Regulation of telomerase reverse transcriptase expression in *Schizosaccharomyces pombe*

By

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Submitted to the graduate degree program in Molecular and Integrative Physiology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Eukaryotic cells undergo chromosome shortening during each cell division due to the inability of DNA polymerase to replicate chromosome ends. This end replication problem is counteracted by the ribonucleoprotein telomerase, which functions as a reverse transcriptase to add DNA repeats to chromosome ends. These DNA repeats, called telomeres, delay cellular senescence and recruit DNA binding proteins which protect chromosome ends and distinguish them from double-strand breaks. Telomere attrition is thought to function as a means of tumor suppression as cells can only undergo a limited number of divisions in the absence of telomerase or an alternative mechanism for replenishing DNA at chromosome ends. In multicellular organisms, telomerase is present and active during the early stages of development, but is later downregulated, resulting in little or no activity in most somatic cell types. In contrast, approximately 90% of cancer cells do have detectable telomerase activity. This discovery has identified telomerase components, and other molecules regulating telomerase function, as potential targets for cancer treatment. Unlike multicellular organisms, single-celled organisms, such as the fission yeast *Schizosaccharomyces pombe*, are widely believed to constitutively express telomerase, despite insufficient studies on expression or activity in this species outside of typical laboratory conditions. Surprisingly, we have observed that the level of telomerase reverse transcriptase (Trt1) protein in this organism decreases in cells which have been arrested by entry into stationary phase, which corresponds with a decrease in *in vitro* telomerase activity from these cells. This decrease in Trt1 level is similarly observed in cells which have been starved of nitrogen or glucose, and the rate of turnover appears to be quite rapid. The downregulation of Trt1 protein is independent of trt1 mRNA transcript level, and regions within the protein coding sequence appear to be sufficient for causing the observed decrease in expression. These results indicate that telomerase reverse

transcriptase expression in the fission yeast *S. pombe* is regulated in nutrient-starved cells, likely through degradation of the protein, as triggered by multiple protein sequence or structural elements. Further studies in this model organism will likely reveal great insights into mechanisms and functions of telomerase regulation.

Acknowledgements

Many people have contributed to the production and completion of this thesis and for whom I am extremely grateful. I would like to thank all the amazing people at Stowers and KUMC for providing friendly and helpful environments in which to work and study. While I can never completely express my appreciation of everyone for whom it is owed, I must single out those individuals who were integral components of my graduate career.

Firstly, I must thank my advisor, Peter. You inspire a high level of independence and creativity and you often had more faith in me than I did. I will continue to work to stop "trying" and start "doing." Peter, your ambition and intellect, coupled with the amazing resources and facilities that come with working in your lab, make this a great place to train as a graduate student. Thank you for the opportunity.

I would also like to thank my thesis committee – Lane, Aron, Joan, and Jennifer – for the advice and assistance you provided about graduate school, research, and life. In addition, I would like to thank Mike Werle, Mike Wolfe, and Susan Abmayr for the guidance they offered throughout my graduate career.

I would especially like to thank all members of the Baumann lab, past and present, for creating an immensely fun and helpful atmosphere in which to work. Thanks to Rachel for maintaining a well-organized lab, but mostly for providing friendly assistance and a kind ear. Also a special thanks to Ram, Lili, Aracely, Li, and Wen for the guidance, support, friendship, and coffee times.

Finally, I would like to thank all my family and friends for your unending support and companionship. I am blessed to have so many amazing people in my life.

Table of Contents

I. Introduction

I.1. Telomerase and the end replication problem

DNA, the molecules that encode genetic information, must be copied in a semi-conservative manner in order to pass information from one cell to the next generation. This process, known as DNA replication, requires the use of DNA polymerases to synthesize the new DNA strand. To initiate DNA replication, DNA polymerase requires the presence of an RNA primer, which is later degraded and usually replaced by DNA. However, at the ends of chromosomes – the linear DNA structures in eukaryotic organisms – this primer is unable to be replaced, which results in chromosome shortening with each successive generation. This is known as the end replication problem [5-7]. Most eukaryotic organisms counteract this problem with the reverse transcriptase telomerase, which reverse-transcribes an RNA template to add DNA repeats, called telomeres, to the ends of chromosomes [8, 9]. Cells which lack sufficient telomerase activity undergo telomere shortening following each cell division. When telomeres reach a critical length, they can trigger DNA damage responses and lead to cellular senescence, unless an alternative method of chromosome end maintenance is established [10-12]. In addition to their role in preventing chromosome end shortening, telomeric DNA can recruit specialized proteins which protect chromosome ends from being recognized as DNA double-strand breaks [13-15].

At its core, telomerase consists of a reverse transcriptase subunit (TERT in mammals, Trt1 in *S. pombe* [2]) and an RNA subunit (TR in mammals [16], Ter1 in *S. pombe* [17, 18]), which provides a template for reverse transcription and telomeric repeat addition. Both subunits are limiting and haploinsufficiency has been demonstrated in mammals [19, 20] and yeast [21], resulting in telomere shortening. In addition, levels of the telomerase complex must be limiting

in order to prevent over-elongation of telomeres [22]. These observations indicate that telomerase expression and activity must be highly regulated in order to maintain a desired telomere length.

I.2. Regulation of telomerase activity

Due to the defects observed when cells over- or under-express telomerase, it seems necessary that cells try to maintain tight control over telomerase activity. Indeed, multiple mechanisms have been implicated in the regulation of telomerase activity (reviewed in [23]). In multicellular organisms, telomerase activity is required in all tissues early in development, but is later lost in most somatic cell types [24]. In addition, telomerase activity is thought to be regulated under various cellular conditions, such as through the cell cycle or when the cells are under stress induced by DNA damage [25, 26]

In most adult tissues, telomerase activity is thought to be downregulated through the expression of core subunits [23]. While the telomerase RNA subunit can be detected in many human primary cells lacking telomerase activity [27], hTERT expression often mirrors telomerase activity, and ectopic expression of hTERT is often sufficient for restoring activity and elongating cellular life-span [28]. Together, these results suggest that hTERT expression is likely a key determinant for the presence of telomerase activity.

Due to its essential role in telomerase function, many labs have tried to understand the mechanisms controlling hTERT expression. The hTERT promoter region has been extensively analyzed and is presumably regulated by many transcription factors, including inducers involved in cell proliferation and tumor suppressors, suggesting that multiple mechanisms likely

contribute to the controlled expression of this protein [29-31]. In addition to promoter regulatory elements, heterochromatin modifications likely play a significant role in telomerase expression, including histone modifications and CpG methylation [31-33] (For reviews on hTERT and hTR gene expression, refer to [34] and [35]). Despite the apparently high level of regulation of telomerase gene expression, telomerase activity can still be absent in cells expressing both hTR and hTERT mRNA, suggesting that transcription is not the sole point of telomerase regulation [36]. One such mechanism may involve multiple alternative splice variants of hTERT. Many of these produce inactive forms of the protein and may act as dominant negative isoforms, a regulatory mechanism that may be linked to differences in activity observed in various cell types [31, 37-40].

In addition to the control observed in embryonic development, telomere repeat addition has been observed to be linked to the cell cycle, as telomerase activity occurs concurrently with telomere replication in late S-phase [25, 41]. This cell cycle-dependent activity at telomere ends is thought to be regulated largely through intranuclear trafficking of telomerase [42], likely through telomerase-associated accessory factors [43, 44]. Expression of telomerase components may also be regulated through the cell cycle. For example, the promoter region of the telomerase RNA component in budding yeast, TLC1, appears to recruit transcription factors which appear to alter expression throughout the cell cycle, increasing expression during S-phase [45]. In humans, TERT appears to associate with the degradation machinery in a cell cycle-dependent manner [46]. Sub-nuclear trafficking may also play a role in regulating telomerase activity in response to DNA damage, which is thought to prevent the *de novo* addition of telomere repeats to double-strand breaks [47] resulting in chromosome instability [48], as hTERT appears to be sequestered to nucleoli under these conditions [26]. Telomerase activity is also thought to be

modulated through phosphorylation by kinases linked to cell proliferation or DNA damage responses [49, 50].

