mM Tris HCl pH 8.0, 1 mM MgOAc, 5% glycerol, 1 mM spermidine, 1 mM DTT, 100 mM KOAc, 0.2 mM dATP, dCTP, and dTTP, 5 µM telomerase assay primer (Table S7), and 0.3 µM 3000 Ci/mmol [α -³²P] dGTP (Perkin Elmer, BLU512H250UC). The reaction was stopped with the addition of 0.5% SDS, 10 mM EDTA pH 8.0, 20 mM Tris HCl pH 7.5, 2 µg/µL Proteinase K (Sigma), and 10000 cpm [γ -³²P] ATP-labeled 100-mer oligonucleotide and incubation at 42°C for 15 minutes, followed by phenol: chloroform extraction and ethanol precipitation. RNase A-treated samples were pre-incubated with 20 ng RNase A (Invitrogen, AM2271) at 30°C for 10 minutes. Extension products were separated on a 10% urea-TBE polyacrylamide sequencing gel at 60 W for 90 minutes, dried and exposed to a PhosphorScreen, and imaged with a Typhoon imager.

Genomic DNA extraction, southern blotting, and chromosome fusion PCR

Genomic DNA was extracted from logarithmically growing cells according to¹³. For southern blotting, 15 µg genomic DNA was digested overnight with EcoRI and separated on a 1% agarose gel, transferred to positively charged nylon membranes (Perkin Elmer, NEF988001) by capillary transfer in 10X SSC, and probed with a ³²P γ -ATP-labeled telomere probe (probe sequence provided in Table S7). The chromosome fusion PCR was adapted from¹². Briefly, 50 µL PCR reactions contained 1 µL genomic DNA, 0.4 µM forward and reverse primers (sequences provided in Table S7), 200 µM dNTPs, 1X Taq ThermoPol Buffer (NEB), and 1.25 U Taq Polymerase (NEB, M0273). The Trt1 gene was amplified as a loading control in an identical PCR

reaction containing the corresponding primers. The PCR reaction consisted of an initial denaturation at 95°C for 5 minutes, followed by 32 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 68°C for 3 minutes, with a final extension at 68°C for 10 minutes.

Structure prediction

The structure of *S. pombe* Bin3 was predicted using Phyre2⁶² and aligned with the co-crystal structure of the *H. sapiens* MePCE methyltransferase domain bound to 7SK snRNA and SAH (6DCB³⁰) in PyMol⁶³. Primary sequences were aligned with the Clustal Omega Multiple Sequence Alignment tool⁶⁴.

Phylogenetic tree construction and search for Bin3-like and Pof8-like sequences

Protein sequences (GeneCatalogs) of 472 fungal species were downloaded from the JGI website (<https://mycocosm.jgi.doe.gov/>) in fasta format. 5614520 proteins were downloaded and merged into a single multifasta file. A blast (Blastp) against all proteins was performed using diamond blastp⁶⁵ with a minimum e-value of 1e-5 and outfmt 6 (for tabular output). In order to identify homologous proteins, proteins were clustered together if they shared at least 50% similarity over 70% of coverage using SiLiX⁶⁶. When a cluster contained two or more paralogous genes derived from duplications, the species was removed from the cluster. Finally, only clusters with a minimum of 100 species were retained for further analysis. Following these cleaning steps, we obtained 5328 clusters containing one gene per species. Proteins sequences of each cluster were aligned using MAFFT (v7.271) with default parameters (<https://mafft.cbrc.jp/alignment/server/>). Multiple alignments were cleaned using trimA1

(v1.4.rev22 build [2015-05-21]) with -st 0.01 option. Fasttree (version 2.1.10 SSE3) with default parameters were used to build phylogenetic trees⁶⁷. The consensus fungi tree was obtained using ASTRAL (astral.5.6.3) software with default parameters⁶⁸. To detect the presence of genes coding for Bin3-like and Pof8-like proteins, blast searches (blastp and tbalstn) were performed on each studied fungus genome, using initially either the full-length *S. pombe* Bin3 protein sequence or the *S. pombe* Pof8 xRRM conserved motif. This procedure was repeated in each fungal clade using clade-specific Bin3-like and Pof8-like sequences detected by the initial search using *S. pombe* sequences. Finally, new blast searches were performed all over again, this time using Bin3-like or Pof8-like “probes” made of a collection of sequences representing the diversity of these two proteins in fungi.

