DETERMINING THE INDIVIDUAL ROLES OF TELOMERE-ASSOCIATED PROTEINS IN TELOMERE REGULATION IN *ARABIDOPSIS*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Determining the Individual Roles of Telomere-Associated Proteins in Telomere Regulation in *Arabidopsis*

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Telomeres consist of repeating sequences of nucleotides at the end of linear chromosomes and associated proteins that together serve to protect chromosome ends from degradation or fusion. Misregulation of telomeres leads to issues such as replication stress, DNA double-strand breaks, and other biological consequences that affect normal DNA replication and organismal viability. Telomerase—an essential enzyme for telomere function and maintenance—is responsible for telomere replication and the maintenance of telomere length. In *Arabidopsis*, studies have led to the discovery of a highly conserved and essential telomere protein complex CTC1/STN1/TEN1 (CST), which caps and protects the end of the chromosomes. The CTC1 and STN1 components of CST complex in *Arabidopsis* interact with telomere-associated proteins POT1a and TEN1, which positively and negatively regulate telomerase activity, respectively. This study will investigate how the individual telomere-associated proteins CTC1, STN1, TEN1, and POT1a interact with each other, with the telomere, and with telomerase *in vivo*. Mutations in amino acids important for mediating the physical interactions between these proteins will be introduced, and their effects on telomere regulation will be tested. Analyzing the biological

consequences of these mutations, and their effect on telomere integrity, will give insight to the individual roles of telomere-associated proteins *in vivo* and advance understanding of how telomeres provide genome stability. As events caused by telomere dysfunction occur in a myriad of diseases related to defects in DNA and cell replication, the study of telomeres, specifically the CST complex, may provide insight into stem cell-related diseases and cancer.

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I also want to extend my gratitude to the Arnold and Mabel Beckman Foundation, for providing me the opportunity to fully immerse myself in research and enabling me to witness the incredible feats that research can accomplish. In addition, I would like to thank the head of the Texas A&M Beckman Research Scholars Program, Dr. Datta, for always giving me advice and encouraging me during my time as a Beckman Research Scholar.

A special thanks to my family for their support during this research experience. Thank you, Mom and Dad, for always supporting me and giving me advice when I needed it most. Thank you, Henry, for your boundless knowledge and willingness to help out (especially for the dozens of emails full of information when I ask for advice). Finally, thank you so much, Amelia and Samantha, for always encouraging me and reminding me of my potential. I appreciate it and love you all very much.

NOMENCLATURE

Co-IP	Co-immunoprecipitation
CST	CTC1/STN1/TEN1 Telomere binding complex
Y2H	Yeast Two-Hybrid Assay

CHAPTER I

INTRODUCTION

Telomeres

The evolution of linear chromosomes has enabled organisms to increase genetic diversity through recombination. However, two major problems in chromosome replication arise as a result of linear chromosomes: the end-protection problem and the end-replication problem (Ishikawa & Naito, 1999). In the end-protection problem, the physical similarity of chromosome ends to double-strand breaks elicits a DNA damage response. The end-replication problem is defined as the inability of conventional DNA replication to fully replicate linear chromosomes. The incomplete replication of the DNA template leads to the shortening of chromosomes in every cycle of DNA replication. Figure 1-1 demonstrates the aforementioned complications with linear DNA replication. Telomeres play an important role in maintaining genome stability by protecting the chromosome ends from these issues.

