Proteomic investigation of protein interactions and post-translational modifications of the
Pfh1 helicase and yeast telomerase holoenzymes

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A DISSERTATION
PRESENTED TO THE FACULTY
OF PRINCETON UNIVERSITY
IN CANDIDACY FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

RECOMMENDED FOR ACCEPTANCE
BY THE DEPARTMENT OF MOLECULAR BIOLOGY
Advisers: Virginia Zakian and Ileana Cristea

November 2012
Abstract

In this thesis I describe a targeted proteomics approach to study in vivo protein interactions and post-translational modifications of protein complexes involved in the maintenance of chromosome stability. Specifically I have used mass spectrometry to study the Schizosaccharomyces pombe PIF1 family helicase, Pfh1 and the telomerase enzyme of S. pombe and Saccharomyces cerevisiae.

Similarly to higher eukaryotes, S. pombe encodes a single PIF1 family protein, Pfh1. Nuclear and mitochondrial isoforms of Pfh1 play essential roles in the maintenance of chromosomal and mitochondrial DNA, respectively. Immunoaffinity purification (IP) of Pfh1-GFP and quantitative mass spectrometry establish a functional role of Pfh1 in DNA replication and telomere regulation. Mass spectrometry revealed S-phase enrichment of Pfh1 protein interactions that have a critical role in DNA replication including the DNA leading strand replication polymerase (Pol2), and telomere regulation including the RecQ family helicase Rqh1. Furthermore, cells overexpressing Pfh1 display longer telomeres compared to cells expressing only the mitochondrial isoform of Pfh1. These findings support a model that Pfh1 facilitates replication fork progression at the telomeres and positively regulates telomere length.

Second, I have purified telomerase from S. pombe and S. cerevisiae with the goal of identifying novel protein components and post-translational modifications. The telomerase holoenzyme is composed of a templating RNA, a catalytic reverse transcriptase protein subunit, and associated proteins important for its biogenesis, activity, and recruitment to the telomere. Immunopurification of telomerase was accomplished by overexpression of multiple protein components with a GFP epitope tag. In both organisms, overexpression of telomerase resulted in
a lengthening of telomeres, suggesting a functional overexpressed complex. In *S. pombe*, I have confirmed several protein associations known to play a functional role in telomere maintenance as well as identified a novel interaction with an essential component of the mRNA nuclear export pathway, Ptr1. A *ptr1-l* mutation revealed shorter telomeres, suggesting a functional role in telomere maintenance. Data supports a model that Ptr1 functions in telomerase RNA nuclear export and assembly with telomerase protein components. *S. cerevisiae* proteomic experiments are in progress and have thus far identified a novel phosphorylation site of the telomerase holoenzyme protein Est1 at S644.

Supplemental Tables 1-5: MS protein interaction data
<table>
<thead>
<tr>
<th>Table of Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract ........................................................................................................ iii</td>
</tr>
<tr>
<td>Chapter 1: Introduction .................................................................................. 1</td>
</tr>
<tr>
<td>Chapter 2: Methods ........................................................................................ 17</td>
</tr>
<tr>
<td>Chapter 3: Post-translational modification of Pfh1 as revealed by MS and studied by genetics</td>
</tr>
<tr>
<td>Results ........................................................................................................... 28</td>
</tr>
<tr>
<td>Discussion ......................................................................................................... 36</td>
</tr>
<tr>
<td>Figures ............................................................................................................ 41</td>
</tr>
<tr>
<td>Chapter 4: Proteomic characterization of Pfh1 protein interactions</td>
</tr>
<tr>
<td>Results ........................................................................................................... 58</td>
</tr>
<tr>
<td>Discussion ......................................................................................................... 67</td>
</tr>
<tr>
<td>Figures ............................................................................................................ 70</td>
</tr>
<tr>
<td>Chapter 5: Role of Pfh1 at the telomeres</td>
</tr>
<tr>
<td>Results ........................................................................................................... 83</td>
</tr>
<tr>
<td>Discussion ......................................................................................................... 87</td>
</tr>
<tr>
<td>Figures ............................................................................................................ 89</td>
</tr>
<tr>
<td>Chapter 6: Protein interactions of <em>Schizosaccharomyces pombe</em> telomerase</td>
</tr>
<tr>
<td>Results ........................................................................................................... 93</td>
</tr>
</tbody>
</table>
Chapter 7: Protein interactions of \textit{Saccharomyces cerevisiae} telomerase

Results ................................................................. 115

Discussion ............................................................. 120

Figures ................................................................. 122

References .............................................................. 130
List of Figures

Figure 1. Sites of Pfh1 phosphorylation identified outside of the conserved helicase motifs 41

Figure 2. Pfh1 post-translational modification in response to DNA damage 42

Figure 3. Sites of Pfh1 phosphorylation detected using a nanoLC LTQ-Orbitrap Velos with ETD and a MALDI LTQ-Orbitrap XL instrument configurations 43

Figure 4. Protein expression of the Pfh1-GFP mutants is stable but variable, and the response of non-phosphorylatable mutants to CPT treatment is similar to wild-type 48

Figure 5. Strains expressing Pfh1 phospho-mutations are viable 50

Figure 6. Telomere length variable in Pfh1 single phospho-mutants 51

Figure 7. Pfh1 wild-type and phospho-mutants co-localize with Rad22 52

Figure 8. Growth of Pfh1 S81D is improved at 30°C in the presence of hydroxyurea 54

Figure 9. Growth of Pfh1-S764D S81D-GFP worse than wild-type Pfh1-GFP in the presence HU 55

Figure 10. Growth of Pfh1- S81 S256 S764-GFP nonphosphorylated and phosho-mimetic better than Pfh1-GFP on HU, and growth of nonphosphorylated mutant worse on bleomycin (BM) than Pfh1-GFP 56
Figure 11. Growth of Pfh1-S81D T84D S256D S764D-GFP better than Pfh1-GFP on BM

Figure 12. Pfh1-GFP solubility optimal in lysis buffer 1 conditions of 250 mM NaCl and 1% TritonX-100

Figure 13. Immunoaffinity-purification of Pfh1-GFP nuclear isoform and wild-type with conditions of 250 mM NaCl, 1% Tx.

Figure 14. Immunoaffinity-purification of Pfh1-GFP nuclear isoform and wild-type in the presence of DNA damage induced by camptothecin (CPT)

Figure 15. Nuclear isoform of Pfh1 is phosphorylated at S764 in the presence or absence of DNA damage induced by CPT

Figure 16. Interaction of Pfh1-GFP with yKu70/80 and Rpa1 observed with conditions of 150 mM NaCl, 1% Tx, and phosphatase inhibitors.

Figure 17. Pfh1-GFP solubility optimal in lysis buffer 2 conditions of 300 mM potassium acetate and 0.1% NP-40

Figure 18. Interaction of Pfh1-GFP with DNA replication proteins Pol2 and the Mcm helicase complex observed in asynchronous cells with optimized conditions of lysis buffer 2

Figure 19. Immunoaffinity-purification of Pfh1-GFP not affected by DNaseI treatment of the cell lysate
Figure 20. Immunoaffinity-purification of Pfh1-GFP nuclear isoform in the presence and absence of DNA damage induced by CPT reveals similar protein interactions of wild-type Pfh1-GFP

Figure 21. Strain yKM333 expressing Pfh1-GFP in a cdc25-22 strain background displays normal cell cycle progression

Figure 22. Pfh1 protein expression is not cell cycle regulated

Figure 23. Identification of Pfh1 cell cycle-specific interactions

Figure 24. Cytoscape visualization of *S. pombe* proteins involved in telomere maintenance

Figure 25. Overexpression of Pfh1 causes telomere lengthening

Figure 26. Overexpression of Pfh1 causes telomere lengthening in a recombination deficient strain background

Figure 27. Telomere lengthening effect observed upon over-expression of Pfh1

Figure 28. Functional analysis of *S. pombe* endogenously expressed Trt1- GFP epitope tagged protein

Figure 29. Nuclear localization of overexpressed *S. pombe* telomerase protein components
Figure 30. The telomerase holoenzyme is stabilized by TER1, the telomerase RNA component 104

Figure 31. *S. pombe* telomere lengthening observed with telomerase overexpression 105

Figure 32. Immunoaffinity-purification of GFP-Trt1 and GFP-Est1 106

Figure 33. Deletion of mlo3, a protein involved in mRNA nuclear export, results in stable, short telomeres 108

Figure 34. The mRNA nuclear export factor ptr1-1 mutant displays shorter telomeres than wild-type at partially nonpermissive temperature 109

Figure 35. The mRNA nuclear export factor ptr1-1 mutant displays stably shorter telomeres than wild-type at partially nonpermissive temperature 110

Figure 36. RNA FISH experiment reveals TER1 nuclear localization in ptr1-1 cells and cytoplasmic localization in mlo3Δ cells 111

Figure 37. Functional analysis of *S. cerevisiae* endogenously expressed Est2 GFP epitope tagged protein 122

Figure 38. Immunoaffinity-purification of GFP-G8-Est2p expressed at endogenous levels 123

Figure 39. Overexpression of *S. cerevisiae* GFP-Est1, GFP-Est2, and GFP-Est3 125
Figure 40. Immunoaffinity-purification of GFP-Est1, GFP-Est2, and GFP-Est3 overexpressed by the GAL1 promoter 126

Figure 41. Telomere lengthening observed with *S. cerevisiae* telomerase overexpression of GFP-Est1, GFP-Est2, and Est3 127

Figure 42. Immunoaffinity-purification of GFP-Est2 and GFP-Est1 overexpressed by the GAL1 promoter 128
List of Tables

Table 1.  *S. pombe* and *S. cerevisiae* stains used in this thesis 27

Table 2.  Pfh1 sites of phosphorylation characterized in this study 46

Table 3.  Observed spectrum counts of Pfh1 phosphopeptides over four experiments at two different time points in the cell cycle, S and G2 47

Table 4.  Summary of Pfh1 phospho-mutant data 49

Table 5.  Top hits of *S. pombe* telomerase protein interactions 107
Acknowledgements

I would like to thank my advisers, Ginger Zakian and Ileana Cristea, for your guidance and encouragement throughout my graduate career at Princeton. I greatly appreciate the time you generously spent with me planning experiments, discussing results, and preparing presentations. Your enthusiasm for research has been a wonderful source of inspiration to me.

Thank you to the members of both the Zakian and Cristea labs. While at Princeton, I have learned so much from my lab mates as well as gained valuable friendships. I greatly respect the hard work and dedication of the scientists with whom I have been fortunate to work. A special thanks to my lab mates and collaborators whose generous help has made possible many experiments: Angela Chan, Todd Greco, Preeti Joshi, Nasim Sabouri, and Christopher Webb. Chris, you’ve also been a wonderful mentor to me in the lab and I greatly appreciate being able to learn from you.

Greatest thanks to my family. I am very fortunate to have a big family with all members offering support in their own special way. To my parents Carolyn and Donald Rainey for their unconditional love and support. To my siblings Tanya, Kristine, Tadhgh, Kendra, and Steven, brothers and sisters-in-law Jon, Dan, Sybil, Kelley and Tom, and mother-in-law Ruth- thank you so much for always being there for me. To my nieces and nephews-thank you for always reminding me of what’s most important in life. Last, but certainly not least, thank you to my wonderful husband Bill who has been there for me every step of the way, always offering me confidence and always believing in me. Our beautiful baby girl Pearl is just turning one, and as I’ve written my thesis I’m proud to remember her being with me for some of my most important experiments. I can never give enough thanks to my Mom, Kristine, Madeline, Samantha, and
Ruth who have helped greatly to take care of Pearl during this past year. I’m certain she will hold
on to her happy memories of this special time spent with you.


**Chapter 1: Introduction**

Portions of this introduction are taken directly from the review, Paesche, K., McDonald, K.R., and Zakian, V.A., Telomerese: *Structures in need of Unwinding*. FEBS Letters, 2010. 584 (17): p. 3760-3772. I co-wrote this review with a postdoctoral fellow in the lab. The only parts that are used are sections that I was responsible for writing.

This thesis describes the application of a proteomics/mass spectrometry based approach for studying the *Schizosaccharomyces pombe* PIF1 family helicase, Pfh1 and the telomerase enzyme of both *S. pombe* and *Saccharomyces cerevisiae*. The following introduction is organized in two sections: helicases and telomeres.

**Helicases**

Helicases utilize the energy of ATP hydrolysis to catalyze the unwinding of duplex nucleic acids and can act upon a variety of structures including DNA, RNA, DNA/RNA duplexes, forked or bubbled replication intermediates, Holiday junctions, and non-Watson-Crick base paired structures such as G-quadruplexes. This flexibility of substrates consequently makes helicases critical for virtually all processes of the cell involving DNA or RNA. While all helicases function to unwind base paired nucleic acids, some can additionally translocate along a single nucleic acid strand before they reach the duplex region, and in this process can displace bound proteins. Perhaps owing to a great versatility of cellular functions, organisms encode for many helicases. For example, 2% of the open reading frames in the *Saccharomyces cerevisiae* genome encode predicted helicase proteins [1].
Two superfamilies of helicases, SFI and SFII, are defined by seven conserved amino acid motifs. The conserved motifs are located in a domain of approximately 300-500 amino acids that are observed by structural analysis to cluster in space (reviewed in [2]). Within the two superfamilies, helicases are classified into families based on higher levels of sequence similarity, both within and outside of the helicase motifs. Helicases are further divided into DNA or RNA helicases based on the chemical identity of the strand onto which they load and by the direction of unwinding. Helicases typically unwind duplexes in a unidirectional manner, moving either 3’ to 5’ or 5’–3’ along the strand onto which they load. The number of nucleic acid base pairs unwound before the helicase dissociates from its substrate defines its processivity. Helicases vary in their in vitro determined processivity, which may reflect the enzyme’s preferred substrate and/or necessary co-factors. For example, the S. cerevisiae Pif1 DNA helicase is poorly processive on conventional duplex DNA but is highly processive on forked RNA/DNA duplexes [3] and G-quadruplex structures [4]. Of particular interest to the Zakian lab’s research of telomere regulation and genome integrity, several helicase families, PIF1, RecQ, FANC-J, and DNA2, have demonstrated functions at telomeres.

**PIF1 family helicases**

The SFI PIF1 family of 5’-3’ DNA helicases is well conserved in eukaryotes over a centrally located 400-500 amino acid helicase domain (reviewed in [5]). Family members, however, do not share size or sequence similarity at their amino or carboxyl termini. PIF1 family helicases were first identified in S. cerevisiae in which there are two homologs, ScPif1 and ScRrm3. Although these two family members are 40% identical within their helicase domains, they have distinct cellular functions. In contrast to S. cerevisiae and several other yeasts that also encode two distinct PIF1 family proteins, S. pombe encodes a single and essential PIF1 family
member, Pfh1. Similarly to *S. pombe*, metazoans encode a single PIF1 family helicase that has approximately equal similarity to the two *S. cerevisiae* proteins. In most organisms, from yeasts to humans, the nuclear PIF1 genes encode at least two isoforms, one localized to mitochondria and one to nucleus [6-10].

**S. cerevisiae Pif1 and Rrm3**

*S. cerevisiae* Pif1 is a distributive enzyme on most DNA substrates but processively unwinds both forked RNA/DNA molecules and G-quadruplex structures [3, 11]. ScPif1 (petite integration frequency 1), the founding member of the PIF1 family, was first identified for its role in promoting recombination in mitochondrial DNA [12]. It was subsequently rediscovered in a mutant screen for genes affecting telomeres [6]. ScPif1 is an inhibitor of telomerase and therefore, negatively regulates telomere length [6, 13]. ScPif1 has been shown to have multiple states of phosphorylation; with some modifications playing a role in regulating telomerase activity at telomeres, and other modifications induced by DNA damage signaling, which regulate telomerase activity at double-stranded breaks (DSBs) [14]. ScPif1 inhibits telomerase at DSBs to prevent aberrant addition of telomeres. In *pif1*Δ cells, telomere addition to DSBs is dramatically increased ~600-fold and gross chromosomal rearrangements (GCRs), similar to those observed in human tumor cells, increase ~1000-fold [6, 15, 16]. Virtually all of the GCR events recovered in Pif1 deficient cells are due to telomere addition at DSBs.

*In vitro* and *in vivo* experiments indicate that ScPif1 inhibits telomerase by physically disrupting its interaction with DNA. *In vitro*, mutations of Est2 (the catalytic subunit of the telomerase holoenzyme) that disrupt interaction with ScPif1 also disrupt the ability of ScPif1 to inhibit telomerase [17]. *In vivo*, chromatin immunoprecipitation (ChIP) experiments reveal that
ScPif1 overexpression reduces telomerase binding at telomeres, while ScPif1 depletion increases telomerase binding at both telomeres and DSBs [18, 19].

ScPif1 additionally plays a role in chromosomal DNA replication as revealed by genetic and biochemical studies. ScPif1 functions with DNA polymerase delta to generate and lengthen 5′ flaps on Okazaki fragments that are cleaved by Dna2 [20-22].

ScRRM3 (rDNA recombination mutant) was first identified because its mutation increases rDNA recombination [23]. At the same time, the Zakian lab discovered ScRrm3 because of its similarity to ScPif1[24]. As revealed by 2D gel analyses and ChIP-chip microarray studies, ScRrm3 plays a critical role in facilitating replication fork progression throughout the genome at sites bound by stable protein complexes including tRNA genes, inactive replication origins, centromeres, sites within rDNA repeats, and telomeres [24-27]. Dissociation of stable protein complexes at these specific sites eliminates Rrm3 dependence for replication fork progression, suggesting that disruption of bound proteins by the helicase activity of Rrm3 is responsible for facilitating replication fork progression [25, 28]. Co-localization of Rrm3 with the DNA leading strand polymerase Pol2, observed by ChIP analysis throughout DNA replication, revealed that Rrm3 travels with the replication fork [27]. Rrm3 has a dramatic effect on replication fork progression at the telomere; in the absence of Rrm3, replication fork pausing within the telomeric DNA is exacerbated 10-fold [26].

_S. pombe_ Pfh1

Like higher eukaryotes, the genome of _S. pombe_ encodes only one PIF1 family helicase, Pfh1 (Pif1 homologue 1). Nuclear and mitochondrial isoforms of Pfh1 play essential roles in the replication of chromosomal and mitochondrial DNA, respectively and also function in DNA
repair [7]. Similar to its *S. cerevisiae* homolog, Pfh1 is most active at unwinding forked substrates [29]. The essential nuclear function of Pfh1 can be supplied by ScRrm3, suggesting that Pfh1 similarly promotes replication fork progression through sites bound by stable protein complexes [7]. Indeed, Pfh1 has recently been shown to be required for efficient fork movement in the ribosomal DNA, the mating type locus, tRNA, 5S ribosomal RNA genes, and genes that are highly transcribed by RNA polymerase II (I am second author on this paper with minor contributions to this work) [30]. The importance of Pfh1 function during replication is further demonstrated with the accumulation of converged replication forks at all of these sites in the absence of Pfh1, as observed by 2D gel analysis [30]. Furthermore, genetic assays suggest that Pfh1, like ScPif1, may also play role during DNA replication in Okazaki fragment maturation [29, 31, 32].