Data availability

Mass spectrometry proteomics and RNA Seq data will be supplied prior to publication.

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774 **Figure Legends:**

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776 **Figure 1: Bin3 interacts with U6 snRNA and the telomerase RNA TER1**

777 A) Scatterplot of normalized and log-transformed Control IP TPM against Bin3 IP TPM. Bin3
778 targets of interest are indicated.

779 B) Northern blot analysis of the mature and intron-containing U6 from total RNA and PrA
780 immunoprecipitates from untagged (wild type, wt) and PrA-tagged Bin3 strains.

781 C) Same as (B), for the telomerase RNA TER1.

782

783 **Figure 2: Bin3 interacts with the same TER1 species as well-established components of the**
784 **telomerase holoenzyme**

785 A) The 5' and 3' ends of Bin3-, Trt1-, Pof8-, and Lsm3-associated TER1 were identified by cRACE.
786 The results of 10 independent clones per immunoprecipitation are shown below a schematic of
787 the architecture of TER1.

B) RNase H northern blots of RNase H generated 5' and 3' ends of TER1 immunoprecipitated by various telomerase components.

C-D) Northern blot of α -TMG flow through (FT) and immunoprecipitated (IP) transcripts from total RNA (C) and Bin3-associated transcripts (D).

Figure 3: Bin3 interacts with components of the mature telomerase holoenzyme

A) Gene ontology analysis (biological process) of top 50 Bin3 protein interactors.

B-C) Examination and nucleic acid-dependence of interactions between Bin3 and Pof8 (B) and Bin3 and Trt1 (C) by co-immunoprecipitation and western blotting. Blots were reprobed for beta actin as a loading control.

Figure 4: Bin3 is recruited to the active telomerase holoenzyme by Pof8

A) Northern blot of TER1 and U6 in PrA immunoprecipitates for an untagged wild type (wt), PrA-tagged (Bin3 PrA), and PrA-tagged Pof8 knockout strain (Bin3 PrA Δ Pof8). Bin3-PrA was detected in input and immunoprecipitated samples by western blots probing for PrA (bottom panel). Possible cleavage products are indicated with an asterisk.

B) Northern blot of mature and intron-containing U6 in PrA- and myc-tagged immunoprecipitated RNA.

C) Telomerase assay of myc- (left) and PrA-tagged (right) proteins. A 32 P-labeled 100-mer oligonucleotide was used as a loading control. Telomerase extension products were compared to a terminal transferase ladder, with +1 and +6 extension products indicated.

D) Proposed model of the fission yeast telomerase holoenzyme. TER1 structure and binding locations are based on models constructed by Hu and colleagues¹⁵ and Mennie and colleagues¹³.

Figure 5: Bin3 depletion results in an increase in TER1 levels and telomere length

A) qRT-PCR of Bin3 mRNA normalized to his1 mRNA (mean $\pm$ standard error, paired *t* test * at $p > 0.05$, ** at $p < 0.01$, *** at $p < 0.001$, and **** at $p < 0.0001$).

B) qRT-PCR and a representative northern blot of TER1 from total cellular RNA (mean $\pm$ standard error, paired *t* test * at $p > 0.05$, ** at $p < 0.01$, *** at $p < 0.001$, and **** at $p < 0.0001$).

C) Southern blot comparing telomere length in wild type, *pof8Δ*, and *bin3Δ* strains transformed with an empty vector or GST-Bin3 following 2 re-streaks on minimal media with or without 15 μ M thiamine (B1), as indicated.

Figure 6: Phylogenic distribution of Bin3 and Pof8 in fungi

Consensus cladogram describing the phylogenic relationships of 472 species representative of fungi phylum and classes and highlighting (using a color code) the distribution of Bin3 and Pof8 in these species. The cladogram is a consensus tree of 5328 distinct protein coding gene trees resulting from a genome-wide, against all, protein comparison (see Materials and Methods).