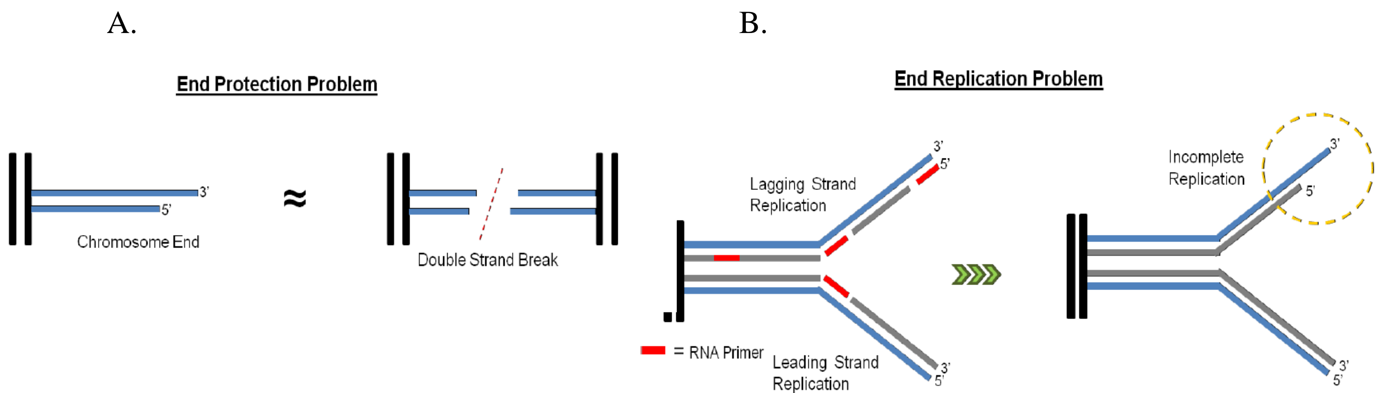


Figure 1-1: (A) The resemblance of chromosome ends to double-strand breaks triggers one of two pathways of DNA damage response: non-homologous end joining (NHEJ) and homology directed repair (HDR). These mechanisms lead to the unwanted extension or fusion of chromosome ends and complications in DNA replication and expression further down the cell cycle. (B) In conventional DNA replication, the lagging strand is unable to be extended fully by conventional DNA polymerases. This limitation leads to a loss of chromosome length with each replication cycle (Renfrew, 2014).

Telomeres fulfill a similar role to the ends of a shoelace: they protect chromosome ends from degradation and preserve the chromosome as a whole. They consist of both double- and single-strand DNA regions comprised of short G-C rich repeats (Grandin & Charbonneau, 2008). The repetitive nucleotide sequences of vertebrate and most plant telomeres consist of repeats of TTAGGG (humans) and TTTAGGG (*Arabidopsis thaliana)* (de Lange, 2004). Telomeric DNA is associated with multiple proteins and protein complexes. Most notably, telomeres are synthesized and maintained through the action of telomerase, a ribonucleoprotein that consists of a reverse transcriptase component (TERT) and an RNA subunit (TER) (Cifuentes-Rojas & Shippen, 2012). One of the most conserved features of telomeric structure is the G-overhang, located on the extreme 3' end of the chromosome, which is selectively extended by telomerase. G-strand invasion into double-strand region of telomere DNA forms a closed structure called a T-loop, which assists in chromosomal conservation by shielding the ends of the telomeres. Throughout the cell cycle, the G-overhang shifts to move between this closed configuration and an open one, where the single-strand overhang does not invade the double-strand DNA. For replication to occur in the S phase, exposure of G-overhang is necessary because it enables maximum telomerase activity (de Lange, 2004). In Figure 1-2, we see the correlation between the formation of T-loops and telomerase activity.

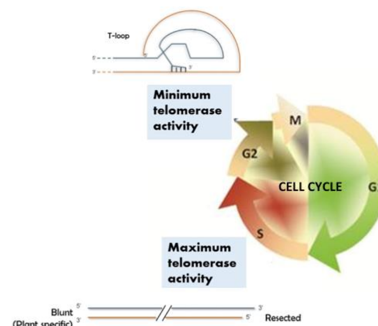


Figure 1-2: The G-overhang—located on the 3' end of the telomere—is involved in the formation of T-loops at chromosome ends. Shifts in this conformation help regulate telomerase activity, with T-loops forming during periods of minimal telomerase activity, while the open configuration enables telomerase to interact with and extend the telomeric sequences (Renfrew, 2014).

A balance between telomerase expression and telomere protection must occur in order to maintain telomere length and, in turn, organismal viability. Telomeres must be maintained at a sufficient length in order for cells to distinguish the end of the chromosome from a double-strand break. Telomeres that are too short suspend the cell cycle and lead to genome instability. In humans, the reduction of telomere length also results in replicative senescence, in which the cell loses the ability to proliferate (Greenberg, 2005; Greider, 1990). However, this replicative senescence prevents the formation of tumors by limiting the ability of cells to replicate (Cifuentes-Rojas & Shippen 2012). The balance necessary for maintenance of proper telomere length has led to the hypothesis that telomeres have two functional states. Telomeres switch between a structural conformation that is accessible to the telomerase, allowing it to extend the telomere, and a non-extendable, closed conformation (Teixeira et al, 2004). The telomerase enzyme engages telomeres when they are in an extendable conformation to replicate telomeric DNA. Once DNA replication is completed, the telomere is converted into a non-extendable state to allow for chromosome end protection (Teixeira et al, 2004). The molecular basis for these conformational changes are unknown and are a main focus for current research.