The apparent complexities of telomerase regulation open the door for many potential problems. In fact, misregulation of telomerase activity can result in a series of complex disorders.

I.3. Telomerase-associated disorders

A spectrum of telomere disorders have been identified, which can occur as autosomal-dominant, recessive, or X-linked mutations to telomerase components or genes involved in the biogenesis or function of telomerase (reviewed in [51]). One of the best characterized telomere defect disorders is dyskeratosis congenita. This disorder is initially characterized by nail dystrophy, skin hyperpigmentation, and oral leukoplakia, and typically results in premature mortality which often resemble features of age-related diseases [52]. Other telomerase-related diseases include pulmonary fibrosis, aplastic anemia, and liver cirrhosis [53-56]. Furthermore, telomere length has been observed to be a heritable trait that can increase the chance of developing telomere defect-associated disorders [57]. These disorders are suggestive of defects in highly proliferative tissues, including epidermal and hematopoietic stem cells as well as germline cells [24, 51, 58-60]. The telomere defects that contribute to these disorders have also been linked to an increased cancer rate in these patients [61].

Telomerase function is linked to development, when cell proliferation is high. Most cell types eventually lose activity [24], and because telomeres have also been observed to shorten in successive generations of replicating cells [62], it has been suggested that telomere shortening

may serve as a molecular clock, triggering replicative senescence [63] and effectively limiting the replicative capacity of cells. These inherent limitations serve an important function in the prevention of tumorigenesis. Indeed, telomerase activity can be detected in ~90% of cancer cells [64]. In fact, exogenous telomerase activity alone can increase the incidence of carcinogenesis [65, 66]. The origin of these cancer cells remains unclear; they may be the result of telomerase reactivation or a transformation of telomerase-positive cells [64]. Despite these challenges, telomerase has become an attractive diagnostic marker and potential therapeutic. Unfortunately, effective treatments designed to restrict telomerase activity have thus far remained elusive [67, 68]. For this reason, studying the molecular processes involved in regulating telomerase activity and expression is essential for understanding and potentially preventing carcinogenesis.

While much progress has been made to better understand how telomerase is regulated mechanistically and temporally, the complexities of these processes suggest that much is left to be done. Identification and characterization of the genes involved in regulating telomerase activity may identify therapeutic targets and reveal insights into the genetic causes for certain cancers and other telomere-related disorders.

I.4. Thesis objectives

The fission yeast *Schizosaccharomyces pombe* serves well as a model system for the study of telomerase function due to a high level of conservation of the telomerase catalytic subunit [2] and telomeric proteins [69], in addition to the increased genetic tractability in this organism compared with mammalian systems [70, 71]. Despite the similarities between processes and components between humans and fission yeast, and the common use of this

organism as a model system, very little is known about telomerase regulation in *S. pombe*. We have hypothesized that telomerase expression is regulated in fission yeast and that the consequential misregulation is harmful to this organism.

The purpose of this thesis is to communicate the significance, methods, results, and analysis of the graduate research performed. Chapter one has outlined the importance of understanding telomerase regulation, as well as the current knowledge on this topic. Chapter two details the materials and methods used to carry out the research described in Chapter three. Chapter three describes the results obtained during the course of graduate study regarding conditions in which Trt1 protein expression is observed to decrease, and insight into the mechanisms that underlie these changes. Finally, chapter four analyzes these results and places them in the context of the current state of the field, as well as the future directions this work invites.

II. Materials and Methods

II.1. Strains and constructs

Table 2.1. Strains used in this study. Strain names and corresponding genotypes used in experiments for this study are shown.

Strain name	Genotype
PP60A	h^+/h^- *leu1-32 /leu1-32 ura4-D18 /ura4-D18 his3-D1 /his3-D1 ade6-M210 /ade6-M216 trt1* $^+$ */trt1 ::ura4*
PP138	h^- *ade6-M216 leu1-32 ura4-D18 his3-D1*
PP265	h^-
PP298	h^- *ade6-M210 leu1-32 ura4-D18 his3-D1 trt1-Cmyc9*
PP573	h^- *trt1-Cmyc9*
PP889	h^+ *leu1-32 ura4-D18 his3-D1 ade6-M210 trt1::ura4 aur1 :aur1* ^{r} *:nda2-trt1-Cmyc9*
PP903	h^- *aur1 :aur1* ^{r} *:nda2-trt1-Cmyc9*
PP922	h^- *aur1 :aur1* ^{r} *:trt1-Cmyc9*
PP924	h^- *aur1 :aur1* ^{r} *:trt1aa290-988-Cmyc9*
PP925	h^- *aur1 :aur1* ^{r} *:trt1aa323-625-Cmyc9*
PP926	h^- *aur1 :aur1* ^{r} *:trt1aa490-988-Cmyc9*
PP927	h^- *aur1 :aur1* ^{r} *:trt1aa625-882-Cmyc9*
PP928	h^- *aur1 :aur1* ^{r} *:trt1aa1-210-Cmyc9*

Genotypes of strains used in these studies are listed in Table 2.1. *nda2-trt1* construct was created by PCR amplifying *trt1* cDNA from pRH5 (lab stock) using primers BLoli3661 (GCATTTACATTCGTTATTATCACAAATTATGACCGAACACCATACCCC) and BLoli3662 (CGTATAAAAGCGATTATTGGATAAATTAGCGGCCGCCTGTCG). This product was then annealed to ~500 nt of upstream and downstream sequences from *nda2*, which were amplified from PP138 genomic DNA using BLoli3678 (CAGGGGCCCTCTTTTGTCGGGCTAAACGC) and BLoli3664 (GGGGTATGGTGTTCGGTCATAATTTGTGATAATAACGAATGTAAATGC) for upstream sequence and

BLoli3680 (CAGCTCGAGAGGCTAATTCCGAGCAATCG) and BLoli3665 (CGACAGGCGGCCGCTAATTTATCCAATAATCGCTTTTATACG) for downstream sequence. These regions have been previously characterized to contain sufficient promoter and terminator sequences for expression of α-tubulin [72]. These products were digested with ApaI, XhoI (underlined in sequences above) restriction enzymes and ligated into digested pCST159 plasmid (courtesy of Julia Cooper Lab). This plasmid was then digested with XcmI and inserted into the genome at the *aur1* locus of sporulated haploid PP60 cells containing *trt1::ura4* by lithium acetate transformation (as described in [73]) to create PP889 strain. This strain was later crossed with PP265 strain on Malt Extract plates (BIO 101) and sporulated to generate PP903 strain.

Truncated Trt1 constructs were created by PCR or synthesized by GENEWIZ, along with 700 nt of *trt1* promoter sequence (determined to be sufficient for transcription in [74]) and C-terminal 9-myc tag similar to that in PP298 strain, and ligated into KpnI, XhoI digested pCST159 plasmid. Plasmids were then integrated into the genome of PP265 cells at the *aur1* locus as above. Inserted sequences were verified by PCR and sequencing following strain construction.

II.2. Culture conditions

All liquid cultures were incubated at 32 °C with shaking. Cell density values were determined by counting via hemocytometer. Unless otherwise indicated, "log phase" denotes cells which have grown in liquid culture for a minimum of 8 hrs and were harvested at a cell density of between 0.5-2x10^7 cells/ml. "Stationary phase" denotes cells which were harvested 24 hrs after log phase cells were harvested and were generally at a cell density of 1-3x10^8 cells/ml.