Only posterior probabilities inferior to 1 are shown. The Bin3 and Pof8 distribution is recapitulated in Supplemental Table 8 with corresponding protein sequences. A cartoon presenting structural domains of Pof8 is presented. Pof8 La module and RRM1 are only inferred

from secondary structure predictions and are represented by *La** and *RRM1** in the corresponding cartoon. In four species (colored in orange in the cladogram) only an N-terminal truncated version of Pof8 (Pof8*) that cannot accommodate the La module is present. Full cladogram with species names and statistical supports of the different nodes is presented in Figure S5.

Supplementary figure captions:

Supplementary figure 1: Bin3 is the essential *S. pombe* methyl phosphate capping enzyme (MePCE) homolog

A) Clustal alignment of the methyltransferase/SAM-binding domains of *S. pombe* Bin3, *H. sapiens* MePCE, and *D. melanogaster* Bin3. SAM-binding residues are indicated in red and nucleotide-binding residues are in blue.

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C) Wild type and Bin3 knockout strains transformed with pRep4x GST-Bin3 grown on minimal media (EMM-ura) (left) and counter-selected on minimal media with 5'-fluoroorotic acid (right).

D) Bin3 overexpression results in a slow growth phenotype that is partially rescued with the addition of thiamine (B1). 10-fold serial dilutions were plated on EMM-ura and grown at 32°C.

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A

B

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Figure 1: Bin3 interacts with U6 snRNA and the telomerase RNA TER1

A) Scatterplot of normalized and log-transformed Control IP TPM against Bin3 IP TPM. Bin3 targets of interest are indicated.

B) Northern blot analysis of the mature and intron-containing U6 from total RNA and PrA immunoprecipitates from untagged (wild type, wt) and PrA-tagged Bin3 strains.

C) Same as (B), for the telomerase RNA TER1.

A

B

C

D

Figure 2: Bin3 interacts with the same TER1 species as well-established components of the telomerase holoenzyme

A) The 5' and 3' ends of Bin3-, Trt1-, Pof8-, and Lsm3-associated TER1 were identified by cRACE. The results of 10 independent clones per immunoprecipitation are shown below a schematic of the architecture of TER1.

B) RNase H northern blots of RNase H generated 5' and 3' ends of TER1 immunoprecipitated by various telomerase components.

C-D) Northern blot of a-TMG flow through (FT) and immunoprecipitated (IP) transcripts from total RNA (C) and Bin3-associated transcripts (D).

A

GO biological process	Fold enrichment	Raw P value	False discovery rate
Telomerase holoenzyme complex assembly	49.39	2.04E-08	5.33E-05
Telomere maintenance via telomerase	31.75	2.54E-04	4.74E-02
RNA-dependent DNA biosynthetic process	31.75	2.54E-04	4.58E-02
Ribonucleoprotein complex assembly	5.47	6.70E-06	2.50E-03
Ribonucleoprotein complex subunit organization	5.29	9.01E-06	2.77E-03
mRNA cis splicing, via spliceosome	5.25	6.76E-05	1.54E-02
mRNA splicing, via spliceosome	4.78	5.48E-05	1.43E-02
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	4.78	5.48E-05	1.43E-02
RNA splicing, via transesterification reactions	4.72	6.08E-05	1.45E-02
RNA splicing	4.52	8.60E-05	1.80E-02

B

C

Figure 3: Bin3 interacts with components of the mature telomerase holoenzyme

A) Gene ontology analysis (biological process) of top 50 Bin3 protein interactors.

B-C) Examination and nucleic acid-dependence of interactions between Bin3 and Pof8 (B) and Bin3 and Trt1 (C) by co-immunoprecipitation and western blotting. Blots were reprobed for beta actin as a loading control.

Figure 4: Bin3 is recruited to the active telomerase holoenzyme by Pof8

A) Northern blot of TER1 and U6 in PrA immunoprecipitates for an untagged wild type (wt), PrA-tagged (Bin3 PrA), and PrA-tagged Pof8 knockout strain (Bin3 PrA *pof8Δ*). Bin3-PrA was detected in input and immunoprecipitated samples by western blots probing for PrA (bottom panel). Possible cleavage products are indicated with an asterisk.

B) Northern blot of mature and intron-containing U6 in PrA- and myc-tagged immunoprecipitated RNA.

C) Telomerase assay of myc- (left) and PrA-tagged (right) proteins. A ³²P-labeled 100-mer oligonucleotide was used as a loading control. Telomerase extension products were compared to a terminal transferase ladder, with +1 and +6 extension products indicated.