CST Complex

Telomere maintenance not only relies on telomeric DNA and telomerase activity, but also on other associated proteins. These proteins function to both help with the end-protection problem as well as promote telomere replication and maintenance. Specialized single-stranded and double-stranded DNA binding proteins work together with the T-loop to protect the chromosome end and to prevent its recognition as a double-strand break (De Boeck et al, 2009; Lewis & Wuttke, 2012).

In Arabidopsis, the CST complex is formed by the proteins CTC1, STN1 and TEN1. As demonstrated by Figure 1-3, this trimeric structure associates with 3'end of the telomere. CTC1 binds directly to the G-overhang of the single-stranded portion of the telomere through an oligosaccharide-oligonucleotide binding domain (OB-fold) and anchors the complex to the DNA (Bhattacharjee et al, 2016).

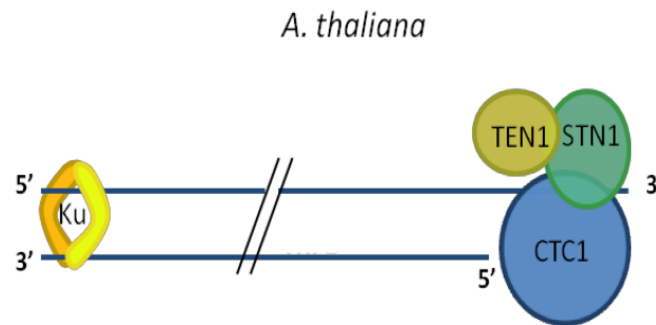


Figure 1-3: The CST complex binds to the single strand portion of the telomere. CTC1 directly interacts with STN1 in *Arabidopsis* to preserve telomere integrity. It also interacts with POT1a to positively regulate telomerase activity (Renfrew, 2014).

The proteins STN1 and TEN1 are necessary for telomere integrity and through their interaction with CTC1 assist in preserving the telomere (Price et al, 2010). In addition to TEN1, CTC1 and STN1 also interact with POT1a exclusively from TEN1, as well as directly with telomerase, to positively regulate telomerase activity (Leehy et al, 2013; Miyake et al, 2009; Surovtseva et al, 2007). These variable functions have led to the idea that the CST complex is required for end protection during the telomerase un-extendable state and is involved in the molecule switch from extendable to un-extendable state.

A current hypothesis is that opposing functions of POT1a and TEN1 cause the switch from telomerase extendable to telomerase un-extendable state throughout the cell cycle. During the G_1 phase of the cell cycle, the heterotrimeric CST complex binds the single-strand portion of

the telomere in the un-extendable state. After DNA replication in the S phase, POT1a (Pa) contacts STN1 (S) and CTC1 (C) and replaces STN1 (S) to form the CTC1/STN1/POT1a (CSP) complex, which promotes the transition to the telomerase extendable state (Renfrew et al, 2014) . Figure 1-4 exhibits the proposed model for the conformational switch of telomeres during the cell cycle.

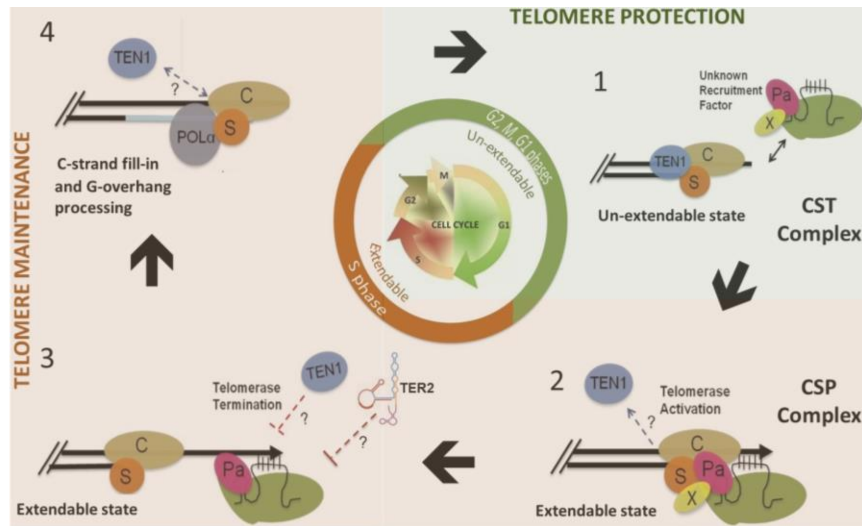


Figure 1-4: A current hypothesis is that opposing functions of POT1a and TEN1 cause the switch from telomerase extendable to telomerase un-extendable state throughout the cell cycle (Figure adapted by Ana V. Suescun from Renfrew, 2014).