"Nitrogen starved cells" denotes cells which had grown into log phase in EMM, then washed once and resuspended with EMM without ammonium chloride. "Glucose starved cells" denotes cells which had grown into log phase in EMM, then washed once and resuspended with EMM containing 0.2 g/l dextrose.

Media recipes:

Yeast Extract with Supplements (YES) – 5 g/l yeast extract (Difco, VWR), 30 g/l glucose (VWR), 225 mg/l adenine, 225 mg/l histidine, 225 mg/l leucine, 225 mg/l uracil (Sigma), distilled water to 1 L

Edinburgh Minimal Media (EMM) – 3 g/l potassium hydrogen phthalate, 4.15 g/l $Na_2HPO_4x7H_2O$, 20 g/l dextrose, 5 g/l ammonium chloride, 1.05 g/l $MgCl_2.6H_2O$, 14.7 mg/l $CaCl_2.2H_2O$, 1 g/l KCl, 0.04 g/l Na_2SO_4, 1 mg/l pantothenic acid, 10 mg/l nicotinic acid, 10 mg/l myoinositol, 1 mg/l biotin, 0.5 mg/l boric acid, 0.4 mg/l $MnSO_4$, 0.4 mg/ml $ZnSO_4.7H_2O$, 0.2 mg/l $FeCl_2.6H_2O$, 40 μg/l molybdic acid, 0.1 mg/l KI, 40 μg/l $CuSO_4.5H_2O$, 1 mg/l citric acid (mix, BIO 101), 940 ml H_2O (Ozarka)

II.3. Denatured protein extract preparation

S. pombe cells ($1x10^8$) were lysed by vortexing with glass beads (0.5 mm diameter) in 10% trichloroacetic acid for 8 min at 4 °C. Beads were washed with 10% trichloroacetic acid and precipitated proteins were collected by centrifugation at 16,000 g for 2 min. Samples were then washed once with acetone, dried, and proteins were suspended in 1x protein sample buffer (1x

Nu-PAGE LDS sample buffer, 50 mM dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS). Samples were heated to 75 °C for 5 min, centrifuged at 16,000 g for 30 sec, and the soluble fraction was stored at -80 °C (adapted from [75]).

II.4. Western blotting

Myc-tagged Trt1 was visualized by western blot by first loading protein extracts onto Novex NuPAGE 6% Tris-Glycine Gels (Life Technologies) and electrophoresis was performed with 1x Tris-Glycine SDS Running Buffer at 120 V for 1.5 hrs. Proteins were transferred to Bio-Rad Zeta-Probe nylon membrane in a Bio-Rad Mini Trans-Blot Cell at 0.5 A for 1.5 hrs in transfer buffer containing a 1:50 dilution of a 6.8 pH mixture of 1 M sodium dihydrogen phosphate and sodium hydrogen phosphate. The membrane was then blocked in 1X PBS (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, 0.24 g/l KH_2PO_4, pH 7.4) with 1% v/v Tween 20 and 5% w/v milk for 45 min. Blots were then probed with anti-c-Myc (A14) rabbit polyclonal IgG antibody (Santa Cruz Biotechnology) at a concentration of 0.5 μg/ml in 1X PBS with 1.5% milk for 1.5 hrs. Blots were washed with 1X PBS + 1% Tween 20 and probed with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Thermo Scientific) at a concentration of 0.2 μg/ml in PBS containing 1.5% milk. Blots were washed with PBS + 1% Tween 20 and PBS and visualized by chemiluminescence via Pierce ECL 2 Western Blotting Substrate (Thermo Scientific) on Amersham Hyperfilm ECL (GE Healthcare Life Sciences).

Western blots for proteins with molecular weights of less than ~60 kDa, including α-tubulin, Cdc2, and truncated Trt1 constructs, were performed as above with the following modifications: samples were loaded onto NuPAGE 4-12% Bis-Tris gels and ran with 1x

NuPAGE MOPS SDS Running Buffer. Proteins were then transferred to Protran BA85 Nitrocellulose Membrane (Whatman) in transfer buffer containing 3.03 g/l Tris 14.4 g/l glycine, and 20% methanol at 100 V for 1 hr. Blots were probed with one of the following primary antibodies: mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich) diluted 1:20,000; mouse monoclonal anti-CDK1 (Cdc2) antibody (Abcam) at 0.1 μg/ml; rabbit polyclonal anti-Ccq1 antibody produced by Open Biosystems from purified protein diluted 1:10,000; or anti-c-Myc antibody as above. Secondary antibodies used included HRP-conjugated anti-rabbit antibody described above or horse radish peroxidase-conjugated goat anti-mouse secondary antibody (Thermo Scientific) at 0.2 μg/ml. Blots were visualized with ECL 2 substrate on a Typhoon 8600 Scanner (GE Healthcare Life Sciences). Blots could be stripped with stripping buffer (15 g/l glycine, 1% SDS, 1% Tween 20, pH to 2.2 with HCl), washed with PBS and PBS + Tween 20 and reprobed. Western blotting procedures adapted from protocols described in [75].

II.5. RNA preparation

Cultures (400 ml) were grown to log phase and cells were centrifuged for 4 min at 5000g, washed once with cold water, and dripped into liquid nitrogen to make frozen beads. Cells were lysed in a 6850 Freezer/Mill (SPEX SamplePrep) with eight 2 minute cycles at a rate of ten per second with 2 min cooling time between cycles. Lysed cells were then transferred to 10 ml 50 mM sodium acetate (NaOAc), 1% sodium dodecyl sulfate mixed with 10 ml of phenol/chloroform/isoamyl alcohol (25:24:1, equilibrated with 50 mM NaOAc pH 5.2). Samples were then mixed, incubated at 65 °C for 2 min, and centrifuged at 7500 rpm, 4 °C for 5 min. RNA samples were extracted three more times with phenol/chloroform/isoamyl alcohol, and

once with chloroform/isoamyl alcohol. The RNA-containing supernatant was subjected to ethanol precipitation and centrifuged at 7500 rpm, 4 °C for 10 min in a Beckman Coulter Avanti J-20 centrifuge with a JS-7.5 swinging bucket rotor. The pellets were then dried and resuspended in 50 mM sodium acetate (pH 5.2) (adapted from [17]).

II.6. Real-time reverse-transcription polymerase chain reaction

10 μg (as determined by spectrophotometry) of DNase-treated (Qiagen) RNA from log and stationary phase cells were reverse transcribed in 15 μl samples with SuperScript III Reverse Transcriptase (RT) (Life Technologies) in 1x First Strand Buffer with 6.7 mM DTT, 40 u RNasin Plus (Promega), and 0.67 mM dNTP mix (Roche) at 55 °C for 1 hr using gene-specific primers at 1.67 μM: BLoli3677 (trt1 exon 1) (CGTTTGTACATCGCTTCTCAAG); BLoli3723 (trt1 exon 5) (GGAGTCGCTTTGCTGTTTG); BLoli3675 (nda2 exon 2) (CCTTAGCGATTCCACTACCAG); BLoli3733 (cdc2 exon 3) (TTTACACCGTTCACTAGCTGG). Samples were then treated with 5 u RNase H (New England Biolabs) and polymerase chain reactions (PCRs) were performed with 2 μl of reverse-transcribed sample in 12.5 μl samples using Power SYBR Green PCR Master Mix (Applied Biosystems) and 100 nM final primer concentration. BLoli3677 and BLoli3676 (CCAAAAGCAGGATTCTTCGC) were used to amplify trt1 exon 1; BLoli3723 and BLoli3722 (AGGCAATTTGGACTTATAAACGC) were used to amplify trt1 exon 5; BLoli3675 and BLoli3674 (TGTTGATTGGTGCCCTACTG) were used to amplify nda2 exon 2; and BLoli3733 and BLoli3732 (CATGGACCGAATTTCAGAAACTG) were used to amplify cdc2 exon 3. Reactions were performed with 10 min at 94 °C followed by 40 cycles of 15 sec at 94 °C, 15 sec at 58 °C, and 20 sec at 72 °C. A melting curve was then collected to identify the presence of a single, expected melting