D) Proposed model of the fission yeast telomerase holoenzyme. TER1 structure and binding locations are based on models constructed by Hu and colleagues¹⁵ and Mennie and colleagues¹³.

A

B

C

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Chromosome fusion PCR (top) from wild type and *pof8Δ* strains overexpressing the pRep4x empty vector or pRep4x Bin3. The Trt1 gene was amplified from genomic DNA as a loading control (bottom).

- Bin3-like and Pof8-like
- Bin3-like only
- Pof8-like only (shorter version)*
- None of the two

Supplementary figure 5: Phylogenetic distribution of Bin3 and Pof8 in fungi

Complete consensus cladogram describing the phylogenetic relationships of 472 species representative of fungi phylum and classes and highlighting (using a color code) the distribution of Bin3 and Pof8 in these species. Only posterior probabilities inferior to 1 are shown.

List of supplementary tables:

Supplementary table 1: Bin3-associated RNAs were detected by RIP-Seq (attached as spreadsheet)

Transcripts with 4 or more reads in at least one of the replicates, as well as a minimum log of odds value of 3.17, are presented.

Supplementary table 2: Results of Bin3-immunoprecipitated proteins from 2 independent mass spectrometry experiments

Proteins present exclusively in both replicates and absent from control immunoprecipitates were considered. The top 11 interacting proteins are presented, ranked by total peak intensity. Interactions persisting in the presence of benzonase are indicated. Experimentally validated and speculative components of the *S. pombe* telomerase holoenzyme are shaded.

Supplementary table 3: Top 50 Bin3-associated proteins used for GO analyses.

Supplementary table 4: Gene ontology analysis (cellular component) of the top 50 Bin3 protein interactors

Supplementary table 5: *S. pombe* strains used in this study

Supplementary Table 6: Primers used to generate tagged strains

Supplementary table 7: probe sequences

Supplementary Table 8: Distribution of Bin3 and Pof8 in the 472 species used to construct the cladogram of Figure 6 and corresponding protein sequences. (attached as spreadsheet)

Supplementary Table 9: Distribution of Bin3 and Pof8 in basal eukaryotic lineages. Presence of a Bin3- or LARP7-family member= Y, absence= N.

Supplementary Table 2

Protein	Gene	Bin3 PrA IP Replicate 1	Bin3 PrA IP Replicate 2	Bin3 PrA IP + benzonase
LARP7 family protein Pof8	pof8	24	24	22
Vigilin KH domain RNA binding protein	vgl1	17	14	20
Uncharacterized RNA binding protein	SPBC1861	16	24	0
RNase P and RNase MRP subunit Pop100	pop100	16	17	0
Telomerase reverse transcriptase Trt1	trt1	13	12	0
Bin3 family protein (bait)	SPBC2A9	12	12	11
WD repeat/ BOP1NT protein Erb1	erb1	9	9	1
Uncharacterized protein	SPCC18B5	7	7	7
Sec24/Sfb2 subunit	sec24	7	3	0
Telomerase regulator Est1	est1	6	9	0
Lsm2-8 complex subunit Lsm6	lsm6	6	6	0

Supplementary Table 3

Protein	Gene
LARP7 family protein Pof8	pof8
Bin3 family protein (bait)	SPBC2A9
Uncharacterized protein	SPCC18B5
Lsm2-8 complex subunit Lsm8	lsm8
Lsm2-8 complex subunit Lsm7	lsm7
Lsm2-8 complex subunit Lsm2	lsm2
Lsm2-8 complex subunit Lsm6	lsm6
Lsm2-8 complex subunit Lsm5	lsm5
Threonine tRNA ligase	ths1
RNase P and RNase MRP subunit Pop100	pop100
Periodic tryptophan protein 2 homolog	SPBC713