The current study aims to determine the individual roles of telomere-associated proteins in this complex *in vivo* and to advance understanding of how telomeres provide stability for chromosomes. By elucidating the specific purpose of each of the aforementioned proteins, insight can be given into the validity of the proposed model and lead to greater understanding of telomere mechanisms and their role in biological processes and dysfunctions.

CHAPTER II

METHODS

This study aimed to elucidate the individual roles of telomeric proteins in the CST complex through separation of function analysis. Amino acid residues in STN1 and CTC1 were identified as potential candidates for mediating interaction between CTC1 and STN1. Single-point mutations of these residues were introduced into these sites, and the mutant constructs were cloned into expression vectors. The constructs were tested for proper expression, followed by testing of protein interaction through co-immunoprecipitation.

Conserved amino acid survey

A survey of the conserved amino acids for the E-CTC1 region (residues 700-1000 of AtCTC1) was conducted using ClustalW2. This program is designed to compare orthologous protein sequences from different species and, using an algorithm with parameters adjustable by the user, align the sequences to determine conserved residues between the orthologs and similar regions of sequences (Goujon et al, 2010; Sievers et al, 2011). Conserved amino acids were used in deciding which residues to use in the study because high conservation of a residue indicates the amino acid plays an important role in the protein, whether through function, structure, or interprotein interaction. For this study, orthologous sequences of flowering plants, yeast, and human were compared using ClustalW2 for CTC1 and Clustal Omega (the updated version of ClustalW2) for STN1 at the portal http://www.ebi.ac.uk/Tools/msa/clustalo/. The alignment used 23 sequences for CTC1 and 19 for STN1, including different species of flowering plants, yeast,

chicken, and human sequences. The results were analyzed for the most highly conserved residues, taking special note of residues that occurred in highly conserved regions.

Protein structure prediction

Using the PHYRE2 Protein Fold Recognition Server (Kelley et al, 2015), AtSTN1 protein structure was predicted using studied *Schizosaccharomyces pombe* and *Homo sapiens* STN1 structures at the following portal: http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index. The full STN1 protein sequence was used to generate this structure. A predicted structure for AtSTN1 was projected using the structure formed by *H. sapiens* STN1/TEN1 complex using the molecular structure visualization and analysis program UCSF Chimera (RBVI, NIH). Localization of the chosen residues was conducted following user instructions (Pettersen et al, 2004). CTC1 did not have enough studied orthologous protein structure to predict a structure and conduct this analysis.

Cloning

STN1 WT and CTC1 constructs were cloned each in pET28a(+) and pCITE4a vectors (Novagen). The full STN1 cDNA sequence was included in the construct, while the CTC1 construct was cloned from the corresponding cDNA for residues 700 to 1000 (E-CTC1), with no extra additions. This region was chosen due to experiments that indicated that the binding region of CTC1 to STN1 is between residues 717-990 (Surovtseva et al, 2009). The referenced region was extended for use in this study. Single-point mutations were introduced into the constructs through site-directed mutagenesis using PfuTurbo DNA Polymerase (Agilent), following PCR procedure indicated by the manufacturer's instructions with changes to the annealing

temperature depending on primers used (Sarkar et al, 1990). Alanine was substituted in the chosen residues due to the concept of alanine scanning (Lefèvre et al, 1997). Constructs were used for cell-free protein expression and co-immunoprecipitation.