In the literature, there are conflicting reports about whether or not *S. pombe* Pfh1 affects telomere length. The first report found that deletion of *pfh1*+ is associated with telomere shortening [9]. In this study, telomere length was analyzed in a population of *pfh1* delete cells derived by sporulation of a *pfh1*+/*pfh1Δ* diploid, which can germinate and divide one or more times before growth arrest. More recently, it has been reported that there is no effect of nuclear Pfh1 depletion on telomere length [7]. In this study nuclear Pfh1, expressed from the thiamine-repressible nmt promoter, was depleted from the cell by growth in thiamine containing medium. Although no nuclear Pfh1 was detectable in this experiment by western blot analysis, a low level of Pfh1 might be present in the nucleus due to incomplete repression of the nmt promoter as it has been shown that even a low level of Pfh1 is sufficient for cell viability [9]. (Both of these published studies are from the Zakian lab.) Indeed, the Zakian lab has found it difficult to deplete cells completely of nuclear Pfh1 by functional assays, despite multiple experimental approaches.
Mammalian PIF1 family proteins

Similar to *S. pombe*, mouse and humans encode a single Pif1 family helicase, mPIF and hPIF, respectively. However unlike in *S. pombe*, mPIF is not essential most likely due to redundancy with that of other helicases or translocases [33]. Like the yeasts PIF1 proteins, hPIF is most active at unwinding forked structures that resemble replication intermediates [34-36]. Furthermore similar to ScPif1, expression of hPIF is cell cycle regulated by ubiquitin-mediated degradation with highest protein expression observed late in the cell cycle [37, 38]. This abundance pattern is unlike what is seen for ScRrm3, which is stably expressed throughout the cell cycle [27]. A role of hPif at telomeres has been suggested by *in vitro* experiments in which hPif reduces telomerase processivity and binds preferentially to DNA containing the telomeric repeat sequence TTAGGG [36]. Two *in vivo* studies of hPif overexpression, show conflicting results of whether or not hPif plays a role in telomere maintenance: results published by the Zakian lab show no effect on telomere length upon hPif overexpression [37], while results of a second group a show telomere shortening [36]. Suggestive evidence supporting a role for mammalian PIF at telomeres comes from experiments in humans and mice, in which Pif1-like proteins co-immunoprecipitate with telomerase [33, 37].

In this thesis, I have studied *in vivo* protein interactions and post-translational modification of the *S. pombe* Pfh1 helicase by a targeted proteomics approach in order to elucidate further its essential role in nuclear DNA replication and telomere regulation. Immunoaffinity purification of Pfh1-GFP and subsequent analysis of co-isolated proteins by mass spectrometry has revealed S-phase enrichment of protein interactions that play a critical role in DNA replication, including the leading strand replication polymerase (Pol2) and subunits of the mcm helicase heptamer ring, and telomere regulation, including the Ku70/Ku80
heterdimer and the RecQ family helicase Rqh1. The functional role of Pfh1 at telomeres has been further analyzed by overexpression of Pfh1 using a high copy plasmid. Telomere lengthening was observed in response to Pfh1 overexpression in the Pfh1-mt* background in which nuclear Pfh1 is absent. Pfh1 positive regulation of telomere length may be explained by its role in promoting replication fork progression through the telomeres, a hard-to-replicate regions of the genome [30].

The enrichment of Pfh1 enabled the use of use of multiple mass spectrometry (MS) instrument configurations, nanoLC LTQ-Orbitrap Velos with ETD and MALDI LTQ-Orbitrap XL, to detect PTMs, which allowed for a comprehensive analysis of Pfh1 phosphorylations. As revealed by MS, I have characterized 10 Pfh1 phosphorylation sites, eight of which are novel, localized primarily on the N-terminus of the protein outside of the conserved helicase domains. Genetic analysis of phospho-mimetic or non-phosphorylatable mutants of Pfh1 have unfortunately not led to a conclusive functional assignment of Pfh1 modifications. However, there is precedence for phosphorylation playing a functional role in regulating ScPif1 at telomeres and double stranded breaks [14].

Telomeres

Telomeres are conserved nucleoprotein complexes at the ends of linear chromosomes that protect the eukaryotic genome. Telomeres protect the DNA from degradation, distinguish normal chromosome ends from broken ends, and facilitate complete replication of the genome. The protection of genetic information is essential for cells, as the accumulation of chromosomal aberrations leads to genomic instability.
In the 1930s Herman Müller working in flies and Barbara McClintock working in maize were the first to observe that the ends of linear chromosomes are special. In the fruit fly *Drosophila melanogaster*, Müller observed that following X-ray irradiation terminal deletions or terminal inversions of the chromosomes could not be recovered [39]. Muller coined the term telomeres from the Greek words ‘telos’ meaning end and ‘meros’ meaning part to describe the specialized structures that he reasoned must exist at the ends of chromosomes. In maize, Barbara McClintock observed that following X-ray irradiation a broken chromosome could fuse with its sister chromatid creating a breakage-fusion-bridge cycle [40]. Furthermore, McClintock reported that a broken end could permanently heal during the reproductive cycle of the chromosome and lose its tendency to fuse with other broken ends [41]. It was not until the late 1970s that the true nature of the telomere structure began to emerge by research done in a simple eukaryote, the ciliated protozoan *Tetrahymena thermophila*. Work by Elizabeth Blackburn in the laboratory of Joe Gall showed that the telomere is a repeated sequence of nucleotides [42, 43]. Later under the guidance of Blackburn, Carol Greider identified the telomerase enzyme, which functions to maintain telomere length [44].

The structure and function of eukaryotic telomeres is highly conserved. The basic structure of almost all eukaryotic telomeres consists of double-stranded repetitive arrays of guanine (G)-rich sequences that runs 5’-3’ towards the chromosome end and terminate in a 3’ single stranded overhang. The length of the double stranded telomere repeat varies greatly between cells of different organisms with yeasts *S. pombe* and *S. cerevisiae* measuring approximately 300 bps, laboratory mouse *Mus musculus* measuring 40-80 kb, and human measuring 10-15 kb [45-47]. The telomeric DNA sequence of *S. pombe* is degenerate with the most frequently occurring motif being TTACAGG, *S. cerevisiae* is heterogeneous with repeats of
TG (1-3), and the vertebrates human and mouse is TTAGGG. A conserved feature that is essential for telomere function is the single stranded 3’ guanine-rich overhang, known as the G-tail [48-52]. As a result of a high concentration of guanines residues, the telomere structure has high thermal stability in the duplex region due to G-C base pairing. Furthermore, G-quadruplex (G4) structures have been demonstrated to form in vivo in the telomeric G-tail of ciliates [53-55]. G4 structures are an example of a non-Watson Crick base paired secondary structure. If G-tails form G4 structures in vivo, then thermal stability of folded tails might also be high. In vitro intra- and intermolecular G4 structures have been demonstrated to form in G-rich single stranded telomeric DNA (reviewed in [56] [57]).

An additional higher-order structure called T-loops is observed by electron microscopy to form at the telomeres of human, mouse, trypanosomes, ciliates, and nematode [58-66]. T-loops are a duplex lariat structure formed by the looping and incorporation of the telomeric 3’ single stranded G-tail in the double stranded region of the same chromosome. T-loops are thought to protect telomeres by sequestering the chromosome end, however it is not understood how they are regulated during the cell cycle to allow for replication of the telomere. It is also possible that t-loops are recombination intermediates rather than capping structures.

Telomeric DNA is bound constitutively by a core group of proteins that form a complex called shelterin. The conserved shelterin complex functions to recruit telomerase to DNA while also distinguishing natural chromosome ends from DNA breaks. Components of the shelterin complex include the double stranded telomere binding proteins Rap1 in S. cerevisiae, Taz1 in S. pombe, and TRF proteins in mammals, as well as, the single stranded telomere binding proteins Cdc13 in S. cerevisiae, and Pot1 in S. pombe and mammals [50, 67, 68]. In mammals and S. pombe, there are shelterin components that bridge the proteins complexes at the duplex region to
those at singles strand tails. In *S. pombe* the shelterin component Ccq1 is required to recruit the telomerase reverse transcriptase to telomeres [69].

In the absence of telomere maintenance, linear chromosomes shorten progressively with every round of DNA replication, a phenomenon referred to as the end replication problem. The end replication problem, first theoretically described by Alexy Olovnikov in 1971 and James Watson in 1972, refers to the difficulty posed by conventional DNA polymerases to completely replicate linear chromosomes [70, 71]. In each S phase of the cell cycle, semi-conservative replication of a linear chromosome results in an 8-12 bp gap in the lagging strand due to the removal of a terminal 8-12 bp RNA primer required for conventional DNA polymerases. In the absence of special telomere maintenance mechanisms, linear chromosomes shorten progressively with every round of DNA replication eventually reaching a critically short length that results in cellular senescence or apoptosis. Therefore, there is a limit for the number of times that a cell can divide; this is known as the Hayflack limit. Abnormal cancer cells circumvent this problem in order to divide indefinitely by taking advantage of a natural cellular mechanism to maintain telomere length, the telomerase enzyme, or more rarely, recombinational telomere lengthening.

Most somatic cells of the human body lack telomerase activity. In these cells, senescence induced by short telomeres may serve as a tumor suppressor mechanism, by most cells undergoing growth arrest before multiple genetic mutations are acquired that may cause a cell to become cancerous. In contrast, greater than 85% of human cancer cells maintain stable telomere lengths by up-regulating telomerase activity [72, 73]. In these cancerous cells, elongation of the telomere promotes cell proliferation and immorality. There are promising potential treatment methods that target cancer cells with up-regulated telomerase activity that display high
specificity and low toxic side effects [74]. Telomerase inhibitors are also being studied in combinations with other conventional cancer treatments.

The fission yeast *S. pombe* and budding yeast *S. cerevisiae* are unicellular eukaryotic model organisms that serve as valuable tools for gaining basic knowledge of telomere regulation that may also apply to multicellular eukaryotic organisms. Fission yeast is nearly as distant evolutionarily from budding yeast as it is from humans, having diverged from the budding yeast lineage more than 300 million years ago [75]. It is useful to study telomere biology in these two evolutionarily divergent yeast species because each shares distinct genetic similarities with higher eukaryotic organisms.

Telomeres are maintained by the enzyme telomerase, a specialized reverse transcriptase that is capable of extending the 3’ end of chromosomes by reverse-transcribing the template region of a tightly associated RNA. Elongation of the 3’ strand by telomerase allows conventional DNA polymerases to fill in the complementary strand for complete replication. The telomerase holoenzyme is composed of a catalytic reverse transcriptase protein subunit (TERT in mammals, Est2 in *S. cerevisiae*, Trt1 in *S. pombe*) and an essential telomerase RNA component (TR in mammals, TLC1 in *S. cerevisiae*, TER1 in *S. pombe*). The carboxyl-terminal half of the telomerase catalytic protein subunit shares sequence homology with reverse transcriptases (RTs) of retro-elements and retroviruses, with seven defined RT motifs in this conserved region [76]. The amino-terminal half of the telomerase reverse transcriptase protein contains several conserved sequence motifs that function as binding domains for the telomerase RNA. Telomerase RNA components are highly divergent in size and sequence among eukaryotes. The size of telomerase RNAs varies from ~150 nucleotides in ciliates, to ~500 nucleotides in vertebrates, to ~1300 nucleotides in budding yeast. The *S. pombe* telomerase RNA, identified
and characterized in the Zakian lab, is similar in size to budding yeast with ~ 1200 nucleotides [77, 78].

In addition to the core components of telomerase, telomerase reverse transcriptase and telomerase RNA, there are a variety of species-specific associated proteins that function in its biogenesis, activity, and recruitment to the telomere. In the ciliate *Tetrahymena*, there are at least four telomerase associated proteins: p75, p65, p45, and p20 [79]. Of the two proteins whose functions have been determined, p65 functions in TR stability and ribonuclear protein (RNP) assembly, while p20 is a component of SCF ubiquitin ligases and negatively regulates TERT stability [79, 80].

In *S. cerevisiae*, telomerase activity *in vivo* requires the association of Est1, Est2, and Est3. These components of the *S. cerevisiae* telomerase holoenzyme were identified by genetics; disruption of any of these proteins results in an est (ever shorter telomere) phenotype, characterized by progressive telomere shortening, senescence, and eventual cell death of the majority of cells in the population [81, 82]. Associations of the *S. cerevisiae* telomerase holoenzyme also include the Ku70/80 heterodimer, Sm proteins, PinX1/Gno1p, p23, and an INO80 subunit Ies3p [83-87]. Of proteins with identified function, Est1 functions in Cdc13-mediated telomerase recruitment, binding of telomeric DNA and telomerase RNA and possibly in activation of telomerase, Ku70/80 plays a role in telomere protection and telomerase recruitment [83], Sm proteins function in telomerase RNA maturation and stability [84], PinX1/Gno1 has been suggested to inhibit biogenesis by sequestering TERT in the nucleolus in an inactive complex lacking TER [85], p23 is a molecular chaperone implicated in telomerase assembly [86], and Ies3 is a subunit of the INO80 chromatin remodeling complex that interacts with Est1 [87].
In *S. pombe*, telomerase-associated proteins include an EST1 homolog, SpEst1 that functions in telomere elongation and telomere capping [88], Sm proteins that functions in TR processing and maturation [78], and Ccq1 that binds Est1 and functions in the recruitment of telomerase to telomeres [69, 89].

In *H. sapiens*, there is a long list of telomerase associated proteins that includes multiple hEst1 proteins, molecular chaperones hsp90, p23, hsp70, p60, and hsp40, as well as PinX1, 14-3-3, MKRN1, and the dyskerin protein complex. The molecular chaperone proteins function in telomerase assembly, with hsp90 and p23 stably associating with the active complex [90]. PinX1 has been suggested to repress telomerase activity by binding to hTERT and hTR [91]. Protein 14-3-3 is involved in nuclear shuttling and localization of hTERT [92]. MKRN1 E3 ligase mediates ubiquitination of hTERT [93]. The dyskerin protein complex binds a Box H/ACA motif of human TR for regulation of RNA processing and maturation [94]. The association of human TR with dyskerin and the determination that mutations in both result in the human disease dyskeratosis congenita, indicate that telomerase-associated proteins have a profound effect on telomerase and telomere regulation.

The telomerase RNA (TR) undergoes several steps of maturation before being assembled with telomerase proteins to form the active holoenzyme. Yeast TR is transcribed by RNA polymerase II, polyadenylated, capped on its 5’ end with a 2,2,7-trimethyl guanosine cap structure, and processed to remove the 3’ polyadenosine tail [77, 95]. In *S. cerevisiae*, the shuttling of TR between the nucleus and cytoplasm plays a critical role in telomerase biogenesis that is not well understood [96-98]. Shuttling between the nucleus and cytoplasm is made possible by the association of TR with the nuclear exportin Crm1, and importins Mtr10-Kap122 [96, 97]. The absence of the telomerase holoenzyme protein components Est1 or Est2, as well as,
the absence of telomere associated proteins Ku70, Tel1 or the MRX complex, which all have roles in recruiting telomerase to telomeres, results in the cytoplasmic accumulation of \textit{S. cerevisiae} TR [52].

Although an active form of \textit{S. cerevisiae} telomerase is present in various stages of the cell cycle in, telomere elongation is cell cycle regulated and restricted to late S/G2 phase [99]. The timing of telomere elongation correlates with the telomere binding of many proteins involved in telomere elongation [99-102].

In the absence of telomerase, homologous recombination (HR) offers an alternative pathway (ALT) for the maintenance of telomeric DNA. There are two ALT pathways in yeast called type I and type II recombination that differ in the substrates and proteins that catalyze the reaction. In \textit{S. cerevisiae}, type I recombination results from the amplification of subtelomeric Y’ elements in a Rad51 dependent manner, while type II recombination results from the expansion of the G-rich telomeric repeats in a Rad50 dependent manner [103-106]. In the absence of telomerase, \textit{S. pombe} cells maintain telomeres by recombination or chromosome circularization [107]. \textit{S. pombe} Rhp51, the homolog of Sc Rad51, is required for telomere maintenance by homologous recombination [108]. Telomere recombination may also contribute to telomere length regulation in telomerase plus cells. For example, telomere rapid deletion (TRD) is an intra-chromatid recombination that can reduce a very long telomere to wild-type length in a single cell cycle.

As described previously, a targeted proteomics approach was adapted for studying protein interactions of the telomerase enzyme in both \textit{S. pombe} and \textit{S. cerevisiae}. Due to extremely low levels of telomerase protein expression, an approach was employed where all
protein and RNA components of the telomerase holoenzyme were overexpressed, with protein components additionally being expressed with a GFP tag. In both organisms, overexpression strains maintained telomeres at a longer length than wild-type cells, suggesting the overexpressed telomerase complex was functional. *In S. pombe* immunoaffinity purification of the telomerase holoenzyme was successful with the identification of several protein associations known to play a functional role in telomere maintenance. One novel interaction identified in my experiments is an essential protein Ptr1, a component of the mRNA nuclear export pathway. I found that a *ptr1-1* temperature sensitive strain had shorter telomeres when grown at the semipermissive temperature compared to the permissive temperature, suggesting a functional role for Ptr1 in telomere maintenance. Experiments are ongoing to determine if Ptr1 may play a role in telomerase RNA processing or stability, which would affect assembly of the telomerase holoenzyme. *In S. cerevisiae*, immunopurification of the telomerase holoenzyme and associated protein complexes in asynchronous cells has met with limited successful. Although I have succeeded in purifying an Est2 complex that I analyzed by MS, this analysis has not identified promising protein interactions. Characterization of a novel site of Est1 phosphorylation at S644 has, however, been confirmed in several experiments. Experiments are ongoing to try telomerase purification in an arrested population of cells at G2, a time point of the cell cycle with highest telomerase activity.
Chapter 2. Methods

*S. pombe* growth conditions and strains

*S. pombe* strains of this study were isogenic to wild-type 972 h- and carried the *ade6-M210/216 his3-D1 leu1-32 and ura4-D18* markers unless otherwise noted (Table 1). Standard molecular genetic techniques and media were used as described [109]. *S. pombe* cells were grown at 30°C in supplemented yeast extract (YES; Difco) or Edinburgh minimal media (EMM; Sunrise Science). Cells treated with camptothecin (+CPT) were grown for 3 hours at 30°C in the presence of 40 µm CPT.

Strains expressing Pfh1-GFP were previously described [7]. Briefly, the pJK148-integrating vector was used to express Pfh1-GFP from the leu1 locus using the endogenous Pfh1 promoter. The control IP strain expressed GFP-NLS from the leu1 locus under the control of the P3nmt promoter. The GFP-NLS construct was generated in pJK148 using the plasmid pFA6a-kanMX6-P3nmt1-GFP tagging construct as a PCR template with the addition of two SV40 nuclear localization signals introduced by PCR primers.