Vigilin KH domain RNA binding protein	vgl1
Uncharacterized RNA binding protein	SPBC1861
Putative glycine tRNA ligase	grs1
Small nuclear ribonucleoprotein-associated protein B	smb1
Pre-mRNA splicing factor Prp12	Prp1
Probable mitochondrial import receptor subunit Tom70	tom70
Nucleic acid binding protein homolog	byr3
Telomerase reverse transcriptase Trt1	trt1
Ribosome biogenesis protein Brx1	brx1
Ribosome biogenesis protein Erb1	erb1
Probable ATP-citrate synthase subunit 2	SPAC22A12

mRNA export factor Elf1	elf1
ATP synthase beta subunit 2	atp2
Sm snRNP core protein Smf1	smf1
Mitochondrial translation elongation factor EF-Tu	tuf1
CUE domain-containing protein	SPBC354
Putative G3BP-like protein	nxt3
Prp19 complex subunit	prp19
Vacuolar carboxypeptidase Y	cpy1
RNA binding protein Nop12	nop12
Putative mitochondrial ribosome recycling factor	tif451
U3 snoRNP-associated protein Utp7	utp7

Chaperonin-containing T-complex eta subunit	cct7
Telomerase regulator Est1	est1
BAR adaptor protein Hob3	hob3
Uncharacterized RNA binding protein	SPCC1827
RNA polymerase I upstream activation factor complex subunit Spp27	spp27
Uncharacterized RNA binding protein	SPBC4F6
Putative leucine-tRNA ligase Lrs1	lrs1
U5 snRNP complex subunit Spp42	spp42
DNA Polymerase V	pol5
Ribosome assembly factor, WD repeat protein Nsa1	nsa1
Translation initiation factor eIF2B epsilon subunit	tif225

26S protease regulatory subunit 8 homolog

let1

Supplementary Table 4

GO cellular component	Fold enrichment	Raw P value	False discovery rate
Lsm2-8 complex	42.34	8.86E-06	1.23E-03
Telomere holoenzyme complex	41.16	6.60E-07	2.44E-04
U6 snRNP	37.04	1.32E-05	1.63E-03
Spliceosomal tri-snRNP complex	15.88	4.49E-06	9.97E-04
U4/U6 x U5 tri-snRNP complex	15.88	4.49E-06	8.31E-04
Catalytic step 2 spliceosome	10.01	2.10E-04	1.46E-02
Spliceosomal snRNP complex	8.50	2.78E-05	2.80E-03
Small nuclear ribonucleoprotein complex	8.10	3.69E-05	3.42E-03
Sm-like protein family complex	7.98	4.05E-05	3.46E-03
Spliceosomal complex	6.12	7.25E-06	1.15E-03

Supplementary Table 5

Figure	Strain	Description	Full genotype	Source
1, Table S1	FY6843	wild type	<i>h- ura4-D18</i>	National BioResource Project
	yJP001	<i>bin3-PrA</i>	<i>h- ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
S1	yAS99	wild type	<i>h- leu1-32 ura4-D18 ade6-704</i>	Intine <i>et al.</i> , 2000 ⁷⁰
	yJP002	<i>bin3Δ + pRep4x GST-Bin3</i>	<i>h- leu1-32 ura4-D18 ade6-704 bin3Δ::natMX6 pRep4x GST-Bin3</i>	This study
2a, b	FY6843	wild type	<i>h- ura4-D18</i>	National BioResource Project
	yJP001	<i>bin3-PrA</i>	<i>h- ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
	TN7706	<i>trt1-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1 trt1⁺::G8-13myc-natMX6</i>	Mennie <i>et al.</i> , 2018
	AM16932	<i>pof8-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1 pof8⁺::13myc-kanMX6</i>	Mennie <i>et al.</i> , 2018
	AM17121	<i>lsm3-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1 lsm3⁺::13myc-kanMX6</i>	Mennie <i>et al.</i> , 2018
2c	FY6843	wild type	<i>h- ura4-D18</i>	National BioResource Project
	yJP001	<i>bin3-PrA</i>	<i>h- ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
3a, Table S2, Table S3, Table S4	FY6843	wild type	<i>h- ura4-D18</i>	National BioResource Project
	yJP001	<i>bin3-PrA</i>	<i>h- ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
3b, S3	yJP001	<i>bin3-PrA</i>	<i>h- ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
	yJP001a	<i>bin3-PrA</i>	<i>h+ ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
	yJP001b	<i>bin3-PrA</i>	<i>h+ ura4-D18 bin3⁺::bin3-PrA-bleMX6</i>	This study
	AM16932	<i>pof8-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1 pof8⁺::13myc-kanMX6</i>	Mennie <i>et al.</i> , 2018