Cell-free protein expression and co-immunoprecipitation

The proteins used in this study were expressed using a T7 Coupled Rabbit Reticulocyte Lysate (RRL) protein expression system (Promega) following manufacturer's instructions with [^{35}S]-methionine (Perkin-Elmer) to label the proteins expressed in both the pCITE4a and pET28a(+) vectors. Expression of proteins was confirmed by adding SDS loading dye, boiling for 3 min, and running the samples on a 12% SDS-PAGE gel. Co-immunoprecipitation (co-IP) assays were conducted to test the effect of mutations on protein interactions using the RRL-expressed proteins. The procedure was conducted as described (Karamyshiva et al, 2004), with the exception of the use of Dynabead Protein G (Thermo Fisher Scientific) for CTC1 mutant testing and Dynabead Protein A (Thermo Fisher Scientific) for STN1 mutant testing. Dynabeads were prepared using 9 µl of beads per reaction, 1 ul of T7 Monoclonal Antibody (Novagen) for every 25 µl of beads, and 100 µl of 1X PBS and 0.1% Tween for every 1 µl of T7 Monoclonal Antibody. The mixture was incubated at room temperature for 10 min, then washed with W100 (20mM Tris OAc pH 8.0, 0.1M glycerol, 1mM EDTA, 5mM $MgCl_2$, 0.2M NaCl, 0.1M KGlu, 0.01M NP40, 2.2 mM 10% Na deoxycholate) and Blocking Buffer (1.5 mg/mL lysozyme, 1.5 mg/mL BSA, 0.2 mg/mL glycogen, dissolve in W100). Co-immunoprecipitation (Co-IP) complexes were washed 3X for 10 min with W400 (0.8425 M W100, 0.315M KGlu, 1mM DTT), 3X for 10 min with 1X PBS in CTC1 mutant testing and 2X for 10 min with 1X PBS in STN1 mutant testing, and 2X with TMG with inversion for both Co-IP tests. The complexes

were eluted by boiling the sample for 3 min in SDS loading dye and then run on 12% SDS-PAGE gels. The gels were dried for autoradiography.

CHAPTER III

RESULTS

Identification of CTC1 residues required for STN1 but not POT1a binding

To determine possible residues important to CTC1-STN1 interaction, a survey of the conserved amino acids for the E-CTC1 region was conducted using ClustalW2. This region was chosen due to experiments that indicated that the binding region of CTC1 to STN1 is between residues 717-990 (Surovtseva et al, 2009). The referenced region was extended and named E-CTC1 for the purpose of this study. Orthologous protein sequences from different species of flowering plants, yeast, and human were compared. Five residues (Fig. 3-1) were selected for further studies: K720, R721, L779, L791, and I793; these residues were chosen due to their high conservation across orthologous proteins, indicating an important role in the protein through involvement in structure, function, etc. Through site-directed mutagenesis, single, double and triple E-CTC1 mutant constructs of the five residues were generated following the concept of alanine scanning (Lefèvre et al, 1997) and inserted into expression vectors pCITE4a and pET28a(+).

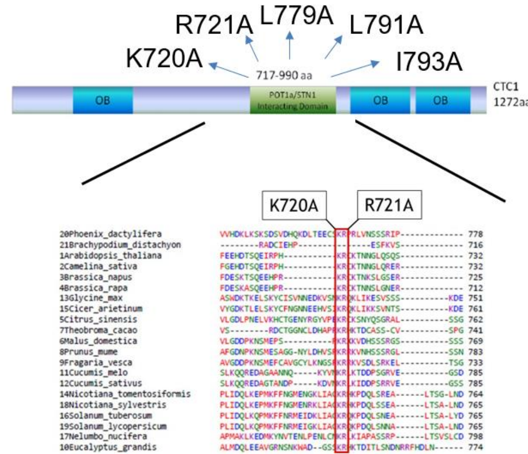


Figure 3-1: Excerpt from results of conserved amino acid survey conducted through Clustal W for E-CTC1. Residues K720, R721, L779, L791, and I793 were chosen for mutation to alanine through site-directed mutagenesis.

The E-CTC1 and STN1 WT constructs were expressed using a T7 Rabbit Reticulocyte Lysate (RRL) protein expression system (Fig. 3-2 A). Co-immunoprecipitation (co-IP) assays were then conducted to test the effect of mutations on protein interactions (Fig. 3-2 B, C).