temperature. Analysis of trt1 exon 1 and nda2 exon 2 was performed in triplicate experiments from biological replicates, and trt1 exon 5 and cdc2 exon 3 was performed in triplicate experiments from the same sample. Data from both trt1 exons were averaged together for trt1 expression analysis. Average values and standard deviations of cycle thresholds (C_t) from replicates were calculated and the difference between log and stationary phases (ΔC_t) were calculated by subtracting the values obtained for stationary extracts from those obtained for log extracts. The log fold change was determined as $E_a^{\Delta Ct}$ where E_a is the amplification efficiency as calculated from a standard curve of serial dilutions for each reaction condition. Protocol adapted from [76].

II.7. Genomic DNA preparation

Cells (~2 x 10^9) were collected by centrifugation and washed once with water and then with Z buffer (50 mM sodium citrate, 50 mM sodium phosphate dibasic, 40 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8 with sodium hydroxide). Cells were resuspended in 2 ml Z buffer containing 0.5 mg/ml Zymolyase (US Biological) and 2 mM dithiothreitol (DTT) and incubated at 37 °C for 1 hr. Sodium dodecyl sulfate (SDS) was added to 4% (w/v) and incubated at 65 °C for 10 min. Sample volume was then raised to 10 ml with 5X TE buffer (50 mM Tris pH 8.0, 5 mM EDTA), proteinase K (Life Technologies) was added to 50 µg/ml, and samples were incubated at 50 °C for 1 hr. 3 ml of 5M potassium acetate solution was added and samples were incubated at 4 °C for 30 min. The soluble fraction was isolated by three rounds of centrifugation at 3700 rpm, 4 °C for 10 min in a Beckman Coulter Allegra 6R centrifuge with a GH-3.8 rotor. One volume isopropanol was added to each sample, and nucleic acids were precipitated at -20 °C for 20 min. Samples were centrifuged at 7500 rpm, 4 °C for 10 min in a Beckman Coulter Avanti J-20 centrifuge with a JS-7.5 swinging bucket rotor.

Precipitates were dried and resuspended in 500 µl 5X TE buffer containing RNase A (Fisher Scientific) at 50 µg/ml and incubated at 37 °C for 1 hr. Nucleic acids were then extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, equilibrated with 5X TE), once with chloroform/isoamyl alcohol, and precipitated with 2.5 volumes of ethanol and 0.25 volumes 10 M ammonium acetate for 1 hr at -20 °C. Samples were then centrifuged, washed with 70% ethanol, dried, and resuspended in 1X TE buffer (adapted from [75]).

II.8. Southern blotting

Genomic DNA was digested for 12 hrs with *Eco*RI (New England Biolabs) and run on a 1% agarose gel prepared with 0.5 X TBE (45 mM Tris, 45 mM borate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.3) at 120 V for ~6 hrs. Gels were stained with ethidium bromide (1 µg/ml) for 30 min and visualized on a Typhoon 8600 Scanner to confirm equal loading. Gels were then washed with 0.25 M hydrochloric acid for 10 min, 0.5 M sodium hydroxide, 1.5 M sodium chloride for 30 min, and 0.5 M Tris-HCl, pH7.5, 1.5 M NaCl for 30 min, with water rinses in between washes. Gels were then washed with 10 X saline-sodium citrate buffer (SSC) (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0 with citric acid) and transferred to a Hybond-N+ nylon membrane (GE Healthcare Life Sciences) by capillary blotting. Membranes were then cross-linked with 120 mJ of ultraviolet light. Probes specific for telomeric sequence and the *rad16* gene were generated by PCR from pTELO (lab stock) and wildtype genomic DNA, respectively, and labeled with [α-^{32}P]dCTP using High Prime DNA Labeling kit (Roche). Hybridizations were performed at 65 °C in Church-Gilbert buffer [77]. Blots were exposed to a storage phosphor screen and visualized by Typhoon Scanner (adapted from [73]).

III. Results

III.1. Trt1 expression is downregulated in response to nutrient starvation

While the expression of telomerase components has been observed to be regulated in different cell types and different conditions in mammalian studies, little is known about if or how the expression of telomerase is altered in the commonly-used model organism *Schizosaccharomyces pombe*. We therefore set out to identify conditions under which expression of the telomerase reverse transcriptase (Trt1) subunit changes in this organism. Our lab had previously observed that cells which had grown into stationary phase – that is, when the cell density in a culture becomes too high to support further growth, largely through lack of nutrients [78] – had reduced *in vitro* telomerase activity compared with cells growing in the logarithmic (log) phase (Baumann Lab, unpublished). To identify the cause of this loss of activity, we analyzed the expression of Trt1 in stationary phase cells – determined by a decrease in cell replication – by western blot, and observed that the level of Trt1 protein decreases as cells grow into stationary phase (Fig. 3.1A). While the cell may undergo many changes that could result in a decrease in telomerase activity, we reasoned that the absence of Trt1 was likely a key factor and decided to first pursue the mechanism by which Trt1 protein expression is regulated.

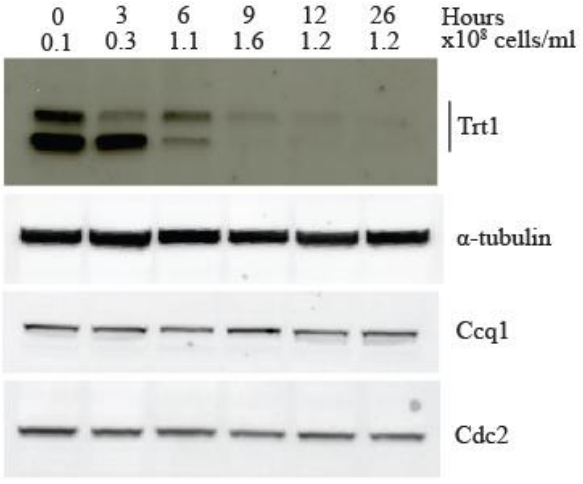

Figure 3.1. Trt1 expression decreases when fission yeast cells enter stationary phase. Western blots were prepared with extracts from cells expressing Trt1 with a C-terminal tag with nine c-Myc repeats (Cmyc9). Blots were probed with anti-c-Myc, anti-α-tubulin, anti-Ccq1, or anti-Cdc2 antibodies. Representative blots are shown from biologically and technically replicated experiments.

Similar to the response observed in cells growing into stationary phase, cells which have been transferred into media lacking nitrogen or glucose exhibit a decrease in Trt1 protein level (Fig. 3.2). This decrease appears to be quite rapid, and corresponds closely with the arrested cell growth (Fig. 3.2B). When glucose-starved cells are transferred to new media containing glucose, the level of Trt1 protein is restored quickly, and before cell replication is resumed (Fig. 3.2D). Similar results were seen in cells recovering from arrest induced by stationary phase or nitrogen starvation (data not shown).