3c	yJP012	<i>bin3-PrA</i> <i>pof8-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>bin3⁺::bin3-PrA-bleMX6</i> <i>pof8⁺::13myc-kanMX6</i>	This study
	yJP001	<i>bin3-PrA</i>	<i>h- ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
	yJP001a	<i>bin3-PrA</i>	<i>h+ ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
	yJP001b	<i>bin3-PrA</i>	<i>h+ ura4-D18 bin3⁺::bin3-PrA-bleMX6</i>	This study
	TN7706	<i>trt1-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>trt1⁺::G8-13myc-natMX6</i>	Mennie <i>et al.</i> , 2018
4a	yJP013	<i>bin3-PrA</i> <i>trt1-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>bin3⁺::bin3-PrA-bleMX6</i> <i>trt1⁺::G8-13myc-natMX6</i>	This study
	FY6843	wild type	<i>h- ura4-D18</i>	National BioResource Project
	yJP001	<i>bin3-PrA</i>	<i>h- ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
	yJP001a	<i>bin3-PrA</i>	<i>h+ ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
	yJP001b	<i>bin3-PrA</i>	<i>h+ ura4-D18 bin3⁺::bin3-PrA-bleMX6</i>	This study
4b	TN12118	<i>pof8Δ</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>pof8Δ::kanMX6</i>	Mennie <i>et al.</i> , 2018
	TN12118a	<i>pof8Δ</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>pof8Δ::natMX6</i>	This study
	yJP011	<i>bin3-PrA</i> <i>pof8Δ</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>bin3⁺::bin3-PrA-bleMX6</i> <i>pof8Δ::natMX6</i>	This study
	FY6843	wild type	<i>h- ura4-D18</i>	National BioResource Project
	yJP001	<i>bin3-PrA</i>	<i>h- ura4-D18 bin3⁺::bin3-PrA-kanMX6</i>	This study
	TN7706	<i>trt1-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>trt1⁺::G8-13myc-natMX6</i>	Mennie <i>et al.</i> , 2018
	AM16932	<i>pof8-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>pof8⁺::13myc-kanMX6</i>	Mennie <i>et al.</i> , 2018

4c	AM17121	<i>lsm3-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>lsm3⁺::13myc-kanMX6</i>	Mennie <i>et al.</i> , 2018
	TN7706	<i>trt1-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>trt1⁺::G8-13myc-natMX6</i>	Mennie <i>et al.</i> , 2018
	AM16932	<i>pof8-myc</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>pof8⁺::13myc-kanMX6</i>	Mennie <i>et al.</i> , 2018
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	yJP001a	<i>bin3-PrA</i>	<i>h+ ura4-D18 bin3⁺::bin3-PrA- kanMX6</i>	This study
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	TN12118	<i>pof8Δ</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>pof8Δ::kanMX6</i>	Mennie <i>et al.</i> , 2018
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5a, 5b, 5c	yAS99	wild type	<i>h- leu1-32 ura4-D18 ade6-704</i>	Intine <i>et al.</i> , 2000
	TN12118	<i>pof8Δ</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>pof8Δ::kanMX6</i>	Mennie <i>et al.</i> , 2018
	yJP002	<i>BinΔ3 +</i> <i>pRep4x GST-</i> <i>Bin3</i>	<i>h- leu1-32 ura4-D18 ade6-704</i> <i>bin3Δ::natMX6 pRep4x GST-Bin3</i>	This study
S4	FY6843	wild type	<i>h- ura4-D18</i>	National BioResource Project
	TN12118	<i>pof8Δ</i>	<i>h- leu1-32 ura4-D18 his3-D1</i> <i>pof8Δ::kanMX6</i>	Mennie <i>et al.</i> , 2018