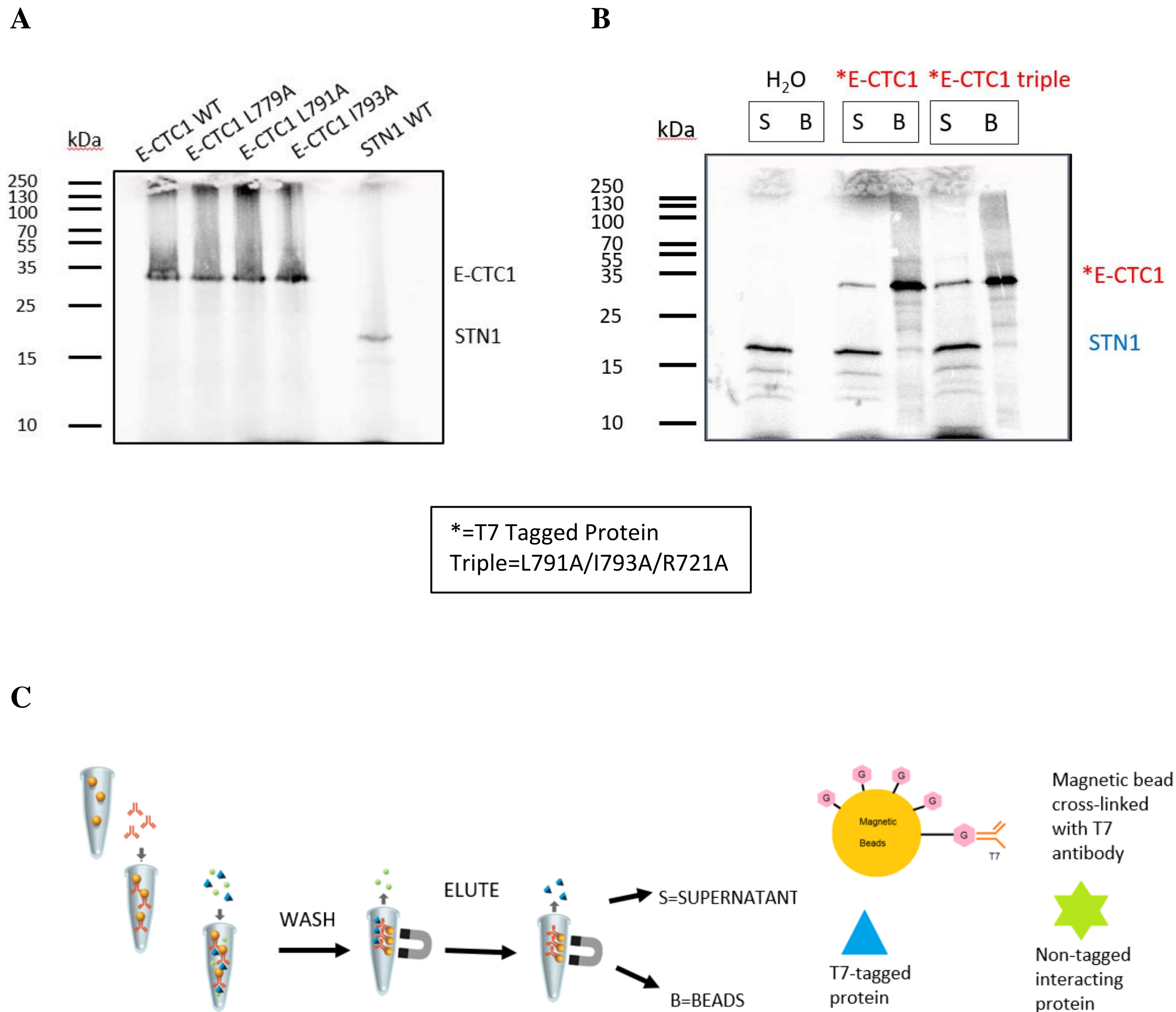
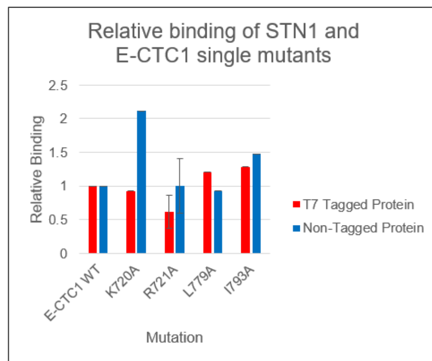


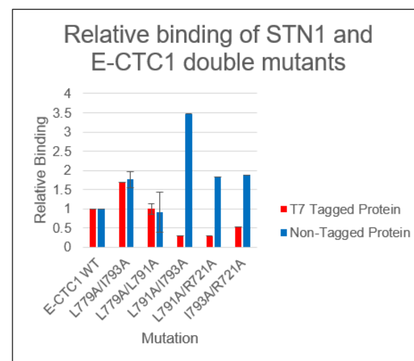
Figure 3-2: (A) Results of E-CTC1 and STN1 expression using T7 Rabbit Reticulocyte Lysate (RRL) protein expression system (B) Result of Co-IP assay using T7 antibody in WT STN1 pCITE4a construct and E-CTC1 WT and L791A/I793A/R721A CTC1 triple mutant pET28a(+) constructs. Supernatant (S) and Bead (B) fractions were analyzed and proteins separated in 12% SDS-PAGE. Water was used to establish beads-untagged protein background. Results were quantified through densitometric analysis with the program Quantity One. (C) Representation of co-IP protocol. Tagged proteins are expressed using RRL protein expression interact with ligand on solid substrate, in this case magnetic beads with T7 antibody attached. Target protein expressed in non-tagged protein, mixed with tagged protein and allowed to interact. Wash and elution steps are conducted, followed by SDS-PAGE gel and autoradiography analysis to check for pull-down of proteins, which indicate interaction or lack thereof.