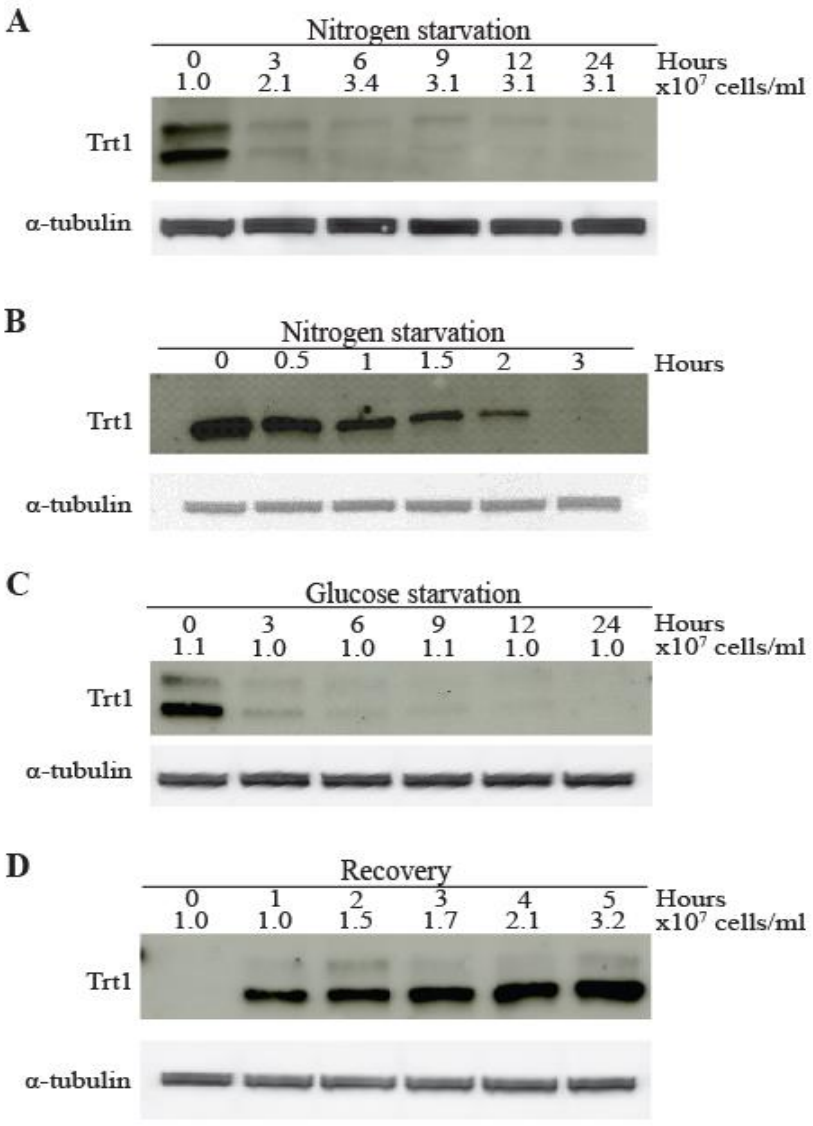

Figure 3.2. Trt1 protein level decreases following nutrient starvation. Trt1-Cmyc9 level was assessed in extracts prepared from cells arrested in media lacking nitrogen **(A and B)** or glucose **(C)**. **D)** Glucose arrested cells were transferred to new, glucose rich media and allowed to continue growth (Recovery). Representative blots are shown from biologically and technically replicated experiments. α-tubulin was used as a loading control.

To analyze Trt1 stability and turnover in rich media, cells were grown to log phase and arrested with the translation elongation inhibitor cycloheximide. By this method, Trt1 half-life was calculated to be approximately 47 min (Fig. 3.3A). This result is similar to the rate of turnover for Trt1 in nitrogen-starved cells (Fig. 3.2A), in which the half-life was calculated to be around 1 hr. Also consistent with the rate of turnover observed in glucose-starved cells (Fig. 3.2C), when cycloheximide-treated cells are washed and resuspended in fresh media lacking the drug, Trt1 protein level is restored to wildtype within one hour (Fig. 3.3B). These results suggest that the turnover of Trt1 in cells arrested by translation inhibition via cycloheximide resemble cells arrested by nutrient starvation or entry into stationary phase, and may indicate a mechanism by which Trt1 is downregulated in these conditions.

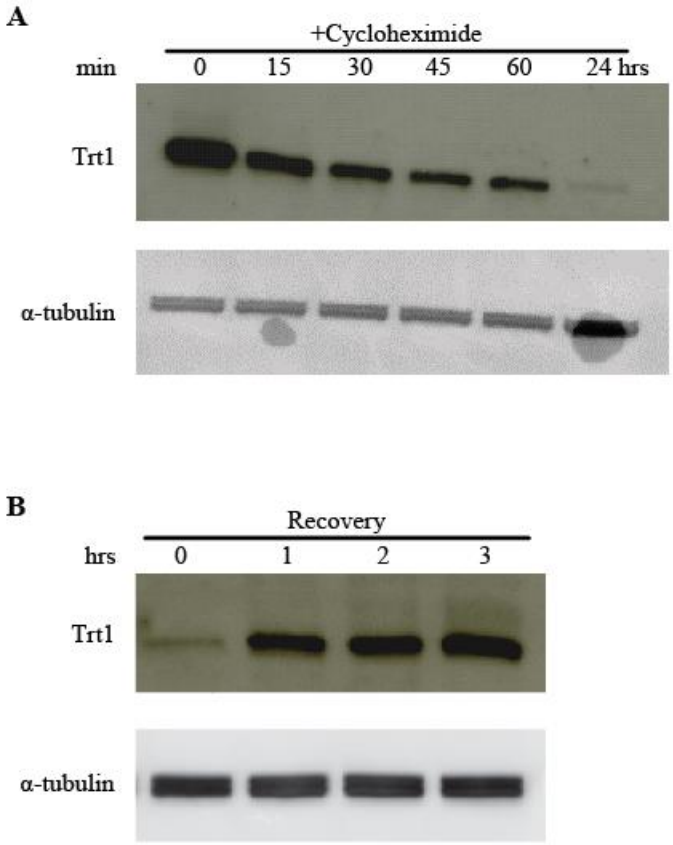

Figure 3.3. Trt1 displays rapid turnover in response to cycloheximide-induced translation inhibition. A) Cells expressing Trt1-Cmyc9 were treated with 160 μg/ml cycloheximide to block translation initiation. Cells were collected at the times indicated and extracts were then analyzed by western blot. **B)** Cells treated with cycloheximide were washed once and resuspended in new media and allowed to resume growth (Recovery). Cells were collected and frozen at the indicated time points and extracts were analyzed by western blot. α-tubulin was used as a loading control.

In addition to the conditions discussed above, others were tested to identify possible environments affecting Trt1 expression. To test whether induced DNA damage could trigger a decrease in Trt1 protein level, cells were treated with hydrogen peroxide, hydroxyurea, or phleomycin at concentrations which slowed cell growth and grown into log phase. Hydrogen peroxide increases oxidative stress in cells, hydroxyurea depletes dNTPs, and phleomycin intercalates into DNA, all of which can also result in DNA breaks [79-81]. Extracts were then analyzed by western blot. Incubation with these drugs did not appear to have any effect on the level of Trt1 protein in log or stationary phase cells (data not shown).

III.2. Decrease of Trt1 protein level is independent of mRNA transcript level

To determine if trt1 mRNA transcription or stability is affected in stationary phase cells, qRT-PCR analysis was performed on RNA extracts from log and stationary phase cells. Transcript levels were normalized to total RNA concentration in each sample and the difference between log and stationary extracts was analyzed (Fig. 3.4). These results indicate that the level of trt1 transcript in these samples changes little, and in fact increases slightly in relative RNA abundance. These results contrast those for nda2 and cdc2 transcripts, in which levels show modest decreases in relative abundance between log and stationary samples, despite no observed change in protein levels (Fig. 3.1). Preliminary RNA-seq data from our lab further indicates similar trt1 transcript levels between log and stationary phase extracts (Baumann Lab, unpublished).