For cell synchronization, *cdc25-22* strains were grown to early mid-log (0.5 x 10^7 cells/ml) at the permissive temperature of 25°C. The cells were collected by filtration and shifted to 37°C for G2 arrest. After 4 hours of incubation at 37°C, the media was quickly cooled (2 minutes by swirling in an ice bath) to 25°C for synchronized growth. Cells harvested at the G2 time point were collected at the end of the 4 hour 37°C incubation. Cells harvested at the S phase time point were collected at 84 minutes, corresponding to the start of replication. Cell cycle progression and the timing of DNA replication were confirmed by FACS analysis.
Mutations of Pfh1 were made in pJK148-pfh1-GFP and were carried out by site-directed mutagenesis (Strategene) of serine (S) or threonine (T) codons to an uncharged alanine (A) residue or a charged aspartic acid (D) residue. Site-directed mutagenesis was confirmed by sequencing analysis. Insertion of the phospho-mutation at the leu1 locus in strain ySP421 (Table 1) was confirmed by Southern blot. Expression of endogenous Pfh1, which was marked with kanMX6 and flanked by loxP sites, was removed by transformation of the cells with plasmid pREP82-Cre. Following Cre expression, removal of the endogenous pfh1+ locus was confirmed by PCR.

The mitochondrial only Pfh1 allele, labeled pfh1-mt* was described previously [7]. Briefly, this allele contains mutations of the methionine codons M265 and M320 to alanine, and M170 to leucine, as well as, the addition of a carboxy-terminal nuclear export sequence. In overexpression experiments, Pfh1 was expressed from plasmid pVS117 [9], which carries the pfh1+ gene under the control of its endogenous promoter in vector pBG2 [110].

The telomerase protein components Trt1 (the telomerase reverse transcriptase) and Est1 were tagged with GFP at the amino terminus and over-expressed by the P3nmt promoter at their endogenous locus by integration of a PCR construct. The telomerase RNA component, TER1, was over-expressed on a plasmid, pSP1-TER1. Correct integration at the gene loci was confirmed by Southern blot analysis.

**S. cerevisiae growth conditions and strains**

*S. cerevisiae* strains of this study were performed in W303 (Rad5+ leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15) background. Standard molecular genetic techniques and
media were used. *S. cerevisiae* cells were cultured in YEP rich media containing 2% galactose. Components of the telomerase complex were over-expressed by the gal1 promoter at their endogenous locus by incorporation of a PCR construct using the pFA6 plasmid series. Protein components, Est1 and Est2 (telomerase reverse transcriptase) additionally were over-expressed with an amino terminus GFP epitope tag with a five glycine linker, which has been shown to specifically increase the functionality of epitope-tagged *S. cerevisiae* telomerase proteins [111].

**Immunoaffinity purification (IP)**

Two liters of *S. pombe* or *S. cerevisiae* cells were grown to mid-log and harvested by centrifugation at 4°C for 10 minutes at 4,000 rpm (2704 x g). Cell pellets were resuspended in freezing buffer (20 mM Na-HEPES, 1.2% polyvinylpyrrolidon (W/V), pH 7.4, and 1/100 protease inhibitor cocktail (sigma)) and frozen as cell droplets by dripping into liquid nitrogen. Cell droplets were cryogenically ground using a Retsch MM301 Mixer Mill (20 steps x 2.5 min at 30 Hz) (Retsch, Newton, PA) to achieve greater than 85% cell lysis, as assessed using light microscopy. Approximately 12 grams of ground, frozen cells were resuspended in lysis buffer (100mM Hepes KOH, pH 7.9, 300mM potassium acetate, 10mM magnesium acetate, 10% glycerol, 0.1% NP-40, 2mM EDTA, 2mM B-glycerophosphate, 50mM NaF, 10mM NaVO4, 1mM DTT, protease inhibitor cocktail (Roche)) in a ratio of 5ml of lysis buffer per 1 gram of cells. Cells were gradually added to the lysis buffer with continuous mixing to avoid cell clumps. Lysis buffer conditions of varying salt concentrations (50-900 mM potassium acetate) were optimized for efficiency of Pfh1-GFP purification. Cell lysate was homogenized using a PT 10-35 Poyltron (Kinematica) for 3 sets of 10 seconds (30 seconds total) with 1 minute on ice in between each set. Insoluble material of the cell lysate was removed by centrifugation at 8K rpm (9265 x g) for 10 minutes at 4°C. For immunopurification of Pfh1-GFP, the supernatant of the
cell lysate was incubated for 30 minutes at 4°C with approximately 20 mg of M-270 epoxy magnetic beads (Invitrogen Dynal) conjugated with 50 µg of in-house developed rabbit polyclonal anti-GFP [112, 113]. Following incubation, the beads were collected and washed six times with 1ml of the lysis buffer. Proteins were eluted from the beads by incubation with 40 µl of 1x LDS sample buffer (Invitrogen) by shaking for 10 minutes at room temperature, followed by 10 minutes at 70°C. Eluted proteins were alkylated with 50 mM chloroacetamide for 30 minutes at room temperature in the dark.

The isolated protein complexes were run on a 4-12% bis-Tris NuPAGE precast gradient gel (Invitrogen) and stained with SimpleBlue Coomassie stain (Invitrogen) for protein band visualization. Samples were prepared for in-gel digestion by excising gel bands into 1-mm sections. Gel sections from a single immunisolation were pooled into approximately eight equal fractions and placed in the wells of a 96-well plate. The gel pieces were destained in 50 mM ammonium bicarbonate, 50% acetonitrile at room temperature with gentle shaking for 15 minutes. Next, the samples underwent two rounds of dehydration in 100% acetonitrile (v/v) and rehydrating in 50 mM ammonium bicarbonate. After a final, third dehydration step in 100% acetonitrile, the gel pieces were suspended in 12.5 ng/µl sequencing grade modified trypsin (Promega) for overnight incubation at 37°C. The next morning, peptides were extracted from the gel pieces in 0.5% formic acid at room temperature with gentle shaking for 4 hours, followed by a second extraction in 0.5% formic acid, 50% acetonitrile at room temperature with gentle shaking for 2 hours. The peptides were concentrated by vacuum centrifugation to approximately 12 µl. Half of the sample was used for mass spectrometric analysis.

**Mass Spectrometry**
Parameters for mass spectrometric analysis were described previously in Tsai, et al. 2012 [114]. Briefly, tryptic peptides were separated by nanoscale liquid chromatography tandem mass spectroscopy (nLC-MS/MS) on a Dionex Ultimate 3000 RSLC, directly coupled to an LTQ-Orbitrap Velos electron transfer dissociation (ETD) (ThermoFisher Scientific) instrument. Data was automatically acquired with MS² fragmentation of the top 20 most intense precursor ions by collision-induced dissociation (CID). Parameters for data processing were also followed as described previously [114]. Briefly, raw files containing MS² data were extracted by Proteome Discoverer (version 1.3; Thermo Scientific) and uploaded to SEQUEST (version 1.20) for searching against a compiled database of the yeast protein sequences of \textit{S. cerevisiae} and \textit{S. pombe}. Post-search validation of the SEQUEST data was conducted by an X! Tandem algorithm in Scaffold (version Scaffold_3_00_04; Proteome Software) using the following filter selections to reduce peptide and protein global false discovery rate to < 1%: 99% protein confidence, 95% peptide confidence, and a minimum of two unique peptides per protein.

**Identification of phosphorylation sites on Pfhl**

The enrichment of Pfhl via immunoaffinity purification and the use of multiple mass spectrometry instrument configurations, nanoLC LTQ-Orbitrap Velos with ETD and MALDI LTQ-Orbitrap XL, allowed for a comprehensive analysis of Pfhl phosphorylations. To enable the identification of phosphopeptides from data acquired using a nanoLC LTQ-Orbitrap Velos instrument, specifications of the SEQUEST MS and MS² data search settings included variable modifications of phosphoserine, threonine, and tyrosine of +79.97 Da. Modifications were validated for phosphate group localization by software and manual evaluation of peptide
fragmentation patterns. MALDI MS/MS analysis of Pfh1 phosphopeptides was conducted as described below.

**Matrix-Assisted Laser Desorption Ionization (MALDI)-MS/MS**

Analyses were performed on a MALDI LTQ-Orbitrap XL mass spectrometer as described previously by Luo, et al. 2010 [115]. IP samples were prepared as described above and isolated proteins were eluted in 700 µl 0.5 N NH4OH, 0.5 mM EDTA solution. The elution was frozen in liquid nitrogen and dried overnight by vacuum centrifugation at low heat. The next morning the pellet was suspended in SDS-PAGE sample buffer, separated on a 4–12% NuPAGE Novex Bis-Tris gel (Invitrogen), and stained with the Coomassie stain, Simply Blue (Invitrogen). After cutting of the gel into 1mm segments, pieces were prepared for overnight incubation with trypsin at 12.5 ng/µl in 50 mM ammonium bicarbonate at 37ºC. The next morning peptides were extracted from gel pieces and concentrated by shaking at room temperature with a solution of 2.5% formic acid (FA), 0.1% trifluloroacetic acid (TFA) and reverse phase resin (POROS 20 R2, Applied Biosystems) for 6 hours. The POROS beads were collected and washed with 0.1% TFA. Peptides were eluted beads in 5 µl of 25 mg/ml 2,5-dihydroxybenzoic acid matrix in 50% methanol, 20% ACN, 0.1% TFA, and deposited on the MALDI target. Peptide mass fingerprinting and targeted MS/MS were performed as described [115, 116]. Phosphopeptides were manually evaluated for phosphate group localization by peptide fragmentation patterns resulting from targeted CID MS² and MS³ analysis.

**Assessing specificity of interactions**

Protein interactions were accessed for specificity and enrichment using the computational program SAINT (significance analysis of interactome) for probabilistic scoring using label-free
quantitative data [117]. Interactions of the IP experiments of Pfh1-GFP and GFP alone at the S phase time-point were paired for SAINT analysis as experimental and control data sets, respectively. Similarly, interactions of the IP experiments of Pfh1-GFP and GFP alone at the G2 time-point were paired. Data imported for SAINT analysis included unweighted spectrum counts and size (number of amino acids) of each observed protein hit. Highly significant protein interactions, either in S or G2 phase of the cell cycle, were identified by a SAINT confidence score of greater than 0.80. Additional interactions that did not meet this threshold were identified as significant if they are known to interact in a complex with a protein that did score greater than 0.80 and based on spectral counts, were either not present in the GFP controls or present by more than two-fold in the S or G2 Pfh1-GFP IP experimental conditions compared with the GFP controls. It was accessed how many significant interactions of Pfh1 are also known to be involved in telomere length regulation. *S. pombe* proteins involved in telomere regulation were identified in UniProt.org with the GO term “telomere maintenance.”

**Building protein interaction networks**

The accession numbers of proteins identified as significant interactions were imported into STRING (http://string-db.org/) for performing network analysis using the parameters of “Experimental” and “Databases” evidences at medium (0.4) confidence level [118]. For visualization of STRING networks, data were exported as a PSI-XML format and imported into Cytoscape [119]. With Cytoscape visualization, nodes represent protein interactions, solid dark lines represent protein complexes, solid grey lines represent known interactions of a complex, and dashed grey lines represent known interactions of a single protein. Proteins identified as being involved in telomere regulation were highlighted in red.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and Southern analysis

Protein extracts were prepared and analyzed as described previously [7]. Unless otherwise noted, whole-cell extracts were prepared by glass-bead lysis in HB buffer (25 mM HEPES pH 7.2, 60 mM B-glycerophosphate, 15 mM MgCl₂, 15 mM EGTA, 1mM dithiothreitol, 0.1 mM sodium vanadate, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) containing protease inhibitory cocktail (Roche). Trichloroacetic acid (TCA) extracts were prepared by glass-bead lysis in 20% TCA, with one wash in 5% TCA and a final wash in 100% ethanol. Unless otherwise noted, protein extracts were resolved by SDS-PAGE in 7% polyacrylamide in a protean3 unit (small gel) (Bio-Rad). Nuclear and mitochondrial isoforms of Pfh1 were resolved by SDS-PAGE in 8% polyacrylamide, 1% crosslinking at 30 mA in a SE400 unit (large gel) (Amersham), and protein samples were run until the 50 kDa molecular weight marker (Bio-Rad) was at the bottom of the gel. Protein transfer to nitrocellulose membrane (Amersham) was set-up overnight in buffer (25 mM Tris, 190 mM glycine, 20% methanol) at 120 mA.

Membranes were blocked in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20) with 5% milk. Probes used for western blot analysis included rabbit anti-Pfh1 polyclonal serum (1:1500) raised against the helicase domain of Pfh1 (447-708 aa) [9], mouse anti-GFP monoclonal serum (Roche), rabbit anti-Rdp1 polyclonal serum (1:500) (Abcam), and horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse immunoglobulin G polyclonal serum (1:3000) (Bio-Rad).

For *S. pombe*, genomic DNA was prepared by phenol-chloroform extraction as previously described [120]. ApaI (NEB) digested DNA was resolved on 1.2% agarose gels in
Tris-borate-EDTA. DNA was transferred to a nylon membrane (GE Healthcare). Southern blots were hybridized with a radiolabeled telomere oligonucleotide containing the *S. pombe* Taz1 binding sequence and imaged on a Typhoon Phosphorimager system (Molecular Dynamics).

**Flow cytometric analysis (FACS)**

*S. pombe* cells were collected in 165 mM EDTA, 0.1% sodium azide 70% EtOH. Cells were pelleted, washed in 100% EtOH, and stored at 4°C. In preparation for FACS analysis, approximately 2 x 10^6 cells were washed 3ml of 50 mM Na citrate, pH 7.2, and incubated overnight at 37°C in 0.5 ml 50 mM Na citrate plus 0.1 mg/ml RNaseA. Following sonication, cells were incubated in 1 uM Sytox Green (Molecular Probes) at room temperature for 30 minutes. Cells were analyzed using a FACScan single laser fixed-alignment benchtop analyzer.

**Microscopy**

Phase contrast and fluorescence microscopy of *S. pombe* cells were conducted using a DeltaVision microscope workstation (Applied Precision) using a 100x objective and CCD camera (Roper Scientific-CoolSNAP HQ). Cells were grown in supplemented EMM to mid-log. Exposure times were 0.1 sec (no filter) for phase contrast, 0.4 sec for GFP, and RFP with 100% neutral density filter. Images were acquired in 24 z sections at 0.15 μm intervals, deconvolved for 10 cycles with the ‘conservative’ preset, and single quick projection images visualized by flattening 16 central sections of 2.4 μm with the ‘max intensity’ preset.
<table>
<thead>
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<td>This study</td>
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**Table 1.** S. pombe and S. cerevisiae stains used in this study.
Chapter 3. Post-translational modification of Pfh1 as revealed by MS and studied by genetics

There are multiple isoforms of Pfh1 that are encoded by alternative translational start sites (Figure 1). The first translational start site, methionine codon M1 encodes the mitochondrial isoform, which, after cleavage of a mitochondrial targeting signal (MTS), yields a protein of 83-kDa. The second translational start site, M21 results in a nuclear isoform of 88 kDa. Western blot analysis of Phf1 suggests that post-translational modification occurs in the presence of DNA damage induced by camptothecin (CTP) [7], a result that was repeated in my hands (Figure 2A). To follow up on this result, I used a MS approach to identify post-translational modifications (PTM) of Pfh1 in the presence and absence of CPT from asynchronous cells. Given the known role of Pfh1 in DNA replication, I also searched for PTMs in synchronized cells harvested at G2 and S phase time points.

For cell synchronization, S. pombe cells containing the cdc25-22 temperature sensitive mutation were arrested in G2 at the non-permissive temperature of 37ºC. The phosphatase Cdc25 acts in tyrosine dephosphorylation and activation of Cdc2 required for the onset of mitosis [121]. Cells were released from G2 arrest and grown at the permissive temperature of 25ºC for synchronized growth. Cells harvested at the G2 time point were collected at the end of the arrest, and cells harvested at the S phase time point were collected 84 minutes after release, corresponding to the start of replication. Cell cycle progression and the timing of DNA replication were confirmed by FACS analysis.

The enrichment of Pfh1 enabled the use of use of multiple mass spectrometry instrument configurations, nanoLC LTQ-Orbitrap Velos with ETD and MALDI LTQ-Orbitrap XL, to detect
PTMs, which allowed for a comprehensive analysis of Pfh1 phosphorylations. Phosphopeptides were identified from the nanoLC LTQ-Orbitrap Velos instrument by MS and MS$^2$ data search settings, which included variable modifications of phosphoserine, threonine, and tyrosine of +80 Da. Phosphate group localization was confirmed by software and manual evaluation of peptide fragmentation patterns. MALDI MS/MS analyses of Pfh1 phosphopeptides were manually evaluated for phosphate group localization by peptide fragmentation patterns resulting from targeted CID MS$^2$ and MS$^3$ analysis (Figure 3, Table 2).

The two phosphorylation sites, S256 and S764, that were previously reported in the literature [122] were identified in a global MS study to identify protein phosphorylation sites in fission yeast. In this large-scale study modified proteolytic peptides were enriched for detection by affinity capture and acid/base chemistry, using methods of immobilized metal ion affinity chromatography (IMAC) and titanium dioxide (TiO$_2$), respectively [122]. Although these methods of phosphopeptide enrichment were not employed in my experiments, the two previously identified phosphorylation sites were verified in my results along with much more sensitive detection of additional novel sites of modification.

In total, ten phosphorylation sites were identified in Pfh1 (Table 2). Of these, two were previously reported in the genome-wide MS study [122], while the other eight sites have not been previously reported. Each of the ten phosphorylation sites is located within the N- and C-terminal domains of Pfh1, outside of the conserved helicase motifs (Figure 1). All of these modifications were observed in S phase cells. One site, S764 was detected in studies of the Pfh1 nuclear isoform. Two sites of phosphorylation, S81 and S764, were also detected in G2 phase cells and in asynchronous cells treated with CPT. However, no sites identified were specific to CPT treatment. Additionally, in the presence of phos-tag acrylamide [123], a compound that
inhibits the migration of phosphorylated proteins, the upper migrating band of Pfh1 observed by treatment of asynchronous cells with CPT was not impeded (Figure 2B). Thus, MS and western blot results suggest that, in the presence of DNA damage induced by CPT treatment, Pfh1 may have other PTMs in addition to phosphorylation, such as ubiquitination or sumoylation.

The relative abundance of specific Pfh1 phosphorylations was accessed by comparing the unweighted spectrum counts of the modified vs. unmodified peptide in multiple experiments (Table 3). The most abundant and readily detectable modifications were S81, T84, S256, and S764. To examine the biological function of Pfh1 phosphorylation, I focused on analysis of the effects of mutation of these four residues using site-specific mutagenesis. Mutations of serine or threonine to alanine (non-phosphorylatable mutant) or aspartic acid (phospho-mimetic) were generated in the coding sequence of Pfh1-GFP within the pJK148 integrating plasmid. Following confirmation of the correct mutation site and the entire open-reading frame of Pfh1-GFP by sequencing analysis, the linearized plasmid containing the Pfh1-GFP phospho-mutant under control of the pfh1+ native promoter was integrated at leu1-32 in strain ySP421. In this strain background, loxP sites flank the endogenous pfh1+ locus. Transformants with correct integration were confirmed by Southern blot analysis and subsequently transformed with a Cre expressing plasmid for the removal of endogenous Pfh1 by recombination at the loxP sites. Independent isolates of Pfh1-GFP phospho-mutants were chosen after the removal of endogenous Pfh1 expression.