Results for each co-IP were quantified and recorded (Fig. 3-3). No significant reduction of interaction occurred between wild type STN1 and E-CTC1 single mutants when compared to wild type interaction (Fig. 3-3 A). Double mutants containing pairs of the five selected mutations were constructed and tested through co-IP. While there was some reduction in several candidates, no significant reduction of interaction occurred between wild type STN1 and E-CTC1 double mutants (Fig. 3-3 B). Mutations that lead to a larger margin of reduction in interaction are desired because the effects of little interaction are going to be tested. So, candidates that lead to an increase or only slight decrease in binding between the two proteins are not good candidates for this study. Constructs of triple mutants of the five selected residues were constructed. Significant reduction of interaction occurred between wild type STN1 and the E-CTC1 mutant L791A/I793A/R721A, indicating residues L791, I793, and R721 as candidates for mediating protein interaction of CTC1 with STN1 (Fig. 3-3 C). To determine if the E-CTC1 triple mutant L791A/I793A/R721A retains POT1a binding while disrupting STN1 binding, co-IP assays were conducted between wild type POT1a and E-CTC1 constructs (Fig. 3-4 A). No significant change in the interaction with POT1a occurred (Fig. 3-4 B). The significant reduction of interaction between the triple E-CTC1 mutant L791A/I793A/R721A and STN1 and the lack of significant reduction with POT1a indicate that there is a POT1a binding function of CTC1 that is separate from its binding to STN1.

A



B



C

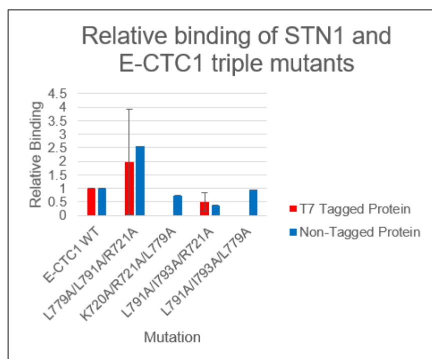
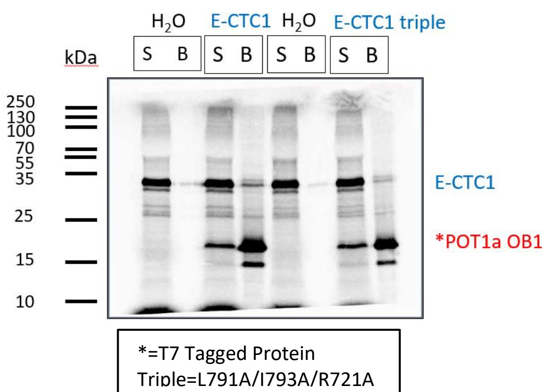


Figure 3-3: Protein interaction analysis between STN1 and E-CTC1 wild type using densitometric analysis with the program Quantity One. Bars with error indicate wild type mutant pairings that were tested three times. (A) No significant reduction of interaction occurred between wild type STN1 and E-CTC1 single mutants. (B) No significant reduction of interaction occurred between wild type STN1 and E-CTC1 double mutants. (C) Significant reduction of interaction occurred between wild type STN1 and the E-CTC1 mutant L791A/I793A/R721A.

A



B

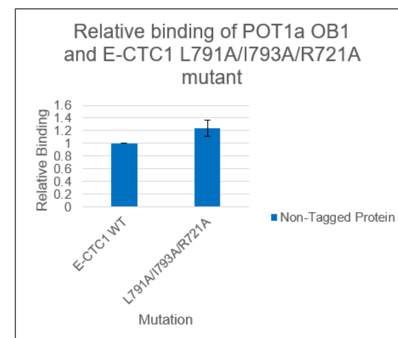


Figure 3-4: (A) Result of Co-IP assay using T7 antibody in WT and triple mutant E-CTC1 pCITE4a constructs and POT1a OB1 pET28a(+) construct. Supernatant (S) and Bead (B) fractions were analyzed and proteins separated in 12% SDS-PAGE. Water was used to establish beads-untagged protein background. Results were quantified through densitometric analysis with the program Quantity One. (B) No significant reduction of interaction occurred between wild type POT1a OB1 domain and the E-CTC1 mutant L791A/I793A/R721A in the non-tagged protein of E-CTC1.