Supplementary Table 6

Primer	Sequence
Bin3 PrA For (tag integration)	5'CATTTGCTTAATCAAGCAGGACTAGTGCTTGAATACAGTATCGAACCTCA AGTAAATAACTCTGAGTACAAGAATTTTGCCAAACGAACAATGTATAAAAA AAAAGGAATTGGAATCATAAAACCTATTAACCTATTAACCTCTACTCGTACGCT GCAGGTCGAC 3'
Bin3 PrA Rev (tag integration)	5'CTACTTCATTCCGCTTTTCTTTCTTTCCTCATATGTTGAATTAGAGAATTTAAT AAAAAATGAGACACGTCCTTTTGGGACATTATGCTTTATAGTTCCAGTCATTGT CTATTCTTAACGTTTACTAGACAAATCGATGAATTCGATGAATTCGAGCTCG 3'
MX6 For (antibiotic marker swap)	5' GACATGGAGGCCCAAGAATAC 3'
MX6 Rev (antibiotic marker swap)	5' TGGATGCGCGCGTTAGTATC 3'
Bin3 KO For (Bin3 gene disruption)	5'TTCGATTCGTAAGTTTAAATTCCATTGCAAAATTTATACTTTTATAAAAAATA GGGGTGATTTGAAAAACCTCCTCGACTTGGGCGGATCCCCGGGTAAATTA 3'
Bin3 KO Rev (Bin3 gene disruption)	5'GAGAATTTAATAAAAAATGAGACACGTCCTTTTGGGACATTATGCTTTATA GTTCCAGTCATTGTCTATTCTTAACGTTTACTAGACAAGAATTCGAGCTCGTT TAAAC 3'

Supplementary Table 7

Figure	Probe	Sequence
1c, 1d, 2b, 3c, 2d, 4a, 4b	U6	5' GTGTCATCCTTGTGCAGGGGCCAT 3'
1c, 4b	U6 intron	5' CAACGAGTTAGTATGACTCGAACCTTGG 3'
1d	Ter1	5' CCCGTTCTTTTAACATTTTGCTCAGACCAAGTG 5'
1d, 2c, 5b	U5	5' CTGGTAAAAGGCAAGAAACAGATACG 3'
2a	Ter1 cRACE For	5' TCTTGAGCGCGTTTTAGGTTTTTTTCACTT 3'
	Ter1 cRACE Rev	5' AACACGGACGAGCTACACTCAAATATTA 3'
2b, 4a, 4b, 5b	Ter1 3' RNase H	5' GGGTAGTCAACTATTTTACTAGCG 3'
	Ter1 5' RNase H	5' TTTCAAATCAATCACCAAGCCC 3'
	Ter1 3' RNase H northern	5' GCTCAGACCAAGTGAAAAAAACC 3'
	Ter1 5' RNase H northern	5' TCCTTGTC AACCTTCTAAGCATG 3'
2c	tRNA Thr ^{CGU}	5' TGCTCCGGCAGTGAAGTCAACA 3'
4c	Telomerase assay primer	5' GTTACGGTTACAGGTTACG 3'
5a	Bin3 mRNA 5' qPCR	5' GAGGAACCTCAATTATCGATCCAAGACTTAAATGTTTGC 3'
	Bin3 mRNA 3' qPCR	5' TCGAGCTTTTTGGATGAGAACATGATCGATATCCAATCC 3'
	His1 5' qPCR	5' TGTCCACCTCGGAATCACTG 3'
	His1 3' qPCR	5' CGAAGACGTGCTTCAGCGA 3'
5b	Ter1 5' qPCR	CAGTGTACGTGAGTCTTCTGCCTT
	Ter1 3' qPCR	CAAAAATTCGTTGTGATCTGACAAG
5c	Telomere southern probe	5' GGGTTACATGTTACAGGTTACA 3'
S3	Chromosome fusion For	5' GGGTTGCAAAGTATGATTGTGGTAA 3'
	Chromosome fusion Rev	5' TGTTGAATGTCAGAACCAACTGTTGCAT 3'
	Trt1 For	5' GCAAAGAAGTTTCCTGGAATAGC 3'
	Trt1 Rev	5' GATGTAATAAAGGGTCGGCAC 3'