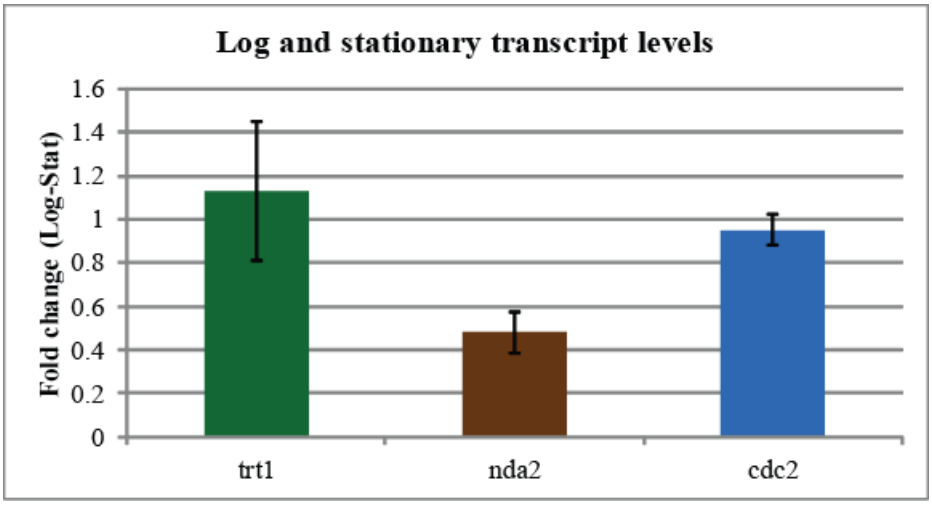

Figure 3.4. Relative trt1 transcript abundance does not decrease between cells in log and stationary phases. RNA was extracted from cells collected in log or stationary phase and analyzed by qRT-PCR to detect the abundance of trt1 transcripts. RNA levels were normalized to total RNA as quantified by spectrometer and the ratio of stationary to log phase samples is shown. nda2 and cdc2 transcript levels are shown for comparison. Error bars indicate standard deviation from the mean.

To further characterize the role of *trt1* transcription and the roles of upstream and downstream sequences in Trt1 expression regulation, *trt1-Cmyc9* cDNA was placed under the control of the α-tubulin (*nda2*) promoter and inserted into an unrelated region of the genome (*aur1* locus) (Fig. 3.5A). This sequence was selected because the endogenous α-tubulin protein has been observed to be stable in the conditions tested (see Fig. 3.1). This construct results in a large increase in Trt1 protein in log phase cells (Fig 3.5B), but the level is still reduced to a similar degree as that seen in wildtype cells (Fig 3.5C). Similar downregulation of wildtype Trt1 in nitrogen- or glucose-starved cells were seen in cells expressing *nda2-trt1* (data not shown). Despite this reduction, the amount of Trt1 protein present in stationary phase *nda2-trt1*-expressing cells appears to greater than expression of the wildtype protein in log phase cells (Fig 3.5B). Cells containing only *nda2-trt1-Cmyc9* are also capable of maintaining telomeres to a

length similar to that seen in cells expressing wildtype Trt1-Cmyc9, with perhaps a modest increase in length (Fig. 3.5D). These data suggest that the trt1 coding sequence is sufficient for the decrease of Trt1 protein level in stationary phase cells, indicating that sequences within the protein coding region may be responsible for Trt1 downregulation.

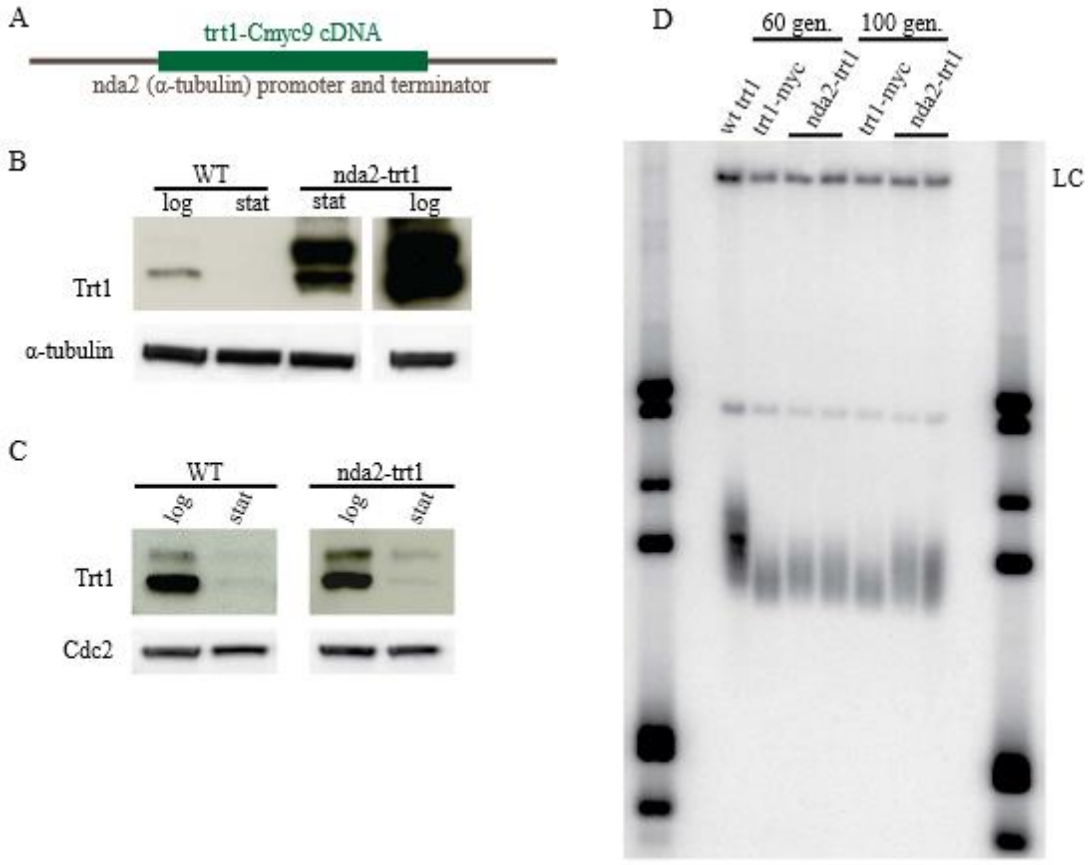

Figure 3.5. Trt1 over-expression with the α-tubulin promoter results in regulation and function similar to that of the endogenous protein. A) Schematic of construct containing the Trt1-Cmyc9 protein coding sequence (cDNA) with ~500 nt of α-tubulin (*nda2*) up- and downstream sequences, referred to as *nda2-trt1*. **B)** Western blot analysis of wildtype (WT) Trt1-Cmyc9 and *nda2* promoter-driven Trt1-Cmyc9 cDNA (*nda2-trt1*) in extracts prepared from cells in log or stationary (stat) phases. Juxtaposition of lanes from the same western blot are shown. α-tubulin was used as a loading control. **C)** Long and short exposures of the same anti-c-Myc western blot are shown for wildtype and *nda2-trt1* extracts (respectively) prepared from cells collected in log or stationary phases. Cdc2 was used as a loading control. **D)** Southern blot analysis of DNA samples from cells expressing endogenous (untagged) Trt1 (wt trt1), Trt1-Cmyc9 (trt1-myc), or overexpressing Trt1-Cmyc9 via the *nda2* promoter (nda2-trt1) after 60 or 100 generations. Probe for *rad16* was used as a loading control (LC).