First, I determined if these mutant proteins were stable, using western blot analysis (Figure 4, lanes 1-12). Protein was prepared from asynchronous cells of two independent isolates for each mutation. Each of the Pfh1-GFP mutant proteins was stably expressed, but the level of expression varied from mutant to mutant and sometimes from isolate to isolate (summary of
phospho-mutant data Table 4). The non-phosphorylatable mutant Pfhl-S81A T84A S256A S764A-GFP appeared to have higher expression than wild-type Pfhl in two independent isolates (Figure 4, lane 1 compared to lanes 3 and 5), while the non-phosphorylatable mutant Pfhl-S81A S256A S764A-GFP shows higher expression in isolate 1 (Figure 4, lane 7) but lower expression in isolate 2 (Figure 4, lane 8). This western blot analysis was performed one time. Given the observed variability especially for isolates that didn’t give the same answer, this experiment should be repeated multiple times.

I also examined if there is a modified, slower migrating band in the non-phosphorylatable mutant proteins isolated from CPT-treated cells. The response of the non-phosphorylatable Pfhl-S81A T84A S256A S764A-GFP mutants to CPT treatment was the same as compared to wild-type (Figure 4, lane 2 compared to lanes 4 and 6), with an upper, slower migrating band presumably due to the post-translational modification of Pfhl in response to CPT treatment. This result with the alanine, non-phosphorylatable mutant of Pfhl further suggests that the upper migrating band observed by western blot analysis is not due to phosphorylation of S81, T84, S256, or S764.

At least three isolates of each phospho-mutant was examined for viability and growth phenotypes. Strains expressing single Pfhl phosphorylation mutations of S81, T84, S256, or S764 were viable (Figure 5A). Strains expressing the single mutation Pfhl-S764A-GFP and Pfhl-S81D-GFP showed a negative growth phenotype at 30º C in preliminary results (Figure 5A arrows). However, this negative growth phenotype was not consistent among multiple isolates. For example, eight of ten Pfhl-S81D-GFP isolates grew slower than wild-type cells (80%), while only six of 13 Pfhl-S764A-GFP isolates displayed slower growth than wild-type cells (45%) (Figure 5B). However, mutation of Pfhl-T84A/D alone or in combination with S81A/D
consistently resulted in a negative growth phenotype in three out of three isolates (100%). The inconsistency of growth phenotype could be explained if a given phospho-mutation negatively affected growth, giving a growth advantage to suppressors that mask the growth phenotype.

Pfh1 phospho-mutations were also examined for a possible role in telomere length maintenance. In *S. cerevisiae* the founding Pif1 family member, ScPif1, is an inhibitor of telomerase, and therefore, negatively regulates telomere length. Interestingly, ScPif1 has been shown to have multiple states of phosphorylation; with some modifications playing a role in regulating telomerase activity at telomeres and other modifications induced by DNA damage signaling, which regulate telomerase activity at double-stranded breaks [14]. In the literature, there are conflicting reports about whether or not *S. pombe* Pfh1 affects telomere length. The first report found that deletion of *pfh1*+ is associated with telomere shortening [9]. In this study, telomere length was analyzed in a population of *pfh1* delete cells derived by sporulation of a *pfh1+/pfh1*Δ diploid, which can germinate and divide one or more times before growth arrest. More recently, it has been reported that there is no effect of nuclear Pfh1 depletion on telomere length [7]. In this study nuclear Pfh1, expressed from the thiamine-repressible nmt promoter, was depleted from the cell by growth in thiamine containing medium. Although no nuclear Pfh1 was detectable in this experiment by western blot analysis, a low level of Pfh1 might be present in the nucleus due to incomplete repression of the nmt promoter and it has been shown that even a low level of Pfh1 is sufficient for cell viability [9]. (Both of these published studies are from the Zakian lab.)

To determine if any of the phospho-mutants affected telomere length, I prepared DNA from four different single mutants (one isolate of each) and examined them by Southern blot analysis (Figure 6). Telomere length was slightly longer in cells expressing Pfh1 S764A-GFP
and Pfh1 S81D-GFP mutations (Figure 6, lanes 1 and 4), and slightly shorter in the S764D-GFP mutant compared to wild-type (Figure 6, lane 2). Although these results observed in only one isolate of each mutation is by no means conclusive, opposite effects on telomere length upon mutation of S764 to an A or a D residue suggests that phosphorylation of Pfh1 at the C-terminal residue S764 plays a role in telomere length regulation.

Telomeres were very short in cells expressing Pfh1-mt*, which have normal levels of mitochondrial Pfh1 but very low levels of nuclear Pfh1 (Figure 6, lane 6). It should be noted that this Pfh1-mt* strain was struck from a -80°C freezer stock, which prior to freezing, had been grown for many generations with limited nuclear Pfh1. To determine if telomere shortening could be attributed to low levels of nuclear Pfh1, I regenerated the pfh1-mt* but this time covered the mutation with a plasmid expressing wild-type Pfh1. I then streaked this strain on media where only cells lacking the plasmid could grow. Even after 300 cell divisions, the pfh1-mt* strain maintained wild-type length telomeres. I conclude that the absence of nuclear Pfh1 does not have a negative effect on telomere length. The second experimental protocol, in which Pfh1-mt* is expressed in the presence of a wild-type Pfh1 covering plasmid is closer to the one used to study the phospho-mutants.

It was previously described that Pfh1 localizes to sites of DNA damage, suggesting it may play a role in DNA repair [7]. Pfh1-GFP co-localizes with Rad22-RFP, the *S. pombe* homolog of *S. cerevisiae* Rad52. As a key player in double-stranded break repair, Rad22 forms foci in response to DNA damage [124]. To investigate if Pfh1 phosphorylation affects its role in DNA repair, I examined the co-localization of Pfh1-GFP with DNA repair foci (labeled by Rad22-RFP) by fluorescent microscopy. If phosphorylation of Pfh1 is important for repair, then it might be expected that a phospho-mutant would fail to localize to Rad22 foci. The occurrence
of single or multiple DNA repair foci as indicated by Rad22-RFP was not significantly different in Pfh1-S81A/D-GFP or Pfh1-S764A/D-GFP single mutants as compared to wild-type cells (Figure 7A and B). Also, the ability of Pfh1-GFP to co-localize with Rad22-RFP was not affected in any of the phospho-mutant strains examined (arrow in Figure 7A), suggesting that these single phosphorylation events do not influence Pfh1’s function in DNA repair.

The growth of Pfh1 single phosphomutations (one isolate of each) was accessed at both 23°C (to test for cold-sensitivity) and 30°C (normal growth temperature). Due to the essential nuclear functions of Pfh1 in DNA replication and repair, cells expressing cold-sensitive pfh1 alleles and grown at restrictive temperature will arrest at G2 phase of the cell cycle [31]. As expected, Pfh1-mt* cells that express normal levels of mitochondrial Pfh1 but very low levels of nuclear Pfh1 display a cold sensitive phenotype (Figure 8A). Pfh1-S764A and Pfh1-S81D showed a slight negative growth phenotype at 23°C compared to wild-type, suggesting that the modification of either of these specific mutations may impair the nuclear functions of Pfh1 in DNA replication and/or repair (Figure 8A).

Two independent isolates of Pfh1 phospho-mutants were tested in the presence of a variety of DNA damage inducing drugs. Camptothecin (CPT) inhibits topoisomerase and results in cell cycle arrest in G2/M. Treatment of cells with hydroxyurea (HU) leads to stalled replication forks and cell cycle arrest in S phase. Bleomycin (BM) induces both single and double-stranded DNA breaks, while methylmethane sulfonate (MMS) causes double-stranded breaks by stalling replication fork progression. No growth phenotype was observed with expression of any of the mutations in the presence of CPT or MMS (see Table 4 for a summary of phenotypes). However wild-type Pfh1-GFP, which presumably may have slightly impaired function due to its epitope tag, showed a negative growth phenotype on HU and a positive
growth phenotype on BM (see discussion for further interpretation of this result). Consequently when examining phenotypes of Pfh1-GFP phospho-mutants, growth was always compared to wild-type Pfh1-GFP.

In the presence of hydroxyurea (HU) at 30°C, cells expressing the single mutations S81A/D and S764A/D showed a positive growth phenotype compared to Pfh1-GFP (Figure 8B). However, cells expressing the double mutation S81D S764D showed a negative growth phenotype (Figure 9), while cells expressing the triple mutation S81A/D S256A/D S764A/D showed a positive growth phenotype (Figure 10) compared to Pfh1-GFP in the presence of HU.

In the presence of BM, cells expressing the triple mutation S81A S256A S764A showed a negative growth phenotype compared to Pfh1-GFP (Figure 10). While cells expressing the quadruple mutation S81D T84D S256D S764D showed a positive growth phenotype compared to Pfh1-GFP in the presence of BM (Figure 11). These growth phenotypes that are not straightforward for interpretation, suggest that multiple sites of Pfh1 phosphorylation may function in combination, making it difficult to assign specific function to select Pfh1 phosphorylation sites. However, several phospho-mutants (with two independent isolates each) display a growth phenotype different from that of Pfh1-GFP in the presence of HU or BM, leading me to conclude that phosphorylation of Pfh1 does play a functional role in DNA replication and/or repair.

Discussion

Nuclear and mitochondrial isoforms of Pfh1 play essential roles in the replication of chromosomal and mitochondrial DNA, respectively and also function in DNA repair [7]. A previous graduate student in the Zakian lab, Stefan Pinter demonstrated that the nuclear isoform
of Pfh1 is localized to sites of DNA damage and that in the presence of exogenous DNA damage
induced by CPT treatment, a post-translationally modified form of the protein is detected by
Western blot analysis. Phosphorylation of a PIF1 family homolog in *S. cerevisiae*, ScPif1, has
been previously characterized in response to DNA damage [14].

By enrichment of Pfh1-GFP by immunoaffinity purification and subsequent mass
spectrometric analysis, I have identified ten phosphorylation sites in Pfh1, eight of which are
novel (Table 2). Two sites confirmed in my study, Pfh1-S256 and –S764, were previously
identified in a large-scale proteomic study in *S. pombe* [122]. All ten modified residues are
located in less conserved regions of the Pfh1 protein, with nine sites residing in the N-terminal
domain and one site residing in the C-terminal domain; all of the characterized phosphorylation
sites lie outside of the highly conserved helicase domains (Figure 1).

Targeted MS analysis was used to characterize phosphorylation of Pfh1 under various
conditions: wild-type and the nuclear only Pfh1 in the presence and absence of DNA damage
induced by CPT in asynchronous cells, and wild-type Pfh1 in S and G2 synchronized cells
(summary of data, Table 2). The C-terminal phosphorylation site, S764 was identified in IP
experiments of the Pfh1 nuclear isoform, confirming that this modification occurs in nuclear
localized Pfh1. In S cells, all ten characterized phosphorylation sites were identified in wild-type
Pfh1, while in G2 cells two sites of modification, S81 and S764, were identified. Modifications
of S81 and S764 were additionally confirmed in asynchronous cells treated with CPT.

Under normal Western blot conditions, a slower, upper migrating band of Pfh1 is
observed from cells treated with CPT, suggesting post-translational modification in the presence
of CPT induced DNA damage (Figure 2A). Western blot analysis using Phos-tag acrylamide to
further impede phosphorylated species showed no difference in Pfh1 migration with or without CPT (Figure 2B). Suggesting global phosphorylation levels of Phf1 do not change in the presence of CPT. Furthermore, Western blot analysis of the non-phosphorylatable mutant Pfh1-S81A T84A S256A S764A displayed the same slower, upper migrating band as seen in wild-type Pfh1 in the presence of CPT (Figure 4).

Based on MS and Western blot analysis data, I conclude that Pfh1 has a basal level of phosphorylation with at least ten modified residues. As suggested by experiments using Phos-tag acrylamide, overall levels of Pfh1 phosphorylation do not change in the presence of DNA damage induced by CPT. Therefore in the presence of CPT, Pfh1 may incur a different form of post-translational modification such as ubiquitination or sumoylation that is responsible for a slower migrating band observed by Western blot. However, these experiments do not address or eliminate the possibility that levels of phosphorylation may change at specific residues in response to DNA damage. MS spectrum count analysis can only allow for relative quantification of a given peptide between experiments and conditions. In addition, the abundance of different peptides cannot be compared due to differences in ionization efficiency of any given amino acid sequence. Also important for consideration is the fact that ionization efficiency of a phosphorylated peptide is less than that of the same unmodified peptide due to the negative charge of an additional phosphate group. Therefore, it is significantly more difficult to detect the phosphorylated form of a modified residue by MS/MS analysis. In my most current experiments, 10 sites of Pfh1 phosphorylation have been detected. However, more modifications may be present which are undetected due to low ionization efficiency. As technologies and sample preparation improve, the ability to identify phosphorylated residues also improves. Indeed throughout the maturation of these MS experiments, sensitivity of phosphopeptide detection
increased along with the number of identified Pfh1 phosphorylation sites. Absolute quantification of peptides by an experiment employing a technique such as isobaric tagging [125] would be helpful in order to access changes in Pfh1 phosphorylation between conditions.

To elucidate the biological significance of Pfh1 sites of phosphorylation, site-specific mutagenesis studies were conducted. Four of the most abundant and readily detectable phosphorylation sites identified, S81, T84, S256, and S764, were mutated to alanine or aspartic acid either alone or in combinations. Variable growth phenotypes were observed with multiple isolates of the same mutation (Figure 5B). This could perhaps be explained if a given phospho-mutation negatively affected growth, giving a growth advantage to suppressors that mask the growth phenotype.

Preliminary evidence suggests that phosphorylation of Pfh1-S764 may have an effect on telomere regulation. Southern blot analysis of genomic DNA showed longer telomeres in the non-phosphorylatable Pfh1 mutant S764A, while the phospho-mimetic S764D showed slightly shorter telomeres. I conclude from this preliminary data obtained from one isolate of each mutant, that phosphorylation of Pfh1 at residue S764 may have negative effect on telomere length. I present data in chapter five that supports a model in which Pfh1 positively regulates telomere lengthening by the telomerase enzyme. If this data can be replicated in multiple isolates it would support the conclusion that Pfh1 phosphorylation at S764 negatively regulates Pfh1’s role at the telomere.

The growth of Pfh1 phospho-mutant strains was accessed at cold temperature, and in the presence of a variety of DNA damage inducing drugs. Due to the essential nuclear functions of Pfh1 in DNA replication and repair, cells expressing cold-sensitive pfh1 alleles and grown at
restrictive temperature will arrest at G2 phase [31]. Pfh1-S764A and Pfh1-S81D showed a slight negative growth phenotype at 23°C compared to wild-type, suggesting that the modification of either of these specific mutations may impair the nuclear functions of Pfh1 in DNA replication or repair. Two independent isolates of Pfh1 phospho-mutants were tested in the presence of DNA damage inducing drugs (summary of results, Table 4): Camptothecin (CPT) inhibits topoisomerase and results in cell cycle arrest in G2/M, hydroxyurea (HU) leads to stalled replication forks and cell cycle arrest in S, Bleomycin (BM) induces both single and double-stranded DNA breaks, while methylmethane sulfonate (MMS) causes double-stranded breaks by stalling replication fork progression. No growth phenotype was observed in the presence of CPT or MMS. However wild-type Pfh1-GFP, which presumably may have slightly impaired function due to the epitope tag, showed a negative growth phenotype on HU and a positive growth phenotype on BM. It was recently demonstrated that Pfh1, similarly to S. cerevisiae Rrm3, plays an important role in replication fork progression [30]. Thus, in the presence of impaired Pfh1-GFP, it makes sense that cell growth would be negative affected by an increase in replication fork stalling due to HU. I present data in chapter 4 that suggests Pfh1 directly interacts and perhaps migrates with the replication fork. A possible role for Pfh1 as a member of the replication fork machinery could be in facilitating DNA repair at single and double strand breaks. In the presence of BM, growth of cells expressing Pfh1-GFP is dramatically improved compared to normal cells. A possible interpretation of this result is that partially impaired Pfh1-GFP does not facilitate DNA repair and replication is allowed to proceed through single-stranded breaks. In this process it would be expected that many mistakes would be incorporated into the genome during DNA replication. This model could be tested by examining if mutation rates increase with BM treatment of Pfh1-GFP expressing cells. Several phospho-mutants displayed a
growth phenotype different from that of Pfh1-GFP in the presence of HU or BM, leading me to conclude that phosphorylation of Pfh1 does play a functional role in DNA replication and/or repair.
Figure 1. Sites of Pfh1 phosphorylation identified outside of the conserved helicase motifs. Schematic representation of Pfh1 ORF. Phosphorylated serine and threonine residues identified by MS analysis are labeled with an arrow and located outside of the conserved helicase motifs. Novel sites of phosphorylation identified in this study are labeled in red. Pfh1 exists in multiple isoforms that are encoded by alternative translational initiation at the labeled methionine codons M1 or M21. M1 encodes the mitochondrial isoform which, after cleavage of a mitochondrial targeting signal (MTS), yields a protein of 83-kDa. Translation from M21 results in a nuclear isoform of 88 kDa.
Figure 2. Pfh1 post-translational modification in response to DNA damage. (A) Western blot analysis of Pfh1 isoforms from asynchronous cells. In the presence of DNA damage induced by treatment with camptothecin (CPT) there is a slower, upper migrating band suggestive of post-translational modification. (B) In the presence of phos-tag, migration of the upper band present with CPT treatment is not impeded, suggesting that the PTM is not phosphorylation.
Figure 3. Sites of Pfh1 phosphorylation detected using a nanoLC LTQ-Orbitrap Velos with ETD and a MALDI LTQ-Orbitrap XL instrument configurations. Modifications were validated for phosphate group localization by software and manual evaluation of peptide fragmentation patterns. (A) Confirmation of Pfh1 phosphorylation at S81 and T84 by manual MS² and MS³ analysis of a doubly phosphorylated peptide. Evidence of a phosphate group observed by a +80 Da peak in the MS spectra. Loss of phosphoric acid observed by a -98 Da shift in MS² and MS³ spectra. (B) Selected CID MS/MS spectra representing phosphorylated peptides of Pfh1.
Figure 3. (B) Continued

**T212 (R)SRT*LPWAVDPYR(Y)**

![MS spectrum of T212 (R)SRT*LPWAVDPYR(Y)](image)

**S256 (R)SSS*LDSLAK(K)**

![MS spectrum of S256 (R)SSS*LDSLAK(K)](image)

**S288 (K)FSVPLNSASKS*PIGSSLFK(T)**

![MS spectrum of S288 (K)FSVPLNSASKS*PIGSSLFK(T)](image)