Supplementary Table 9

Species	Classification	BIN3	LARP7
<i>Fragilariopsis cylindrus</i>	Stramenopiles, Bacillariophyta	N	N
<i>Pseudo-nitzschia multiseriis</i>	Stramenopiles, Bacillariophyta	N	N
<i>Thalassiosira oceanica</i>	Stramenopiles, Bacillariophyta	N	N
<i>Thalassiosira pseudonana</i>	Stramenopiles, Bacillariophyta	N	N
<i>Ochromonadaceae</i> sp	Stramenopiles, Chrysophyta	Y	N
<i>Nannochloropsis gaditana</i>	Stramenopiles, Eustigmatophyceae	Y	N
<i>Nannochloropsis oceanica</i>	Stramenopiles, Eustigmatophyceae	Y	N
<i>Aplanochytrium kerguelense</i>	Stramenopiles, Labyrinthulomycetes	N	N
<i>Aurantiochytrium limacinum</i>	Stramenopiles, Labyrinthulomycetes	N	N
<i>Schizochytrium aggregatum</i>	Stramenopiles, Labyrinthulomycetes	N	N
<i>Achlya hypogyna</i>	Stramenopiles, Oomycota	Y	Y
<i>Aphanomyces euteiches</i>	Stramenopiles, Oomycota	Y	Y
<i>Aphanomyces astaci</i>	Stramenopiles, Oomycota	Y	Y
<i>Phytophthora cactorum</i>	Stramenopiles, Oomycota	y	y
<i>Phytophthora capsici</i>	Stramenopiles, Oomycota	y	y
<i>Phytophthora cinnamomi</i>	Stramenopiles, Oomycota	y	y
<i>Phytophthora infestans</i>	Stramenopiles, Oomycota	y	y
<i>Phytophthora sojae</i>	Stramenopiles, Oomycota	y	y
<i>Plasmopara halstedii</i>	Stramenopiles, Oomycota	y	y
<i>Pythium brassicum</i>	Stramenopiles, Oomycota	Y	Y
<i>Saprolegnia parasitica</i>	Stramenopiles, Oomycota	Y	Y
<i>Thraustotheca clavata</i>	Stramenopiles, Oomycota	Y	Y
<i>Blastocystis hominis</i>	Stramenopiles, Opalozoa	N	N
<i>Pelagophyceae</i> sp.	Stramenopiles, Pelagophyceae	Y	Y
<i>Ectocarpus siliculosus</i>	Stramenopiles, Phaeophyceae	Y	Y
<i>Tetrahymena thermophila</i>	Alveolates, Ciliophora	Y	Y
<i>Paramecium tetraurelia</i>	Alveolates, Ciliophora	Y	Y
<i>Pseudocohnilembus persalinus</i>	Alveolates, Ciliophora	Y	N
<i>Oxytricha trifallax</i>	Alveolates, Ciliophora	Y	Y
<i>Symbiodinium microadriaticum</i>	Alveolates, Dinophyta	Y	N
<i>Stylonychia lemnae</i>	Alveolates, Ciliophora	Y	Y
<i>Stentor coeruleus</i>	Alveolates, Ciliophora	Y	N
<i>Perkinsus marinus</i>	Alveolates, Perkinsea	Y	N
<i>Vitrella brassicaformis</i>	Alveolates, Chromorida	Y	N
<i>Gregarina niphandrodes</i>	Alveolates, Apicomplexa	N	N
<i>Cryptosporidium muris</i>	Alveolates, Apicomplexa	N	N
<i>Eimeria acervulina</i>	Alveolates, Apicomplexa	Y	N

Toxoplasma gondii	Alveolates, Apicomplexa	Y	N
Neospora caninum	Alveolates, Apicomplexa	Y	N
Cyclospora cayetanensis	Alveolates, Apicomplexa	N	N
Cystoisospora suis	Alveolates, Apicomplexa	Y	N
Plasmodiophora brassicae	Rhizaria, Endomixa	Y	N
Trypanosoma brucei	Excavates, Euglenozoa	N	N
Naegleria gruberi	Excavates, Heterolobosea	Y	N
Naegleria fowleri	Excavates, Heterolobosea	Y	N
Giardia lamblia	Excavates, Fornicata	N	N
Giardia intestinalis	Excavates, Fornicata	N	N
Trichomonas vaginalis	Excavates, Parabasalia	N	N
Emiliana huxleyi	Haptophyta, Isochrysidales	Y	Y
Chrysochromulina tobinii	Haptophyta, Prymnesiales	N	N
Pavlovalas sp	Haptophyta, Pavlovalas	Y	N
Phaeocystis globosa	Haptophyta, Phaeocystales	Y	N