III.3. Sequences within the Trt1 protein coding region are sufficient for downregulation

To attempt to identify regions of Trt1 responsible for the observed decrease in Trt1 level in stationary phase cells, truncated constructs were created based on predicted structural domains suggested by Nicolas Thomä. These constructs were then placed under the control of the *trt1* promoter and expression in log and stationary phase cells was analyzed by western blot (Fig. 3.6). Interestingly, the Trt1 N-terminus (aa1-210) is the only region tested which remains stable in stationary phase cells. All other regions, including non-overlapping regions, appear to be downregulated in stationary phase cells, although not to the same extent: aa490-988 and aa625-882 are downregulated approximately two-fold, while aa323-625 and aa625-829 levels decrease to near the same degree as that of the full-length Trt1 protein. This is interesting because overlapping regions, notably aa625-882 and aa625-829, appear to be regulated differently, while non-overlapping regions, aa323-625 and aa625-829, are regulated similarly. While these results could be a result of the instability of expressing artificial protein constructs, they may indicate that multiple structural domains or sequence elements within Trt1 could be responsible for the decrease in protein level in the conditions analyzed.

To attempt to identify genes or pathways involved in the stability of Trt1 or its downregulation in stationary phase, strains were analyzed which contained knockouts of various candidate genes. Candidate genes were chosen based on gene function, including genes involved in telomere maintenance, protein degradation, or others that may play a role in regulating Trt1 expression (Table 3.1). These strains all appeared to have near-wildtype levels of Trt1 protein in log or stationary phase extracts by western blot (data not shown). While these experiments may

suggest that these genes are not involved in the regulation of Trt1 expression, they cannot rule out the possibility of redundancies in these pathways which may negate any effects of the knockouts on observable differences. Therefore, more experiments are needed to identify genes involved in regulating Trt1 expression.

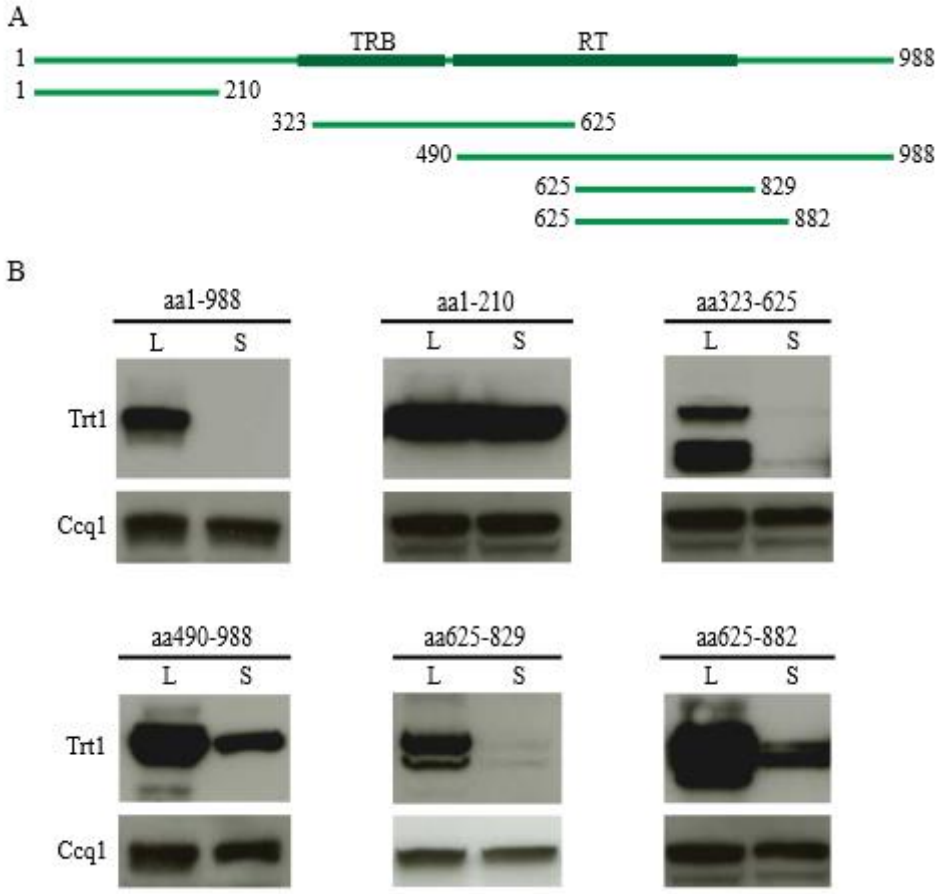

Figure 3.6. Truncated Trt1 constructs are downregulated to varying degrees in stationary phase cells. A) Schematic representing truncated Trt1 proteins. Trt1 truncations were designed to correspond with predicted structural domains (Courtesy of Nicolas Thomä Lab). Constructs were integrated at the *aur1* locus with *trt1* promoter sequence and a C-terminal 9-myc tag. Regions involved in telomerase RNA binding (TRB) and reverse transcription (RT) are indicated in the full length protein [2, 3]. **B)** Extracts were prepared from cells expressing truncated constructs in log (L) and stationary (S) phases and analyzed by western blot. Ccq1 was used as a loading control.

Table 3.1. Gene knockouts analyzed for effects on Trt1 protein expression. Gene knockouts were generated in our lab or created by Bioneer ([1]). Knockout strains were crossed with cells expressing Trt1-Cmyc9 on malt extract plates. Knockouts were verified by determining the presence of the selective gene and the absence of the target gene at the 5' and 3' ends of the gene coding region via polymerase chain reaction. Trt1 expression was analyzed by western blot using extracts from log and stationary phase cells. The genes listed appeared to have little to no effect on Trt1 level compared with expression in wildtype log and stationary phase cells (not shown). Gene descriptions obtained from PomBase [4].

Gene	Function
	Telomere maintenance
ccq1	Telomere maintenence protein
est1	Telomerase regulator
pot1	Telomere end-binding protein
rap1	Telomere binding protein
taz1	Human TRF ortholog
ter1	Telomerase RNA
	Ubiquitin ligases
pub2	HECT-type ubiquitin-protein ligase
pub3	HECT-type ubiquitin-protein ligase
rfp2	SUMO-targeted ubiquitin-protein ligase subunit
rhp6	*rad6* homolog, ubiquitin conjugating enzyme E2
dbl5	Ubiquitin-protein ligase E3
dsc1	Golgi Dsc E3 ligase complex subunit
mug30	Ubiquitin-protein ligase E3
pep3	HOPS/CORVET complex subunit, ubiquitin-protein ligase E3
rad8	Ubiquitin-protein ligase E3
SPAC6B12.07c	Ubiquitin-protein ligase E3
SPAC12B10.01c	Ubiquitin-protein ligase E3
SPAC167.07c	Ubiquitin-protein ligase E3
SPAC16E8.13	Ubiquitin-protein ligase E3
SPAP32A8.03c	Ubiquitin-protein ligase E3
SPAPB17E12.03	Ubiquitin-protein ligase E3
SPBC14F5.10c	Ubiquitin-protein ligase E3
SPBC15C4.06c	Ubiquitin-protein ligase E3
SPBC16G5.03	Ubiquitin-protein ligase E3
SPBP8B7.23	Ubiquitin-protein ligase E3 implicated in transcription
ufd2	Ubiquitin-protein ligase E4
	Miscellaneous
isp6	vacuolar serine protease
pbr1	Human CTD-binding SR-like protein rA9 homolog
pmt3	SUMO (small ubiquitin-like modifier)
pom1	DYRK family protein kinase
rdp1	RNA-directed RNA polymerase

IV. Discussion

The work presented here show that the expression of Trt1, the telomerase reverse transcriptase subunit in *Schizosaccharomyces pombe,* is downregulated when cell growth is arrested by way of entry into stationary phase or starvation of nitrogen or glucose. This downregulation appears to be independent of transcript level, and different regions within the protein coding sequence appear to be sufficient for the decrease in protein level. These results reveal interesting insight into the regulation of telomerase expression in fission yeast, yet more work is needed to fully understand the mechanisms and purposes for these complex processes. Knowledge in *S. pombe* could lead to a better understanding of the same phenomena in normal and cancerous human cells.