**S764 (R)ATTQEGQLQVLNFS*PAK(V)**

![MS spectrum of S764 (R)ATTQEGQLQVLNFS*PAK(V)](image)
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<th>$Z$ b</th>
<th>$\Delta M$ (ppm)</th>
<th>G2</th>
<th>S</th>
<th>CPT</th>
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<td>S75</td>
<td>Y</td>
<td>(R)SSTNDQQTFFSQS*DNLPSSPITLPAK(R)</td>
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<td>(R)SSTNDQQTFFSQS*DNLPSSPITLPAK(R)</td>
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<td>S764</td>
<td>N</td>
<td>(R)ATTQEGLQVLNF*PAK(V)</td>
<td>6.21</td>
<td>2</td>
<td>2.8</td>
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**Table 2. Pfh1 sites of phosphorylation characterized in this study.** Phosphopeptides observed in cells from three different experimental conditions: G2 time-point of the cell cycle, S time-point of the cell cycle, and asynchronous cells treated with camptothecin (CPT). Novel sites of phosphorylation identified in this study are in red. Two sites of Pfh1 phosphorylation, S256 and S764, have previously been reported in a large-scale phospho-proteomics study in fission yeast [122]. (a) $X_c$ - SEQUEST cross-correlation score; (b) Z - charge state
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<th>S mod/ unmodified experiment</th>
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<td>0/0</td>
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<td>S764</td>
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<td>21/93</td>
<td>19/89</td>
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Table 3. Observed spectrum counts of Pih1 phosphopeptides over four experiments at two different time points in the cell cycle, S and G2. For each experiment, spectrum counts of modified peptides are expressed over the spectrum counts of unmodified peptides. Novel sites of phosphorylation identified in this study are in red.
Figure 4. Protein expression of the Pfh1-GFP mutants is stable but variable, and the response of non-phosphorylatable mutants to CPT treatment is similar to wild-type. Western blot analysis of Pfh1 phospho-mutants in the presence and absence of DNA damage induced by CPT. Protein was prepared from asynchronous cells. Two independent isolates of each mutation were analyzed. Lower molecular weight bands on the gel are presumed to be protein degradation products.
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**Table 4. Summary of Pfh1 phospho-mutant data.** + indicates Wt levels of growth or protein expression. Additional + signs symbolize greater than Wt growth or protein expression and - signs symbolize less than Wt. Two independent isolates of each mutation was tested. If data for two isolates was not in agreement, it is presented for both isolates. NG represents no growth. Blank entries represent not tested. Grey shading represents growth phenotypes all similar to wild-type S. pombe. Symbols in red highlight differences observed between a Pfh1-GFP phospho-mutant compared with wild-type Pfh1-GFP control.
**Figure 5. Strains expressing Pfh1 phospho-mutations are viable.** Cell growth of Pfh1 phospho-mutants at 30°C. (A) Smaller, slow growing colonies were observed with mutations S81D and S764A. Pfh1-mt* expresses mitochondrial but has very low levels of nuclear Pfh1. (B) Summary of observed growth phenotypes in multiple isolates of single and double combinations of Pfh1 phosphorylation mutants.
Figure 6. Telomere length variable in Pfh1 single phospho-mutants. Southern blot analysis of telomere length with EcoRI (left) and Apal (right) digestion of genomic DNA probed with radiolabeled oligonucleotides to the S. pombe Taz1 telomere binding sequence. Telomere length observed to be slightly longer in cells expressing Pfh1 S764A and S81D mutants, and slightly shorter in the S764D mutant compared to wild-type. Very short telomeres were observed in cells with the mutation Pfh1-mt* which expresses mitochondrial but has very low levels of nuclear Pfh1.
(A)  
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<th>GFP</th>
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<td>Pfh1 Wt</td>
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<td>Pfh1 Wt + CPT</td>
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<td>pfh1 S764A</td>
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(B)  
- **Cells with Rad22 Foci**  
- **Cells with Multiple Rad22 Foci**

*S81A, n=1
n=3 for all other strains*
Figure 7. *Pfh1* wild-type and phospho-mutants co-localize with *Rad22*. (A) Co-localization of *Pfh1*-GFP was observed by florescent microscopy with *Rad22*-RFP in the presence and absence of DNA damage (CPT treatment) and in a strain expressing *Pfh1 S764A*. Arrow points to an example of a foci in which *Pfh1*-GFP and *Rad22*-RFP are co-localized. (B) Percentage of cells observed with single (left) or multiple (right) *Rad22* foci in single phosphomutation strains.
Figure 8. Growth of Pfh1 S81D is improved at 30°C in the presence of hydroxyurea (HU). Log-phase cells were spotted at 10-fold serial dilutions on plates with and without 5 mM HU (A) At 23°C growth of Pfh1 phospho-mutants is similar to wild-type both with and without HU (B) At 30°C in the presence of HU growth of S81D is better than wild-type. Pfh1- mt* refers to a cold sensitive strain lacking nuclear Pfh1 and expressing only mitochondrial Pfh1.
Figure 9. Growth of Pfh1-S764D S81D-GFP worse than wild-type Pfh1-GFP in the presence of HU. Pfh1-GFP grew significantly worse than wild-type *S. pombe* cells on HU. Growth of double phospho-mutants was not affected by CPT or MMS. Log-phase cells were spotted at 5-fold serial dilutions on plates with the indicated concentrations of HU, CPT, and MMS.
Figure 10. Growth of Pfh1- S81 S256 S764-GFP nonphosphorylated and phosho-mimetic better than Pfh1-GFP on HU, and growth of nonphosphorylated mutant worse on bleomycin (BM) than Pfh1-GFP. Pfh1-GFP grew significantly better than wild-type S. pombe cells on BM. Log-phase cells were spotted at 5-fold serial dilutions on plates with indicated concentrations of HU, CPT, MMS, and BM.
Figure 11. Growth of Pfh1-S81D T84D S256D S764D-GFP better than Pfh1-GFP on BM. Log-phase cells were spotted at 5-fold serial dilutions on plates with the indicated concentrations of HU, CPT, MM, S, and BM.
Chapter 4. Proteomic characterization of Pfh1 protein interactions

To elucidate further the cellular function of the essential *S. pombe* Pif1 family helicase Pfh1, I performed immunoaffinity purification (IP) of Pfh1-GFP to identify associated proteins by mass spectrometry. IP experiments from asynchronous cells were conducted using the previously described *S. pombe* strains ySP207, ySP210, ySP390, and ySP398 [7] (Table 1) in which Pfh1-GFP or Pfh1\textsuperscript{m1}-GFP (nuclear isoform) are expressed from the *leu1-32* locus under its native promoter, with endogenous Pfh1 expression maintained at the *pfh+* locus. As previously described, wild-type Pfh1-GFP localizes to the nucleus and mitochondria while Pfh1\textsuperscript{m1}-GFP, which has a mutation in the translational start site of the mitochondrial isoform and lacks the mitochondrial targeting signal (Figure 1), localizes exclusively to the nucleus [7]. The molecular mass of the nuclear and mitochondrial isoforms of Pfh1 are 88 and 83 kDa, respectively, with the GFP fusions proteins (+27.5kDa) running at approximately 116 and 111 kDa. Control IP experiments designed to identify nonspecific interactions of the GFP epitope tag, were conducted using the *S. pombe* strain yKM346 in which GFP with two SV40 nuclear localization signals (GFP-NLS) are expressed from the *leu1-32* locus under control of the nmt promoter.

With optimized lysis buffer conditions, Pfh1-GFP and GFP control were immunoisolated from asynchronous, S-phase, and G2-phase *S. pombe* cell populations using magnetic beads conjugated with anti-GFP antibodies. Immunoisolated Pfh1-GFP or GFP alone were eluted from the beads in SDS buffer at 70°C with shaking. The eluted target protein and associated interactions of an IP experiment were resolved by SDS-PAGE in a 4-12% gradient gel, with each IP sample run in a single lane with at least two empty lanes next to it to avoid contamination from neighboring samples. Gel lanes were cut into 1mm segments, grouped in approximately eight fractions per lane and prepared for in-gel digestion with trypsin. Peptides were analyzed by
nanoLC-tandem MS (MS/MS) on a LTQ-Orbitrap Velos and MALDI MS (MS/MS) on a LTQ-Orbitrap XL mass spectrometer (see Chapter 1 Methods, for more details regarding instrumentation and MS analysis). LC MS/MS results were analyzed using the SEQUEST database (Proteome Discoverer) and subsequently loaded into Scaffold with filtering conditions were set to ≥ 2 unique peptides, <1% peptide, and protein false discovery rate.

IP of Pfh1-GFP was optimized by examining the solubility of Pfh1-GFP and observed associated protein interactions in a variety of lysis buffer conditions. A first set of experiments was conducted with varying concentrations of NaCl, and the detergents Triton X-100 (Tx) and deoxycholate (DOC) in a lysis buffer (buffer 1) based on that of the Cristea Lab (20 mM HEPES-KOH, pH 7.4, containing 0.1 M potassium acetate, 2 mM MgCl2, 0.1% Tween 20, 1 μM ZnCl2, 1 μM CaCl2, 0.5% Triton X-100, 250 mM NaCl, 1/100 (v/v) protease), which was originally described for the purification of S. cerevisiae nuclear pore complexes [112]. The amount of soluble Pfh1-GFP protein found in the supernatant versus the amount of insoluble protein found in the pellet were compared with lysis buffer containing 150 or 250 mM NaCl and 0.25%- 1.0% Tx, and 0.5% Tx/ 5% (DOC) by western blot analysis (Figure 12). Based on these results, conditions of higher salt, 250 mM NaCl, and higher detergent, 1% Tx, were determined to be optimal for Pfh1-GFP solubility.

With these buffer 1 conditions, IP of the nuclear isoform, Pfh1-m1-GFP (strain ySP210) and wild-type Pfh1-GFP (strain ySP207) were performed with eight grams of cells, 7mg of antibody-conjugated magnetic beads, and elution with ammonium hydroxide. Following separation of the Pfh1-GFP IP sample on a 4-12% gradient gel, the target and associated proteins were visualized by Coomassie staining (Figure 13A). As determined by Western blot analysis, Pfh1-GFP was efficiently isolated from the supernatant and eluted from the beads, and was not
detected in the pellet (Figure 13B). The lower molecular weight degradation product of Pfh1-GFP visualized in both the Coomassie stain gel and the Western blot was more prominent in the IP experiment of the nuclear Pfh1 than wild-type.

Using the optimized conditions described above, IP of the nuclear and wild-type Pfh1 isoforms were conducted in S. pombe cells with DNA damage induced by camptothecin (CPT) treatment (40µM CPT for 3 hours) (Figure 14). CPT inhibits topoisomerase and results in cell cycle arrest in G2/M. Western blot analysis of Phf1 suggests that post-translational modification occurs in the presence of DNA damage induced by camptothecin (CTP) [7], a result that was repeated in my hands (Figure 2A). Strains used in this experiment were the nuclear isoform Pfh1-m1-GFP strain ySP210, and wild-type Pfh1-GFP strain ySP398, which additionally expresses Chk1-3HA, whose mobility was used as a control to confirm cellular DNA damage induced by CPT treatment. In the presence of DNA damage, Chk1 is phosphorylated and this modification can be visualized as a slower migrating band by Western blot analysis [126] (Figure 2B). It was confirmed by MALDI collision induced dissociation (CID) MS/MS and MS3 analysis that in experiments both with and without CPT treatment, that the nuclear isoform of Pfh1 is phosphorylated at amino acid residue S764 (Figure 15). Modification of S764, as well as, S81 and T84 were identified in the wild-type Pfh1-GFP IP samples (Figure 3). No additional sites of modification were detected in the presence of CPT treatment.

Although the IP of Pfh1-GFP was successful under these initial lysis buffer conditions of 250 mM NaCl, 1% Tx, several expected protein interactions were not observed. Therefore, a less stringent lysis buffer condition was employed with lower salt concentration (150 mM NaCl) and the addition of phosphatase inhibitors- 50mM sodium fluoride (NAF) and 10mM sodium orthovanadate NaVO₄ to maintain interactions (Figure 16). With lower salt and the addition of
phosphatase inhibitors, more interactions were detected and multiple members of protein complexes were observed including the Ku70/Ku80 heterodimer required for DNA repair and telomere maintenance in eukaryotic cells, and the DNA replication factor A (RPA) heterotrimer single-stranded DNA binding protein complex (composed of 68, 30, and 12 kDa subunits) that is required for DNA replication, recombination, repair, and telomere maintenance. These interacting proteins were observed in replicate analyses from three independent isolates and characterized on complementary MS instrument configurations, MALDI LTQ-Orbitrap XL and nanoLC LTQ-Orbitrap Velos (Figure 16). Interestingly, these specific interactions were not detected using lysis buffer 1 containing 150 mM NaCl and no phosphatase inhibitors, suggesting that they are dependent on phosphorylation of itself, Phf1, or some other mediating protein.

A second set of experiments was conducted using a lysis buffer containing varying concentrations of potassium acetate in lysis buffer (buffer 2) that was based on that of the Labib lab (100mM Hepes KOH, pH 7.9, 300mM potassium acetate, 10mM magnesium acetate, 10% glycerol, 0.1% NP-40, 2mM EDTA, 2mM B-glycerophosphate, 50mM NaF, 10mM NaVO₄, 1mM DTT, protease inhibitor cocktail (Roche)), which was described for the purification of the S.cerevisiae replisome-associated GINS complex [127] (Figure 17). The efficiency of immunoisolation of Pfh1-GFP and associated proteins were compared under conditions of buffer 2 containing 50 mM, 300 mM, and 900 mM potassium acetate in a small-scale preliminary study using approximately 4 g of cells. IP samples were visualized after separation in a 4-12% gradient gel by Coomassie staining (Figure 17A), while the solubility of the target was accessed by western blot comparison of the abundance of Pfh1-GFP present on the beads after immunopurification, in the buffer flow through after collection of the beads, and in the pellet of cellular debris (Figure 17B). By western blot analysis it was observed that with conditions of
50mM potassium acetate, Pfhl-GFP is less soluble, with more present in the pellet than flow through (Figure 17B, lane 3 versus lane 2). With 300 mM potassium acetate, more Pfhl-GFP was successfully immunoisolated on the beads and less non-soluble protein was present in the pellet (Figure 17, lane 4 versus lane 6). With 900 mM potassium acetate, even less Pfhlp was observed in the pellet (Figure 17, lane 9), but Pfhl-GFP was also not detected in the flow through or bead sample, perhaps suggesting that at this high concentration of salt Pfhl-GFP is not stable or perhaps more likely suggesting a problem with sample loading as the amount of non-specific bands at ~50 kDa are less in the Coomassie stained gel. Ultimately, optimal buffer conditions of 300 mM potassium acetate were chosen based on yield of Pfhl-GFP and co-isolated proteins as determined by MS analysis.

Using the optimized conditions of buffer 2 with 300 mM potassium acetate, Pfhl-GFP was isolated from an asynchronous population of cells (strain ySP398) and the co-isolated proteins were analyzed by MS analysis. Proteins were resolved by SDS-PAGE and visualized by Coomassie stain (Figure 18). Peptides of identified proteins were confirmed by MS/MS with nanoLC LTQ Orbitrap CID analyses. Pfhl interaction was observed with proteins involved in DNA replication and repair including the replicative DNA polymerase Pol2, members of the replicative Mcm helicase complex, and the Ku70/Ku80 heterodimer required for DNA repair and telomere maintenance in eukaryotic cells. The only proteins in common in experiments using buffer 1 or buffer 2 were Ku70/Ku80. Based on these results, buffer 2 appeared to be the better choice for studying Pfhl protein interactions. Thus, for all experiments detailed below, proteins were identified in cells prepared in lysis buffer 2.

To determine if DNA mediates the interactions of Pfhl-GFP, chromosomal DNA of the cell lysate was digested with an excess of DNaseI (640 U/g of cell or ~70 ug/ml, 30 min.
incubation at 4°C) during the IP experiment immediately before the addition of conjugated beads (Figure 19). The IP of Pfh1-GFP and co-isolated proteins was not changed by the addition of DNaseI treatment of the cell lysate as visualized by Coomassie stain (Figure 19A) and subsequently confirmed by MS analysis. To determine if digestion of genomic DNA by DNaseI was successful, DNA precipitated from the cell lysate before and after DNaseI treatment was visualized on an agarose gel by ethidium bromide stain (Figure 19B). Low molecular weight DNA was observed in samples of cell lysate taken before and after DNaseI treatment (Figure 19B, lanes 1 and 2), suggesting that chromosomal DNA was degraded during earlier steps of the IP experiment before the addition of DNaseI. Enzymatic activity of DNaseI was not affected in the cell lysate, as demonstrated by the digestion of plasmid DNA (pDNA) that was added to a sample of the cell lysate prior to DNaseI digestion (Figure 19B, lanes 3 and 4).

Since this study focuses on the nuclear functions of Pfh1, interactions of the Pfh1 nuclear (not the mitochondrial) isoform are of particular interest. In addition, I was interested in interactions that may regulate the nuclear isoform in response to DNA damage. Thus, IP experiments were conducted using the optimized buffer 2 conditions with the ySP210 strain expressing the nuclear isoform of Pfh1, Pfh1-m1-GFP both in the presence and absence of CPT-induced DNA damage (Figure 20). However, no significant differences were detected by MS analysis between nuclear only Pfh1 in the presence or absence of DNA damage compared to samples of wild-type Pfh1 from undamaged cells.

Cell cycle regulated interactions of Pfh1

To assess if any of the Pfh1 interactions were important for DNA replication, I performed an IP of Pfh1 during S phase with the idea that interactions important for Pfh1’s role in DNA
replication might be specific to or enriched in S phase cells. With the optimized lysis buffer 2 conditions, Pfh1-GFP and GFP control were immunoisolated from S-phase, and G2-phase S. pombe cell populations. For cell synchronization, S. pombe cells containing the cdc25-22 temperature sensitive mutation were arrested in G2 at the non-permissive temperature of 37°C. The phosphatase Cdc25 acts in tyrosine dephosphorylation and activation of Cdc2 required for the onset of mitosis [121] . Cell cycle progression and the timing of DNA replication were confirmed by FACS analysis. Strains expressing either Pfh1-GFP (yKM333) or GFP alone (yKM346) from the S. pombe leu1-32 locus in a strain background containing the cdc25-22 mutant were confirmed to progress normally through the cell cycle by FACS analysis (Figure 21). Cells were released from G2 arrest and grown at the permissive temperature of 25°C for synchronized growth. Cells harvested at the G2 time point were collected at the end of the arrest, and cells harvested at the S phase time point were collected at 84 minutes after release from the G2 phase, corresponding to the start of replication.

Pfh1 protein expression during the cell cycle was studied using a strain expressing Pfh1-13MYC (NS103), which migrates at approximately 125 kDa. Pfh1-13-MYC protein expression was not regulated during the cell cycle as seen by western blot analysis of cell fractions obtained every 20 minutes during a cell synchrony experiment (this experiment was conducted by post doctoral fellow Nasim Sabouri in the Zakian lab; Figure 22). Despite unequal loading of protein sample, western blot analysis suggests that Pfh1 abundance is not cell cycle regulated (Figure 22). As expected for the same volume of culture collected, there were more cells present at later time points of the cell cycle due to cell division. I conclude that Pfh1 is not cell cycle regulated, as the protein level of Pfh1 does not increase and decrease again with progression of the cell cycle. However, since we are using a temperature sensitive cdc25-22 strain for cell
synchronization experiments, it should be confirmed that Pfh1 protein stability is not affected by temperature by Western blot analysis of Pfh1 harvested from cells grown at the temperature of arrest, 37ºC, and the temperature of release, 25ºC.

Immunoisolation of Pfh1-GFP or GFP at S or G2 time-points of the cell cycle was conducted with two biological replicates at each S and G2 time-points. IP experiments from S and G2 phase cells were conducted in parallel followed by relative quantification of protein interactions by MS using unweighted spectrum count analysis (Figure 23A). Differences in protein interactions between S and G2 phase IP samples of Pfh1-GFP were first visualized by Coomassie stained gels and could especially be seen in the size range of 75-125 kDa (Figure 23B).

Protein interactions were accessed for specificity and enrichment using the computational program SAINT (significance analysis of interactome) for probabilistic scoring using label-free quantitative data [117]. Interactions of the IP experiments of Pfh1-GFP and GFP alone at the S phase time-point were paired for SAINT analysis as experimental and control data sets, respectively (Supplemental Table 1). Similarly, interactions of the IP experiments of Pfh1-GFP and GFP alone at the G2 time-point were paired (Supplemental Table 2). Data imported for SAINT analysis included unweighted spectrum counts and size (number of amino acids) of each observed protein hit. Highly significant protein interactions, either in S or G2 phase of the cell cycle, were identified by a SAINT confidence score of greater than 0.80. The 51 protein interactions of Pfh1 that make this cut-off are listed in Supplemental Table 3.

More than half of the proteins that meet the stringent ≥ 0.80 SAINT score are involved in DNA replication, repair, and/or telomere biology (Figure 23C proteins in bold). Additional
interactions were identified as significant if they are annotated by the interaction database biogrid (thebiogrid.org/) as a physical or genetic interaction of a protein that did score greater than 0.80, and a specific interaction of Pfh1-GFP compared to GFP alone (Figure 23C proteins not in bold). These protein interactions were considered specific if, with a spectrum count greater than 5, they show greater than 2-fold spectral count enrichment in at least one Pfh1-GFP experiment compared to GFP control experiments. Protein interactions that were detected at low levels with less than five spectral counts were only identified as a specific interaction if it was not present in any of the GFP control experiments. These specific interactions bring the list to a total of 114 (Supplemental Table 4).

The accession numbers of proteins identified as significant interactions were imported into STRING (http://string-db.org/) for performing network analysis. For visualization of STRING networks, data were exported as a PSI-XML format and imported into Cytoscape [119]. With Cytoscape visualization, nodes represent protein interactions, solid dark lines represent protein complexes, solid grey lines represent known interactions of a complex, and dashed grey lines represent known interactions of a single protein. I also determined the number of significant interactions of Pfh1 with proteins known to affect telomere length. I identified such proteins as telomere proteins if they are identified in UniProt.org with the GO term “telomere maintenance.” Proteins identified as being involved in telomere regulation are highlighted in red (Figure 23D). In total 17 of the 91 proteins (19%) that interacted specifically with Pfh1 are involved in telomere maintenance.
Discussion

Immunoaffinity purification of Pfh1-GFP and associated protein complexes was optimized in lysis buffer 2 with 100 mM HEPES, 300 mM potassium acetate, and 0.1% NP-40. Proteomic analysis of Pfh1 was completed under a variety of conditions including wild-type and nuclear only Pfh1 from asynchronous cells in the presence and absence of DNA damage induced by CPT, and wild-type Pfh1 at S and G2 time-points of the cell cycle.

Under asynchronous cell cycle conditions, Pfh1 interaction was observed with proteins involved in DNA replication and repair including the replicative DNA polymerase Pol2, members of the replicative Mcm helicase complex, and the Ku70/Ku80 heterodimer required for DNA repair and telomere maintenance in eukaryotic cells. These interactions were not DNA mediated, as DNaseI treatment of the cell lysate did not change the profile of protein interactions.

Novel Pfh1 interactions identified during S phase further implicate Pfh1 as functioning in DNA replication, repair, and telomere maintenance. Protein interactions were accessed for specificity and enrichment using the computational program SAINT for probabilistic scoring using label-free quantitative data [117]. Highly significant protein interactions, either in S or G2 phase of the cell cycle, were identified by a SAINT confidence score of greater than 0.80. Of the 51 protein interactions of Pfh1 that make this cut-off (Supplemental Table 4), more than half are involved in DNA replication, repair, and/or telomere biology (Figure 23C proteins in bold).

Pfh1 has recently been shown by experiments of Nasim Sabouri, a postdoctoral fellow of the Zakian lab, to be required for efficient replication fork movement in the ribosomal DNA, the mating type locus, tRNA, 5S ribosomal RNA genes, and genes that are highly transcribed by
RNA polymerase II [30]. The importance of Pfh1 function during replication is demonstrated with the accumulation of converged replication forks at all of these sites in the absence of Pfh1, as observed by 2D gel analysis. Furthermore, genetic assays suggest that Pfh1, like its S. cerevisiae homolog ScPif1, may also play role during DNA replication in Okazaki fragment maturation [29, 31, 32].
Figure 12. Pfh1-GFP solubility optimal in lysis buffer 1 conditions of 250 mM NaCl and 1% TritonX-100 (Tx). Western blot analysis of Pfh1-GFP after resuspension of cells in lysis buffer condition 1 with varying concentrations of NaCl, and detergents Tx and deoxycholate (DOC). The soluble (supernatant) and insoluble (pellet) fraction of Pfh1-GFP were compared between conditions. Pfh1-GFP runs at approximately 115 kDa. Lower molecular weight bands on the gel are presumed to be protein degradation products.
Figure 13. Immunoaffinity-purification of Pfh1-GFP nuclear isoform and wild-type with conditions of 250 mM NaCl, 1% Tx. (A) Coomassie stained gel of Pfh1m1-GFP nuclear isoform (left) and Pfh1-GFP wt (right) IP samples. (B) As determined by western blot analysis, Pfh1-GFP was efficiently isolated from the supernatant and eluted from the beads, and was not detected in the pellet. The lower molecular weight degradation product of Pfh1-GFP was more prominent in the IP experiment of the Pfh1 nuclear isoform than wild-type, as seen in both the coomassie stained gel and western blot.
Figure 14. Immunoaffinity-purification of Pfh1-GFP nuclear isoform and wild-type in the presence of DNA damage induced by camptothecin (CPT). (A) Coomassie stained gel of Pfh1\textsuperscript{m1}-GFP nuclear isoform (left) and Pfh1-GFP wt (right) IP samples from cells treated with CPT (B) Western blot analysis of IP efficiency.
Figure 15. Nuclear isoform of Pfh1 is phosphorylated at S764 in the presence or absence of DNA damage induced by CPT. MALDI MS/MS and MS$^3$ analysis of the Pfh1 peptide containing amino acid residue S764 obtained from the IP of the nuclear isoform, Pfh1-m1-GFP. Evidence of a phosphate group was observed by a +80 Da peak in the MS spectra and manually evaluated by targeted CID MS$^2$ and MS$^3$ analysis. Localization of the phosphate group was confirmed by a -98 Da shift in the MS$^2$ spectra, corresponding to the loss of phosphoric acid.
Figure 16. Interaction of Pfh1-GFP with yKu70/80 and Rpa1 observed with conditions of 150 mM NaCl, 1% Tx, and phosphatase inhibitors. Immunoaffinity-purification and MS analysis of Pfh1-GFP and associated proteins under less stringent salt conditions. Proteins were resolved by SDS-PAGE and visualized by Coomassie Stain. Peptides were confirmed by MALDI LTQ CID and nanoLC LTQ Orbitrap CID analyses.
Figure 17. Pfh1-GFP solubility optimal in lysis buffer 2 conditions of 300 mM potassium acetate and 0.1% NP-40. Immunofinity-purification of Pfh1-GFP with buffer 2 containing 50-900 mM potassium acetate and 0.1% NP-40. (A) Proteins were resolved by SDS-PAGE and visualized by coomassie stain (B) The amount of Pfh1-GFP bound on the beads, found in the soluble (flow through) fractions and insoluble (pellet) fractions were compared between conditions.
Figure 18. Interaction of Pfh1-GFP with DNA replication proteins Pol2 and the Mcm helicase complex observed in asynchronous cells with optimized conditions of lysis buffer 2. Immunoaffinity-purification of Pfh1-GFP from asynchronous cells with conditions of buffer 2 containing 300 mM potassium acetate and 0.1% NP-40. Proteins were resolved by SDS-PAGE and visualized by Coomassie stain. Peptides of identified proteins were confirmed by MS with nanoLC LTQ Orbitrap CID analyses. Pfh1 interaction was observed with proteins involved in DNA replication and repair such as the replicative DNA polymerase Pol2, members of the replicative Mcm helicase complex, and the Ku70/Ku80 heterodimer required for DNA repair and telomere maintenance in eukaryotic cells.
Figure 19. Immunoaffinity-purification of Pfh1-GFP not affected by DNase I treatment of the cell lysate. (A) Proteins were resolved by SDS-PAGE and visualized by Coomassie Stain. (B) Ethidium bromide stained agarose gel of precipitated DNA from an aliquot of the cell lysate before and after DNase I treatment (lanes 1 and 2) and with the addition of plasmid DNA (lanes 3 and 4) as a control for DNase I activity in the experimental lysis buffer.
Figure 20. Immunoaffinity-purification of Pfh1-GFP nuclear isoform in the presence and absence of DNA damage induced by CPT reveals similar protein interactions of wild-type Pfh1-GFP. The nuclear isoform Pfh1\textsuperscript{m1}-GFP was isolated from cells grown in the presence and absence of CPT. Proteins were resolved by SDS-PAGE and visualized by Coomassie Stain.
Figure 21. Strain yKM333 expressing Pfh1-GFP in a cdc25-22 strain background displays normal cell cycle progression. FACS analysis of genomic DNA content during cell cycle progression, with 0 minutes corresponding to time of release from G2 arrest.
**Figure 22. Pfh1 protein expression is not cell cycle regulated.** (A) Western blot analysis of Pfh1-13MYC expression in a *cdc25-22* strain background over a synchronized cell cycle. Anti-MYC western blot (top) and anti-Rdp1 (bottom). (B) FACS analysis of genomic DNA content over the same cell synchrony. Cell synchrony and FACS analysis by Nasim Sabouri.
Figure 23. Identification of Pfh1 cell cycle-specific interactions. (A) Experimental design for parallel immunoaffinity purifications of Pfh1-GFP and GFP from cells harvested at G2 or S phase of the cell cycle and relative quantification of Pfh1 specific interactions by MS. (B) Immunoaffinity-purifications of GFP or Pfh1-GFP performed at G2 and S time points resolved by SDS-PAGE gel with the target and associated proteins visualized by Coomassie stain. (C) Diagram displaying highly enriched and specific protein interactions of Pfh1-GFP that are involved in DNA replication, repair, and telomere regulation. Proteins in bold scored a SAINT significant score of enrichment of >0.80. Proteins not in bold are specific interactions of Pfh1-GFP that did not make the SAINT score cut-off but are known to interact with another protein of SAINT score >0.80. (D) Cytoscape visualization of highly enriched and specific protein interactions of Pfh1 that are involved in DNA replication, repair, and telomere regulation. Nodes represent protein interactions, black solid lines connect members of a complex, dashed grey lines represent interactions of a protein complex, while solid grey lines represent interactions of a single protein. *S. pombe* proteins involved in telomere regulation are highlighted in bright pink.
Chapter 5. Role of Pfh1 at the telomeres

As revealed by mass spectrometry (MS), Pfh1 interacts with several proteins involved in telomere maintenance. Immunoaffinity purification (IP) of Pfh1-GFP during S phase of the cell cycle and subsequent MS analysis of associated proteins resulted in the identification of 17 proteins that are involved in telomere maintenance (see Chapter 4- Proteomic characterization of Pfh1 protein interactions). These 17 proteins represent 33% of the 51 total proteins in *S. pombe* that are annotated in the UniProt database with the gene ontology (GO) term, “telomere maintenance” (Figure 24). Pfh1’s interaction with this representative group of proteins suggests a possible functional role of Pfh1 in telomere regulation.

In *S. cerevisiae* the founding Pif1 family member, ScPif1, is an inhibitor of telomerase and therefore negatively regulates telomere length. In the literature, there are conflicting reports about whether or not *S. pombe* Pfh1 affects telomere length. The first report found that deletion of *pfh1+* is associated with very modest telomere shortening [9]. In this study, telomere length was analyzed in a population of *pfh1* delete cells derived by sporulation of a *pfh1+/pfh1Δ* diploid, which can germinate and divide one or more times before growth arrest. More recently, it has been reported that there is no effect of nuclear Pfh1 depletion on telomere length [7]. In this study nuclear Pfh1, expressed from the thiamine-repressible nmt promoter, was depleted from the cell by growth in thiamine containing medium. Although no nuclear Pfh1 was detectable in this experiment by western blot analysis, a low level of Pfh1 might be present in the nucleus due to incomplete repression of the nmt promoter and it has been shown that even a low level of Pfh1 is sufficient for cell viability [9]. (Both of these published studies are from the Zakian lab.)
In my experiments, the effects of Pfh1 on telomere length were studied by its overexpression in a strain that has little or no nuclear Pfh1 expression, in which the mitochondrial only isoform of Pfh1, Pfh1-mt*, is expressed from the *leu1-32* locus and the endogenous *pfh1+* is deleted. This strain was created by loss of the Pfh1 expression plasmid, pVS117 in strain ySP377. The pVS117 *his3*+ marked plasmid was maintained in cells by growth in EMMS (minimal media) minus histidine, and lost by growth in fully supplemented EMMS media. Telomere length was accessed in ySP377 before and after the loss of pVS117. Pfh1 overexpression by plasmid pVS117 was confirmed by Western blot analysis (Figure 25B).

In the presence of pVS117, telomeres were maintained for over 200 generations at a stable length that was approximately 500 bp longer than the wild-type length of approximately 300 bp (Figure 25A lanes 2-10 versus lane 1). When the plasmid was lost, telomeres abruptly shortened to approximately 300 bp and were maintained for over 200 generations at the same length as wild-type cells (Figure 25A lanes 11-17 versus lane 1). When the plasmid was re-transformed into cells, a gradual lengthening effect was observed, with bulk telomeres measuring approximately 600 bp after 3 restreaks which corresponds to ~75 generations. An equilibrium length of approximately 500 bp was reached after 8 restreaks (~200 generations) (Figure 25A lanes 18-25). This lengthening effect is specific to Pfh1 overexpression, as transformation of the plasmid empty vector did not result in a change in telomere length (Figure 25A lanes 26-33).

To determine if telomere elongation from Pfh1 overexpression is due to recombination or a telomerase mediated event, Pfh1 was overexpressed in a recombination deficient strain, *rhp51Δ* cells (strain MCW3) [124]. In the absence of telomerase, *S. pombe* cells maintain telomeres by recombination or chromosome circularization [107]. *S. pombe* Rhp51 is required for telomere maintenance by homologous recombination [108]. In *rhp51Δ* cells, longer telomeres are
observed in the presence of the Pfh1 overexpression plasmid pVS117 in three independent isolates after three restreaks (approximately 75 generations) (Figure 26A lanes 4-6) compared to the *rhp51Δ* strain background (lanes 10-12). Cells transformed with the empty vector plasmid (EV; Figure 26A lanes 1-3) or plasmid expressing the helicase dead Pfh1 K337A mutation (lanes 7-9) had telomere length the same as *rhp51Δ* and did not exhibit telomere lengthening. Thus, the telomere lengthening effect observed upon Pfh1 overexpression in the Pfh1-mt* strain background is also observed in an *rhp51Δ* recombination deficient strain background.

In fission yeast there are multiple recombination pathways that occur through Rhp51-dependent and independent events. To address the possibility that the telomere lengthening effect observed upon overexpression of Pfh1 in the *rhp51Δ* strain background occurs through Rhp51-independent recombination events, the Southern blot of Figure 25A was re-analyzed after probing with an STE1 subtelomere probe (Figure 26B). The *S. pombe* STE1 telomere probe was previously described as demonstrating hyperrecombination events of greater than 2.5 kb in a *taz1Δ* background [128]. *S. pombe* cells lacking the telomere binding protein Taz1 have misregulation of chromosome ends that results in elevated telomeric recombination [128]. No bands greater than 2.5 kb, that are indicative of recombination events, were observed in *rhp51Δ* cells transformed with plasmid pVS117 (Figure 26B), suggesting that telomere lengthening observed with Pfh1 overexpression is not due to Rhp51 independent recombination events.

These results support the model that telomere lengthening by Pfh1 is telomerase mediated. Furthermore, telomere lengthening was not observed upon overexpression of the helicases dead Pfh1 K337A mutant protein. This result indicates that the ATPase/helicase activity of Pfh1 is needed to cause telomere lengthening. However, it should be noted that in the *rhp51Δ* strain background, pVS117 overexpression of Pfh1 and Pfh1 K337A has not yet been
confirmed by western blot analysis to be comparable in the two strains (experiment in progress). However, in subsequent overexpression experiments the abundance of Pfh1 K337A was shown to be similar to that of Pfh1 wild-type (Figure 27B).

To elucidate further the function by which Pfh1 positively regulates telomere length, experiments in the Pfh1-mt* background were repeated with overexpression of the Pfh1 point mutations pfh1-K337A and pfh1-L430P. The pfh1-K337A mutation, which is a residue in the invariant lysine in the Walker A box, abolishes the essential in vivo helicase function of Pfh1. In the human Pif1 homolog, the mutation corresponding to pfh1-L430P is associated with a predisposition for breast cancer [129]. In strain ySP377, in which only the mitochondrial isoform of Pfh1 is expressed, telomere length was examined in three independent isolates over four restreaks (approximately 100 generations) in the presence or absence of pVS117, a plasmid overexpressing wild-type Pfh1, Pfh1-K337A, or Pfh1-L430P. Telomere length was consistently observed to be longer upon wild-type Pfh1 overexpression, in agreement with earlier experiments (Figure 27A lanes 6-9). However, inconsistent results were observed over the three isolates in regard to telomere length in the presence of Pfh1-K337A or Pfh1-L430P overexpression (Figure 27A lanes 10-17). Western analyses show that Pfh1-L430P protein expression was significantly lower than wild-type Pfh1 (Figure 27B), and this difference might explain the inconsistent effects of overexpression of this mutant on telomere length. Thus, plasmid expression of Pfh1-wild-type, K337A, and L430P should be examined in all isolates over the multiple restreaks.
Discussion

Telomere lengthening was observed in response to Pfh1 overexpression in the Pfh1-mt* background in which nuclear Pfh1 is absent. It is interesting to note that the same effect of telomere lengthening upon Pfh1 overexpression is not observed in a wild-type strain background in which endogenous nuclear Pfh1 is present. Telomere lengthening was additionally observed in response to Pfh1 overexpression in the recombination deficient \textit{rhp51}Δ strain background, suggesting that the effect of Pfh1 on telomeres is telomerase mediated. However, in the \textit{rhp51}Δ strain like the wild-type strain, endogenous Pfh1 is expressed in the cell. Perhaps this difference in telomere length phenotype could be the result of levels of DNA damage signaling in cells somehow regulating Pfh1’s positive regulation of telomere length. DNA damage signaling would be expected to be higher in both the \textit{pfh1-mt}* and \textit{rhp51}Δ strains in which telomere lengthening is observed in response to Pfh1 overexpression.