While Trt1 protein level decreases as a result of nitrogen and glucose starvation, the pathways involved in this downregulation have yet to be determined. An intriguing possibility is that this is an example of cell cycle-regulation of telomerase expression. Nitrogen starvation results in arrest in G1, while glucose starvation results in arrest in G2. The Cooper Lab [82] has claimed that Trt1 protein level remains constant through the cell cycle in temperature-sensitive cell cycle mutants, but this idea likely warrants further investigation. Currently, the results shown here do not identify whether Trt1 expression in these conditions is regulated by the same pathways or different pathways. Further analyzing strains that contain knockouts and mutants in other genes involved in regulating cellular quiescence, such as some map kinases or the target of rapamycin complexes [83], or those directly involved in regulating protein expression, including genes involved in translation regulation or protein degradation, will likely identify the genes and pathways involved in the downregulation of Trt1. While the gene knockouts listed in Table 3.1

do not appear to be involved in the regulation of Trt1 expression, these results cannot rule out the possibility of redundancies in these pathways which may negate any effects of the knockouts on observable differences. Therefore, double mutants may be aid in identifying genes involved in regulating Trt1 expression.

The results shown indicate that the mechanisms for Trt1 downregulation may involve protein degradation triggered by multiple sequence or structural elements. Proteins involved in the degradation of hTERT have been identified and suggested to play a role in cell cycle-dependent regulation of telomerase activity [46, 84, 85]. More mutations and deletions to Trt1 would likely reveal regions that are required for Trt1 degradation. In addition, mass spectrometry analysis of Trt1 may be able to identify post-translational modifications that may serve to affect Trt1 expression or function. Similar experiments from Trt1-immunopurified extracts may also reveal other proteins which interact with Trt1 to regulate expression.

The Bähler Lab [86] has previously analyzed global RNA and protein abundance in growing cells and those induced to quiesce by 24 hrs of nitrogen starvation. They observed that overall protein abundance is reduced in nitrogen starved cells, but to a degree that is largely consistent with the decrease in cell volume, which is ~50%. This is in contrast with mRNA abundances in quiescent cells, which uniformly and globally decrease. The authors suggest that this disparity could be due to increased protein stability in these quiescent cells. This is of course in contrast to the observed expression of Trt1, the mRNA of which does not appear to decrease in arrested cells, while the protein level does. In addition, the authors report only a slight decrease in relative Pol II occupancy of trt1 mRNA between log and quiescent cells. Together, this research supports the ideas presented in this thesis: that the observed downregulation of Trt1 is likely through post-translational mechanisms that may be specific to this protein or a small

subset of proteins that require specific regulation distinct from global protein expression and stability.

The decrease in Trt1 expression under the *nda2* promoter suggests that the *trt1* promoter is not a source for downregulating Trt1 in the conditions tested. The stability of the aa1-210 Trt1 construct in stationary phase cells under the *trt1* promoter further indicates that the *trt1* promoter is not involved in Trt1 downregulation in stationary phase cells. These results are consistent with the observation that the level of trt1 mRNA does not decrease in stationary phase cells, as seen in qRT-PCR and RNA-seq data. The *nda2-trt1* construct further suggests that Trt1 protein level may be regulated post-translationally, as many post-transcriptional and translational regulatory mechanisms involve an mRNA's 5' and 3' untranslated regions [87, 88].

Increasing Trt1 expression by use of the α-tubulin promoter still results in a decrease in stationary phase cells, but to a level that is still above that seen in log phase cells expressing the Trt1 with its native promoter. This suggests that the machinery required for downregulating Trt1 is likely being overloaded in these cells. While this does not allow us to probe the mechanisms by which Trt1 level decreases, it does allow us to pursue possible effects on cells that express Trt1 in stationary phase. Preliminary data suggests that exogenously expressing Trt1 in stationary phase cells via the *nda2-trt1* construct (see Fig. 3.5B) does not restore telomerase activity in extracts (data not shown). Similarly, cells overexpressing Trt1 in log phase also do not have increased telomerase activity over cells expressing wildtype levels of Trt1. This is also suggested by the near-wildtype length of telomeres in these cells (Fig. 3.5D). These experiments suggest that the downregulation of telomerase activity in stationary phase is likely more complex than merely caused by changes in Trt1 level. This is not surprising as Trt1 is not the only component required for telomerase activity. Specifically, processed, functional Ter1 is likely

limiting in these samples. While experiments from our lab suggest that the level of processed Ter1 does not decrease in stationary phase, the level of spliced Ter1 appears to increase in these cells (Baumann Lab, unpublished), which may have a dominant negative effect on telomerase activity [89]. Further, these cells overexpressing Trt1 do not appear to have any growth defects when grown under standard laboratory conditions. Once the mechanisms involved in Trt1 downregulation following nutrient starvation have been identified, future experiments could much more effectively focus on the consequences of deregulating Trt1 expression, including effects on telomere length maintenance and cell proliferation.

While telomerase expression and activity appear to be tightly regulated, the implications for misregulation of telomerase, particularly in a single-celled organism, remain unclear. While regulating the expression of telomerase subunits is expected to have implications for telomerase activity at telomeres, other cellular processes may be affected as well. Extra-telomeric roles for telomerase components have been identified in mammals [90], and similar functions may be present in yeast. For example, human TERT has been observed to have a role as a transcriptional modulator in the Wnt/β-catenin signaling pathway, which is involved in many cellular processes including cell proliferation and differentiation [91]. Similar roles in cell growth may exist in fission yeast. In addition, telomerase has been observed to add *de novo* telomeres to DNA double-strand breaks, which can lead to the loss of DNA and genome instability [92, 93], suggesting that this is likely another condition for which maintaining low levels of telomerase could be vital for the cell.

One intriguing observation is the presence of two Trt1-Cmyc9-specific bands on western blots (Fig. 3.1). The expected size of Trt1-Cmyc9 is approximately 132 kDa, and the two bands appear to run between 5 to 20 kDa apart on western blots. In addition, the ratio of the two seems

to change between samples, with no obvious correlation between this shift and the cellular conditions tested. The presence of a second band may be an artifact of the presence of the C-terminal 9-myc tag on Trt1, but it may also be a post-translational modification or degradation product that holds a biological significance involving the function or regulation of Trt1. Based on the apparent size difference, we hypothesized that a second band could be caused by a post-translational modification, such as monoubiquitination, or possibly proteolytic cleavage. This strain was further analyzed by Haering, et. al. who also describe the presence of two Trt1-Cmyc9 specific bands, the larger of which appears more robustly in immunoprecipitation experiments, which lead them to believe the alternative form may be a recombination event in the myc tag repeats [74]. This possibility is unlikely, however, as we have not detected a mixed population of cells with tags of varying lengths as determined by PCR or sequencing. Thus, the cause and potential biological importance of this second band remains elusive.

The results discussed in this thesis reveal the surprising result that expression of the catalytic subunit of telomerase, Trt1, is regulated in the fission yeast *Schizosaccharomyces pombe*, and attempts to identify the mechanisms by which said regulation occurs. Further work will hopefully provide a better understanding of these processes and potentially reveal implications relating to a series of diseases in humans.

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