To confirm that telomere elongation by Pfh1 is telomerase mediated and not a recombination mediated event, Pfh1 overexpression experiments were repeated in a telomerase deficient or \textit{rad22}Δ strain. \textit{S. pombe} Rad22, like its \textit{S. cerevisiae} Rad52 homolog, plays a central role in recombination being required for both Rhp51-dependent and independent events. However, experiments examining telomere length upon Pfh1 overexpression in telomerase deficient (\textit{ter1}Δ) or \textit{rad22}Δ cells were not successful due to the inability to passage these strains for more than one restreak (~25 generations). The telomere length effect of Pfh1 could only be detected in the \textit{pfh1-mt}* strain after three restreaks (~75 generations).

Additional evidence that further defines Pfh1’s function at telomeres comes from experiments conducted by post-doctoral fellow Nasim Sabouri in the Zakian lab. Chromatin
immuno-precipitation (ChIP) of Pfh1-13Myc showed the specific association of Pfh1 with telomeric DNA during S phase of the cell cycle. Further 2D-gel analysis of telomeric DNA before and after Pfh1 deletion in the cell revealed multiple sites of replication fork pausing in Pfh1’s absence. Dr. Sabouri’s data suggests that similar to *S. cerevisiae* Pif1 family member Rrm3, Pfh1 facilitates replication fork progression at the telomeres. However, unlike *S. cerevisiae* Pif1 family member Pif1, Pfh1 positively regulates telomere length. Our data support a speculative model that by Pfh1 facilitating replication fork progression at the telomeres, they may be more accessible or in a more favorable state for telomerase to act, resulting in a telomere lengthening phenotype. A possible way to test this model might be a ChIP experiment in which Trt1 (telomerase catalytic subunit) binding is measured at the telomeres in the presence and absence of Pfh1 overexpression.
Figure 24. Cytoscape visualization of *S. pombe* proteins involved in telomere maintenance. Nodes represent protein interactions, black solid lines connect members of a protein complex. Proteins identified by IP and MS analysis as being highly enriched and a specific interaction of Pfh1-GFP are colored in red. These 17 proteins represent 33% of the 51 total proteins in *S. pombe* that are annotated in the UniProt database with the gene ontology (GO) term, “telomere maintenance.”
Figure 25. Overexpression of Pfh1 causes telomere lengthening. (A) Southern blot analysis of Apal digested DNA from WT (lanes 1 and 34) or independent isolates of cells struck over several generations in the presence or absence of pVS117, a plasmid over-expressing Pfh1. In strain ySP377, in which only the mitochondrial isoform of Pfh1 is expressed (Pfh1-mt*), telomere length is observed to be longer than that of WT cells in the presence of pVS117 (lanes 2-10). When pVS117 is lost, ySP377 telomeres decrease in length to approximately the same as wild-type (lanes 11-17). ySP377 cells that have lost the plasmid and have shorter telomeres were transformed with either pVS117 or EV plasmid. Cells transformed with pVS117 regained longer telomeres (lanes 18-25) while cells transformed with EV plasmid did not (lanes 26-33). (B) Western blot analysis using anti-Pfh1 antibodies showing ySP377 cells in the presence or absence of the overexpressing pVS117 plasmid (top). Protein samples were separated on a 6% acrylamide gel. The membrane was Ponceau S stained to examine loading and transfer of the gel (bottom).
Figure 26. Overexpression of Pfh1 causes telomere lengthening in a recombination deficient strain background. (A) Southern blot analysis of Apal digested DNA from three independent isolates of rhp51Δ cells (strain MCW3) after three restreaks (approximately 75 generations) in the presence or absence of pVS117, a plasmid over-expressing Pfh1. In rhp51Δ cells, telomere length is observed to be longer in the presence of pVS117 (lanes 4-6) than compared to rhp51Δ strain background (lanes 10-12). Cells transformed with the plasmid EV (lanes 1-3) or plasmid expressing the helicase dead Pfh1 K->A mutation (lanes 7-9) had telomere length the same as rhp51Δ and did not exhibit a telomere length effect. (B) Southern blot of (A) stripped and re-probed with Ste1 subtelomeric probe.
Figure 27. Telomere lengthening effect observed upon over-expression of Pfh1. (A) Southern blot analysis of Apal digested DNA from three independent isolates over four restreaks (approximately 100 generations) in the presence or absence of pVS117, a plasmid over-expressing wild-type Pfh1, Pfh1-KA, or Pfh1-L430P. In strain ySP377, in which only the mitochondrial isoform of Pfh1 is expressed, telomere length is consistently observed to be longer upon wild-type Pfh1 overexpression. (B) Western blot analysis using anti-Pfh1 antibodies showing ySP377 cells in the presence or absence of the overexpressing pVS117 plasmid. Protein samples were separated on a 7% acrylamide gel.
Chapter 6. Protein interactions of *Schizosaccharomyces pombe* telomerase

Experimental contributions of postdoctoral fellow Christopher Webb of the Zakian lab are acknowledged within this chapter.

To elucidate further the process of telomerase assembly, activation, and its recruitment to the telomere, *in vivo* protein interactions of the *Schizosaccharomyces pombe* telomerase holoenzyme were analyzed by mass spectrometry. Known components of the *S. pombe* telomerase holoenzyme are the protein components Trt1 (telomerase reverse transcriptase) and Est1, as well as, the telomerase RNA, TER1.

Various Trt1-GFP epitope tagged strains were initially constructed with Trt1 expressed at its endogenous locus and under its native promoter. The GFP tag was incorporated at the Trt1 amino (N) or carboxyl (C) terminus, both with and without a glycine linker, which has been demonstrated to improve the *in vivo* function of tagged telomerase proteins [111]. One copy of the *trt1*+ gene was tagged in a diploid yeast strain by transformation of a PCR construct. Integration of the construct at the desired target sequence was confirmed by Southern blot analysis, diagnostic PCR, and DNA sequencing. Following sporulation and tetrad dissection, all transformants were observed to segregate 2:2 for the selectable marker, supporting Southern data indicating a single integration event per transformant.

To determine if Trt1 expressed with a GFP epitope tag is functional *in vivo*, cell viability and telomere length maintenance were analyzed (Figure 28). *S. pombe* strains expressing C-terminally tagged Trt1 with the linker displayed wild-type cell growth and telomere length (Figure 28 A,B), while *S. pombe* strains expressing C-terminally tagged with no linker and N-
terminally tagged Trt1 displayed wild-type cell growth and stable, but slightly shorter, telomere length (summary of phenotypes, Figure 28C).

However, Trt1 expressed at endogenous levels with a GFP epitope tag was not detectable by Western blot analysis or fluorescent microscopy, presumably due to its low level of protein expression, and attempts at immunoaffinity purification (IP) of Trt1-GFP for proteomic analysis of associated proteins were unsuccessful.

Challenges of low protein abundance were overcome by overexpressing the protein and RNA components of the telomerase holoenzyme. GFP-Trt1 and GFP-Est1 were expressed at their endogenous loci under the nmt promoter, while TER1 was expressed from the previously described high copy number his3+ marked plasmid, pSP1-Ter1+ [77]. Characterization of S. pombe telomerase overexpression strains was completed with the help of a visiting undergraduate student Daniela Garcia (now a graduate student in the Zakian lab), who worked under my guidance. Cells individually overexpressing GFP-Trt1 or GFP-Est1 were visualized by fluorescent microscopy. GFP-Trt1 was localized throughout the nucleus (Figure 29 left panel), while GFP-Est1 was localized to single focus at the nuclear periphery (Figure 29 right panel, arrow).

S. pombe haploid cells individually expressing trt1::nmt-GFP-Trt1 or est1::nmt-GFP-Est1 were mated, sporulated, and tetrad dissected. The two alleles segregated 2:2, supporting Southern data indicating a single integration event per transformant. Two independent spore isolates were obtained that contained both trt1::nmt-GFP-Trt1 and est1::nmt-GFP-Est1 for subsequent transformation with plasmid pSP1-Ter1+. Northern blot analysis conducted by postdoctoral-fellow Christopher Webb of the Zakian lab confirmed that TER1 was
overexpressed by plasmid pSP1 at levels of approximately 20x more than wild-type (Figure 30A). As shown by western blot analysis, the telomerase protein components GFP-Trt1 and GFP-Est1 were more abundant in the presence of TER1 overexpression (Figure 30B, lanes 4 and 5 vs. lanes 1-3), suggesting that stability of the telomerase proteins is dependent on the telomerase RNA.

The overexpression of telomerase protein components Est1-GFP, Trt1-GFP, and the telomerase RNA resulted in a telomere lengthening effect of greater than 150 bp compared to wild-type cells as shown by Southern blot analysis of EcoRI and ApaI digested genomic DNA of two independent spore isolates (Figure 31). The observed telomere lengthening effect suggests that the overexpressed components of the telomerase holoenzyme form a functional complex.

IP of GFP-Trt1 and GFP-Est1 from strain yKM316 in which the telomerase proteins, as well as, the telomerase RNA were overexpressed was conducted in an asynchronous population of cells using a previously optimized lysis buffer for *S. pombe* (described in Chapter 4 for the IP of Pfh1): 100mM Hapes KOH, pH 7.9, 300mM potassium acetate, 10mM magnesium acetate, 10% glycerol, 0.1% NP-40, 2mM EDTA, 2mM B-glycerophosphate, 50mM NaF, 10mM NaVO₄, 1mM DTT, protease inhibitor cocktail (Roche). Proteins were resolved by SDS-PAGE and visualized by Coomassie stain (Figure 32A). Co-isolated proteins were analyzed by MS analysis and peptides of identified proteins were confirmed by MS/MS with nanoLC LTQ Orbitrap CID analyses. Previously conducted *S. pombe* control IP experiments with GFP were used to access nonspecific protein interactions (Figure 23B).

After excluding nonspecific protein interactions that are observed in the GFP control experiments and interactions that were detected at less than five spectral counts, a short list of 15
prominent interactions was revealed (Table 5). Included on this list are two proteins, Pof8 and Ccq1 (a protein directly involved in telomerase recruitment to the chromosome), both of which are known to trigger short telomeres (>150bp shorter than wild-type) when deleted [69, 130]. Ccq1 binds Est1 and functions in the recruitment of telomerase to telomeres [27, 28]. Additionally of interest on this list is a protein involved in mRNA nuclear export, Ptr1. A protein known to indirectly interact with Ptr1, Mlo3, was recently identified by a post doc in the Zakian lab in a RNA three-hybrid experiment as interacting with telomerase RNA (C.J. Webb unpublished results). The deletion of Mlo3 results in short telomeres (50-150 bp shorter than wild-type) [130], a result repeated in the hands of C.J. Webb (Figure 33). Stm1, another protein involved in the pathway of mRNA nuclear export, also affects telomere length as telomeres are 50-150 bp longer than wild-type in a strain deleted for stm1 [130]. The telomere phenotypes of Mlo3 and Stm1 both involved in the S. pombe mRNA nuclear export pathway suggests a possible role of these proteins and Ptr1 in telomerase RNA maturation and/or assembly into the telomerase complex.

As Ptr1 is essential, it was not previously identified as affecting telomere length in the genome-wide screen of S. pombe deletion mutants of nonessential genes [130]. The ptr1 mutation was first identified and studied in a screen of temperature-sensitive (ts) mutants that are defective in mRNA export at the nonpermissive temperature [131]. The ptr1+ gene is characterized as a putative homolog of S. cerevisiae Tom1, a HECT-type ubiquitin ligase E3 [131]. The ts mutant ptr1-1 has a defect in nuclear export of poly(A)+ mRNA but no defect in nuclear import or export of protein [131]. Rae1 is an essential mRNA export factor that localizes to the nuclear periphery. Indicating their functional interaction, the double mutant ptr1-1 rae1-167 displays a synthetic growth defect [131]. Further implicating Ptr1 in the nuclear mRNA
export pathway, Rae1 interacts in a complex with Mlo3, and in *S. cerevisiae* overexpression of Stm1 suppresses the mutation of Tom1 (homolog of Ptr1) [132].

Analysis of telomere length was conducted at the permissive temperature of 25°C and the semipermissive temperature of 30°C in three independent isolates of wild-type and *ptr1-1* mutant cells (Figure 34A). Telomere length was also examined in *rae1-167* and double mutant *ptr1-1 rae1-167* cells at 25°C, but not at 30°C as neither strain grew at the higher temperature. It is important to keep in mind that there is a general temperature effect on telomere length maintenance; it is well known that telomeres are modestly shorter at high temperatures. Telomeres were approximately 100 bp shorter in the *ptr1-1* mutant than wild-type cells at 25°C (Figure 34A, lanes 1-6) and this difference was slightly increased at the semipermissive temperature of 30°C (Figure 34A, lanes 13-18).

To determine if this increase in telomere length is due to telomerase or recombination, the Southern blot in Figure 34A was stripped and reprobed with the Ste1 subtelomeric probe (Figure 34B), which detects telomere hyperrecombination that generate novel telomere band of greater than 2.5 kb in a *taz1Δ* background [128]. *S. pombe* cells lacking the telomere binding protein Taz1 have misregulation of chromosome ends that results in elevated telomeric recombination [128]. No bands indicative of recombination were detected in the *ptr1-1* mutant when grown at 25°C or 30°C (Figure 34B red boxes, lanes 4-6 vs. lanes 16-18). Thus, the increase in telomere length in *ptr1-1* mutant cells is likely due to telomerase, not recombination.

Telomere length maintenance was examined in two independent *ptr1-1* isolates grown for many generations at 30°C (Figure 35). In both isolates, telomeres were approximately 100 bp shorter than in wild-type cells grown at 30°C or *ptr1-1* cells grown at 25°C after five restreaks.
While one isolate showed shorter telomeres out to eight restreaks (approximately 200 generations) (Figure 35, left panel), the second isolate picked up a suppressor after streak 5 (Figure 35, right panel).

One explanation for the effects of Ptr1 and Mlo3, two proteins that function in mRNA nuclear export, on telomere length is that they affect the localization, maturation, and/or stability of TER1 (telomerase RNA). During telomerase assembly, TER1 is shuttled between the nucleus and the cytoplasm. If this process were disrupted, it would be expected to have a negative effect on telomerase holoenzyme assembly and consequently negatively affect telomere length. To test this hypothesis, Northern analysis and RNA FISH of TER1 in ptr1-1 cells grown at 25°C and 30°C and in mlo3Δ cells at 30°C are underway. Christopher Webb in the Zakian lab conducted a FISH experiment using Stellaris RNA probes designed against TER1 to compare nuclear versus cytoplasmic TER1 RNA localization. Preliminary results suggest that ptr1-1 has more TER1 localized in the nucleus when grown at the partially permissive temperature of 30°C (Figure 36C) compared to ptr1-1 cells grown at 25°C (Figure 36B) or TER1 localization in wild-type cells (Figure 36A). Surprisingly preliminary results suggest that mlo3Δ has the opposite effect than anticipated on TER1 localization. In mlo3Δ more TER1 is localized to the cytoplasm than nucleus compared to wild-type cells (Figure 36C). Computational analysis is underway for quantitative assessment of TER1 localization to the cytoplasm and nucleus in ptr1-1 cells grown at 25°C and 30°C and in mlo3Δ cells at 30°C.

Discussion

Proteomic analysis of in vivo S. pombe telomerase interactions was conducted in haploid cells overexpressing the telomerase protein components epitope tagged with GFP as well as the
telomerase RNA, TER1. In the *S. pombe* telomerase overexpression strain, longer telomeres were observed compared to wild-type cells, suggesting that the overexpressed telomerase components form a functional complex (Figure 31). IP of GFP-Trt1 and GFP-Est1 from strain yKM316 in which the telomerase proteins, as well as, the telomerase RNA were overexpressed was conducted in an asynchronous population of cells using a previously optimized lysis buffer for *S. pombe* Pfh1 (see Chapter 4). Co-isolated proteins were analyzed by MS analysis and peptides of identified proteins were confirmed by MS/MS with nanoLC LTQ Orbitrap CID analyses.

After excluding nonspecific protein interactions that are observed in the GFP control experiments and interactions that were detected at less than five spectral counts, a short list of 15 prominent interactions remained (Table 5 and Supplemental Table 5). Known interactions of the *S. pombe* telomerase holoenzyme include the Sm proteins and Ccq1. Sm proteins, which play an important role in RNA processing and stability, have been demonstrated to associate with *S. pombe* TER1 to facilitate its proper processing and association with Trt1 [133]. Ccq1 binds Est1 and functions in the recruitment of telomerase to telomeres [27, 28]. Although Sm proteins associate with the telomerase RNA they were not identified in my studies; this may not be surprising as Sm proteins might only be part of the telomerase complex as it matures or be very loosely associated. Included on this list are two proteins, Pof8 and Ccq1, both of which are known to trigger short telomeres (>150bp shorter than wild-type) when deleted [69, 130].

A novel interaction of interest on this list is a protein involved in mRNA nuclear export, Ptr1. A protein known to interact with the same pathway as Ptr1, Mlo3, was recently identified by a post doc in the Zakian lab in a RNA three-hybrid experiment as interacting with telomerase RNA (C.J. Webb unpublished results). The deletion of Mlo3 results in short telomeres (50-150
bp shorter than wild-type) [130], a result repeated in the hands of C.J. Webb (Figure 33). Stm1, another protein involved in the pathway of mRNA nuclear export, also affects telomere length as telomeres are 50-150 bp longer than wild-type in a strain deleted for stm1 [130]. The telomere phenotypes of Mlo3 and Stm1 both involved in the *S. pombe* mRNA nuclear export pathway suggests a possible role of these proteins and Ptr1 in telomerase RNA maturation and/or assembly into the telomerase complex.

Growth of *ptr1-1* at the partially permissible temperature of 30°C showed telomeres maintained approximately 100 bp shorter than in wild-type cells grown at 30°C or *ptr1-1* cells grown at 25°C over eight successive restreaks (Figure 35A). Telomere shortening appears to be telomerase mediated and not due to recombination (Figure 35B). One explanation for the effects of Ptr1 and Mlo3, two proteins that function in mRNA nuclear export, on telomere length is that they affect the localization, maturation, and/or stability of TER1 (telomerase RNA). During telomerase assembly, TER1 is shuttled between the nucleus and the cytoplasm [96-98]. If this process were disrupted, it would be expected to have a negative effect on telomerase holoenzyme assembly and consequently negatively affect telomere length.

To test the hypothesis that Ptr1 and Mlo3 regulate TER1 biogenesis and/or assembly with the telomerase proteins, Northern analysis and RNA FISH of TER1 in *ptr1-1* cells grown at 25°C and 30°C and *mlo3Δ* cells at 30°C are underway. Preliminary results of RNA FISH suggest that *ptr1-1* has more TER1 localized in the nucleus when grown at the partially permissive temperature of 30°C (Figure 36C) compared to *ptr1-1* cells grown at 25°C (Figure 46B) or TER1 localization in wild-type cells (Figure 36A). Surprisingly preliminary results suggest that *mlo3Δ* has the opposite effect than anticipated on TER1 localization. In *mlo3Δ* more TER1 is localized to the cytoplasm than nucleus compared to wild-type cells (Figure 36C). Computational analysis
of RNA FISH images will allow a quantitative assessment of TER1 localization to the cytoplasm and nucleus in *ptr1-1* cells grown at 25°C and 30°C and in *mlo3Δ* cells at 30°C. Northern blot analysis of TER1 in *ptr1-1* cells grown at 25°C and 30°C and in *mlo3Δ* cells at 30°C is also underway.
Figure 28. Functional analysis of *S. pombe* endogenously expressed Trt1- GFP epitope tagged protein. (A) Five successive restreaks of the parental *S. pombe* wild-type and trt1::Trt1-G8-GFP haploid. Cells were grown on YES media at 30 °C for three days. (B) Southern blot analysis of telomere length in the trt1::Trt1-G8-GFP strain (left) and WT strain (right) after successive restreaks. Streak 0 represents genomic DNA from haploid spore. Bracket, position of wild-type bulk telomere signal. (C) Summary of observed senescence and telomere phenotype with the various Trt1-GFP epitope tagged strains.
Figure 29. Nuclear localization of overexpressed *S. pombe* telomerase protein components. GFP-Trt1 is diffusely localized to the nucleus (left panel) while GFP-Est1 is localized to distinct foci at the nuclear periphery (right panel, arrow).
Figure 30. The telomerase holoenzyme is stabilized by TER1, the telomerase RNA component. (A) Northern blot analysis of *S. pombe* cells with and without TER1 overexpression. Labeled probe against TER1 RNA and loading control U2 snRNP. (B) Western blot analysis of *S. pombe* cells overexpressing GFP-Trt1 (lane 1), GFP-Est1 (lane 2), GFP-Trt1 and GFP-Est1 (lane 3), and two independent isolates overexpressing GFP-Trt1, GFP-Est1, and TER1 (lane 4 and 5). Lower molecular weight bands on the gel are presumed to be protein degradation products.
Figure 31. *S. pombe* telomere lengthening observed with telomerase overexpression. Telomere length longer in two independent isolates of *S. pombe* trt1:: GFP-Trt1 est1:: GFP-Est1 pSP1-TER1 over two restreaks. Southern blot analysis of *S. pombe* genomic DNA digested with EcoRI (left side of gel) and Apal (right side of gel).
Figure 32. Immunoaffinity-purification of GFP-Trt1 and GFP-Est1. (A) Proteins of GFP-Trt1 GFP-Est1 IP were resolved by SDS-PAGE and visualized by Coomassie stain. Peptides of identified proteins were confirmed by MS with nanoLC LTQ Orbitrap CID analyses. (B) Western blot analysis with anti-GFP antibodies of remaining beads and flow through samples after IP experiment.
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Table 5. Top hits of *S. pombe* telomerase protein interactions. Proteins in red were previously reported to affect telomere length [130].
Figure 33. Deletion of mlo3, a protein involved in mRNA nuclear export, results in stable, short telomeres. Southern blot analysis of Apal digested genomic DNA of two individual isolates over eight restreaks. Experiment by C.J. Webb.
Figure 34. The mRNA nuclear export factor ptr1-1 mutant displays shorter telomeres than wild-type at partially nonpermissive temperature. Southern blot analysis of Apal digested gDNA of three individual isolates of wild-type (Wt), ptr1-1, rae1-167, and the double mutant ptr1-1 rae1-167 grown at the permissive temperature (25°C) and partially nonpermissive temperature (30°C) (A) Southern blot analysis using telomere oligonucleotide probe for comparison of telomere length (B) Southern blot analysis using Ste1 subtelomeric probe for detection of recombination events of greater than 2kb that would affect telomere length.
Figure 35. The mRNA nuclear export factor \textit{ptr1-1} mutant displays stably shorter telomeres than wild-type at partially nonpermissive temperature. Southern blot analysis of Apal digested genomic DNA of two individual isolates of ptr1-1 grown at the permissive temperature of 25°C, and for 8 successive restreaks (approximately 200 generations) at the partially nonpermissive temperature of 30°C. Isolate 1- left panel, isolate 2- right panel.
Figure 36. RNA FISH experiment reveals TER1 nuclear localization in \textit{ptr1-1} cells and cytoplasmic localization in \textit{mlo3Δ} cells. RNA FISH experiment employing Stellaris probes against TER1. TER1 localization in: (A) wild-type cells grown at 30°C. (B) \textit{ptr1-1} cells grown at the permissive temperature of 25°C (C) \textit{ptr1-1} cells grown at partially nonpermissive temperature of 30°C cells (D) \textit{mlo3Δ} cells grown at 30°C.
(B) \textit{ptr1-1 25}^\circ\text{C}
(c) *ptr1-1 30°C*

DIC

DAPI

TER1

TER1, DAPI
(d) \textit{mlo3}Δ
Chapter 7. Protein interactions of *Saccharomyces cerevisiae* telomerase

*Experimental contributions of Angela Chan of the Zakian lab and Preeti Joshi of the Cristea lab are acknowledged within this chapter.*

*In vivo* protein interactions of the *Saccharomyces cerevisiae* telomerase enzyme were additionally targeted for immunoaffinity purification (IP) and proteomic analysis by mass spectrometry. Known components of the *S. cerevisiae* telomerase holoenzyme are the protein components Est2 (telomerase reverse transcriptase), Est1, Est3, as well as, the telomerase RNA, TLC1.

Various Est2 GFP epitope tagged strains were initially constructed with Est2 expressed at its endogenous locus and under its native promoter. The GFP tag was incorporated at the Est2 amino (N) or carboxyl (C) terminus, both with and without a glycine linker, which has been demonstrated to improve the *in vivo* function of tagged telomerase proteins [111]. One copy of the EST2 gene was tagged in a diploid yeast strain by transformation of a PCR construct. Integration of the construct at the desired target sequence was confirmed by Southern blot analysis, diagnostic PCR, and DNA sequencing. Following sporulation and tetrad dissection, all transformants were observed to segregate 2:2 for the selectable marker, supporting Southern data indicating a single integration event per transformant.

To determine if the *S. cerevisiae* Est2 GFP tagged proteins are functional *in vivo*, cell viability and telomere length maintenance were analyzed. In a haploid strain containing Est2-GFP, cell viability was analyzed by a senescence assay in which cell growth was observed over approximately five successive restreaks (approximately 200 cell generations). Furthermore, the ability of the strain to maintain telomere length was evaluated by Southern blot analysis. S.
cerevisiae strains expressing N-terminally tagged Est2 displayed wild-type cell growth and slightly shorter but stable telomere length, while an N-terminal tag with an eight glycine (G8) linker displayed wild-type cell growth and telomere length (Figure 37 A,B). Strains expressing C-terminally tagged Est2 displayed immediate senescence at streak1 and very short telomeres, while a C-terminal tag with a G8 linker displayed wild-type cell growth and short but stable telomeres (summary of strain phenotypes, Figure 37C). However, the expression of GFP epitope tagged Est2 could not be confirmed in any of these strains by Western blot analysis or fluorescent microscopy, even in strains with WT or near WT telomere length presumably due to the low levels of Est2 protein, estimated at approximately 40 molecules per cell [134]. For future experiments, I used the GFP-G8-Est2 because it had no apparent deleterious effects on telomeres.

Despite its low level of expression, immunoaffinity purification (IP) of GFP-G8-Est2 for proteomic analysis of associated proteins showed encouraging preliminary results. GFP-G8-GFP was immunoisolated from asynchronous cells (strain yKM76) in lysis buffer (20 mM HEPES-KOH, pH 7.4, containing 0.1 M potassium acetate, 2 mM MgCl₂, 0.1% Tween 20, 1 μM ZnCl₂, 1 μM CaCl₂, 0.25% Triton X-100, 250 mM NaCl, 1/100 (v/v) protease) using magnetic beads conjugated with GFP. Immunoisolated GFP-Est2 was eluted from the beads in 0.5 N NH₄OH, 0.5 mM EDTA solution and precipitated overnight. The eluted target protein and associated interactions were suspended in SDS sample buffer and resolved by SDS-PAGE in a 4-12% gradient gel (Figure 38A). The gel lane was cut into 1mm segments and prepared for in-gel digestion with trypsin. Peptides were analyzed by MALDI MS (MS/MS) on a LTQ-Orbitrap XL mass spectrometer (see Chapter 1 Methods, for more details regarding instrumentation and MS analysis). Peptide mass fingerprinting and targeted MS/MS were performed as previously
described [115, 116]. The presence of Est2 was confirmed with high confidence (Figure 38B). However, other members of the telomerase holoenzyme and known interactions of *S. cerevisiae* telomerase were not identified in this experiment. Furthermore, the detection of GFP-G8-Est2 following IP could not be repeated under identical conditions in subsequent experiments. In an attempt to improve the IP of GFP-G8-Est2, various experimental conditions were tried without success: an increase in lysis buffer stringency (0.25%-1% Triton X), DNase treatment of the cell lysate, Myc tag Est2 for IP with anti-Myc antibody, and IP from G2 arrested nocodazole treated cells. Negative results under these various conditions led to the conclusion that IP of the telomerase holoenzyme and associated proteins via endogenously expressed GFP-G8-Est2 is exceedingly difficult.

Challenges of low protein abundance were overcome by overexpressing the protein and RNA components of the telomerase holoenzyme. A PCR construct containing the GAL1 promoter was integrated at the *tlc1* locus and a construct containing the GAL1 promoter, a five glycine linker (G5) and a GFP epitope tag was integrated at the *est1*, *est2*, and *est3* loci in *S. cerevisiae* haploid cells. Overexpression of GFP-Est3 had a negative effect on cell viability in haploid cells, however in the presence of GFP-Est2 overexpression, the negative growth phenotype was rescued (Figure 39A). By western blot analysis, it was observed that GFP-Est1 and GFP-Est3 protein abundance was significantly greater than GFP-Est2, presumably due to differences in protein stability (Figure 39B). A diploid cell overexpressing GFP-Est1, GFP-Est2, GFP-Est3, and TLC1 was obtained by mating the transformant of lane 4 in Figure 40B with that of lane 5.

The overexpression of TLC1 from the GAL1 promoter in galactose grown cells was confirmed by RT-PCR (Figure 39C, lane 2). As a comparison for expression level and serving as
a positive control, TLC1 was overexpressed from plasmid pYT1204 from Yasumasa Tsukamoto (Figure 39C, lane3). Southern blot analysis of telomere length revealed slightly shorter telomeres in three independent isolates of the diploid strain yKM332 overexpressing GFP-Est1, GFP-Est2, GFP-Est3, and TLC1 (Figure 39D, lanes 4,5,6). IP of GFP-Est1, GFP-Est2, and GFP-Est3 was conducted in an asynchronous population of cells using a previously optimized lysis buffer (described in Chapter 4 for the IP of S. pombe Pfh1): 100mM Hepes KOH, pH 7.9, 300mM potassium acetate, 10mM magnesium acetate, 10% glycerol, 0.1% NP-40, 2mM EDTA, 2mM B-glycerophosphate, 50mM NaF, 10mM NaVO₄, 1mM DTT, protease inhibitor cocktail (Roche). Proteins were resolved by SDS-PAGE and visualized by Coomassie stain (Figure 40A). Co-isolated proteins were analyzed by MS analysis and peptides of identified proteins were confirmed by MS/MS with nanoLC LTQ Orbitrap CID analyses. Disappointingly, the IP experiment of the telomerase complex in strain yKM332 did not result in the identification of proteins that are known to interact with telomerase such as Cdc13, ScPif1, Stn1, or yKu70/80, suggesting that the isolation of an active telomerase complex was not successful. However, sequence coverage of the targeted Est1, Est2, and Est3 proteins did allow for the detection of Est1 phosphorylation at S644 (Figure 40B).

Because GFP-Est3 was not fully functional in vivo (Figure 41A), an overexpression strain was constructed that expressed Est3 under the GAL1 promoter without a GFP epitope tag. Characterization of this strain and subsequent IP experiments were conducted by Angela Chan of the Zakian lab. Four independent isolates of haploid cells that expressed GFP-Est2, GFP-Est1, Est3, and TLC1, with each product overexpressed using the GAL1 promoter, displayed progressive telomere lengthening over four successive restreaks (Figure 41B), strongly suggesting that the overexpressed telomerase complex is functional. IP of isolate 5c (strain
yKM352) in an asynchronous population of cells was conducted with the previously described lysis buffer (100mM Hepes KOH, pH 7.9, 300mM potassium acetate, 10mM magnesium acetate, 10% glycerol, 0.1% NP-40, 2mM EDTA, 2mM B-glycerophosphate, 50mM NaF, 10mM NaVO₄, 1mM DTT, protease inhibitor cocktail (Roche)). Proteins were resolved by SDS-PAGE and visualized by Coomassie stain (Figure 42A). In western blot analysis of a 5% fraction of the IP sample, a less prominent band corresponding to GFP-Est2 can be seen along with GFP-Est1 (Figure 42B).

Co-isolated proteins were analyzed by MS analysis and peptides of identified proteins were confirmed by MS/MS with nanoLC LTQ Orbitrap CID analyses, work by Preeti Joshi of the Cristea Lab. In the IP experiment of strain yKM352, it was again disappointing to not identify known interactions of telomerase. Particularly discouraging is the absence of Est3, which was overexpressed but was not tagged with GFP and therefore would not have been directly targeted in the IP experiment. This could possibly be explained by the fact that Est3 is known to interact only transiently with the telomerase complex [135], and this transient association may be more labile than that of other subunits. However, it may also suggest that the IP of the telomerase complex was not successful. Post-translational modifications of the telomerase components Est1 and Est2 could be characterized in this experiment including the novel phosphorylation of Est1 at S644, which was previously observed in the IP experiment of yKM332. Other sites of modifications observed with less conclusive MS/MS spectra were phosphorylation of Est1 at S77 and Est2 at Y696 (Figure 42C).
Discussion

*In vivo*, telomerase regulation is cell cycle regulated. Therefore, cell cycle changes in protein interactions and post-translation modification are of interest. A new postdoctoral fellow in the Zakian lab, Kah Wai Lin is following up my observations on *S. cerevisiae* telomerase interactions and phosphorylation events to determine if any of the interactions or phosphorylations occur at specific time points of the cell cycle in which telomerase is active, specifically at the end of S phase, or when it is inactive, as in G1 phase. For these experiments, I generated an *S. cerevisiae* strain expressing GFP-Est1, GFP-Est2, Est3, and TLC1 under the GAL1 promoter with the Bar1 protease deleted by the insertion of the NatMX cassette. This strain will be arrested in G1 phase with alpha-factor. For late S/G2 phase cells, the strain will be arrested in nocodazole. Similarly, a strain overexpressing GFP with the GAL1 promoter with *bar1::NatMX* has also been created to be used in parallel control IP and MS experiments to access nonspecific interactions of the GFP epitope tag.

Preliminary experiments using the *S. cerevisiae* strain described above have been competed by Kah Wail Lin of the Zakian lab. An IP experiment of GFP-Est1 and GFP-Est2 has been completed in G1 alpha-factor arrested cells. The phosphorylation of Est1 S644 has been confirmed in G1 of the cell cycle following MS/MS analysis of this IP sample.
Figure 37. Functional analysis of *S. cerevisiae* endogenously expressed Est2 GFP epitope tagged protein. (A) Five successive restreaks of the parental *S. cerevisiae* wild-type and est2::GFP-G8-Est2 haploid strain. Cells were grown on YEPD media at 30 °C for 2 days. (B) Southern blot analysis of telomere length in the est2::GFP-G8-Est2 strain (left) and WT strain (right) after successive restreaks. Streak 0 represents genomic DNA from haploid spore. Bracket, position of wild-type bulk telomere signal. (C) Summary of observed senescence and telomere phenotype with the various Est2-GFP epitope tagged strains.
Figure 38. Immunoaffinity-purification of GFP-G8-Est2p expressed at endogenous levels. (A) Coomassie stained gel of Pfh1-GFP IP sample isolated from 38g of cells with lysis buffer conditions of the Cristea lab containing 0.25% TritonX and 250 mM NaCl. Labeled are proteins identified in different regions of the gel by MS/MS analysis. (B) Est2 MS identification and example of Est2 MS/MS confirmation. Peptides were analyzed by MALDI MS (MS/MS) on a LTQ-Orbitrap XL mass spectrometer.
Figure 39. Overexpression of *S. cerevisiae* GFP-Est1, GFP-Est2, and GFP-Est3. Telomerase components were individually tagged at their endogenous loci under the GAL1 promoter. (A) Cells grown on YM plates plus 2% galactose. (B) Western blot analysis of four isolates overexpressing GFP-Est1, or GFP-Est2 and GFP-Est3. Isolates of Lanes 4 and 5 were mated to obtain a diploid overexpressing GFP-Est1, GFP-Est2, and GFP-Est3. (C) RT-PCR using primers designed for the detection of TLC1. RNA isolated from yK3M32 grown in 2% glucose or 2% galactose. A high-copy plasmid overexpressing TLC1 (pTLC1) served as a positive control for TLC1 detection. ACT1 served as a control for RNA loading. (D) Southern blot analysis of telomere length in the telomerase overexpression diploid strain with and without TLC1 overexpression by the GAL1 promoter or plasmid pYT1204 (pTLC1).
Figure 40. Immunoaffinity-purification of GFP-Est1, GFP-Est2, and GFP-Est3 overexpressed by the GAL1 promoter. (A) Coomassie stained gel of IP sample from *S. cerevisiae* diploid overexpression strain, yKM332. Isolated from approximately 8g of cells with lysis buffer containing 0.1% NP-40 and 300mM potassium acetate. (B) Selected CID MS/MS spectra representing Est1 phosphorylation at S644.
Figure 41. Telomere lengthening observed with *S. cerevisiae* telomerase overexpression of GFP-Est1, GFP-Est2, and Est3. *S. cerevisiae* haploid overexpression strain with fully functional Est3 (no GFP tag). Telomerase components were individually tagged at their endogenous loci under the GAL1 promoter. (A) Western blot analysis using anti-GFP antibodies for the detection of GFP-Est2 and GFP-Est1. Cells were cultured in 2% galactose. (B) Telomere length longer in four independent spore isolates of *S. cerevisiae* strain yKM342 over four restreaks. Southern blot analysis of *S. cerevisiae* genomic DNA. (Data of Angela Chan)
Figure 42. Immunoaffinity-purification of GFP-Est2 and GFP-Est1 overexpressed by the GAL1 promoter. (A) Proteins of GFP-Est2 GFP-Est1 IP from strain yKM352 were resolved by SDS-PAGE and visualized by Coomassie stain. Peptides of identified proteins were confirmed by MS with nanoLC LTQ Orbitrap CID analyses. (B) Western blot analysis with anti-GFP antibodies of remaining beads, supernatant, pellet, and 5% of IP sample. (C) Selected CID MS/MS spectra representing Est1 phosphorylation at S77 and Est2 phosphorylation at Y696.
References


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