STN1 single mutant G107A plays a role in structure or protein interaction of STN1 with CTC1 and TEN1

To determine possible residues important to STN1-CTC1 interaction, a survey of the conserved amino acids for STN1 was conducted using Clustal Omega. Orthologous protein sequences from different species of flowering plants, yeast, and human were compared. Due to the likelihood of highly conserved amino acids being important to protect function, nine conserved amino acids were selected for further study: G107, I106, P136, G45, D62, D63, W73, G114, and L12. Using the PHYRE2 Protein Fold Recognition Server, AtSTN1 protein structure was predicted by comparing *S. pombe* and *H. sapiens* STN1 structures, and the nine conserved residues were localized (Fig. 3-5 A). The predicted structure for AtSTN1 was projected using the structure formed by *H. sapiens* STN1/TEN1 complex, and localization of the nine conserved residues were determined in comparison with the interface of STN1 and TEN1 (Fig. 3-5 B). Of the nine residues, four were determined to be in locations of most probable involvement in STN1 protein interaction and least likely interaction with TEN1: W73, I106, G107, and G114.

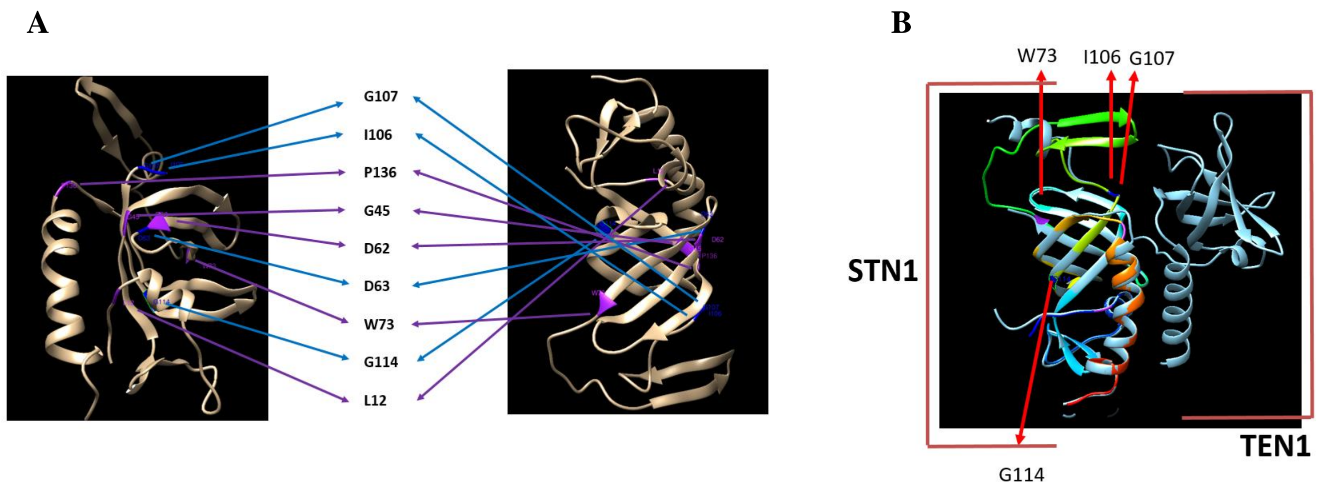


Figure 3-5: Modeling of AtSTN1 using PHYRE2 Protein Fold Recognition Server and UCSF Chimera molecular structure visualization and analysis program (A) Localization of nine conserved residues chosen through Clustal Omega analysis on the AtSTN1 predicted structure (B) Overlay of AtSTN1 predicted structure on the crystal structure of the HsSTN1/TEN1 complex.

Co-immunoprecipitation (Co-IP) assays were conducted to test the effect of STN1 single mutations on protein interaction with CTC1 (Fig. 3-6 A). Results were quantified through densitometric analysis with the program Quantity One and recorded (Fig. 3-6 B). STN1 single mutant G107A showed a significant reduction in interaction with wild type E-CTC1. STN1 single mutant G114A is currently undergoing testing.

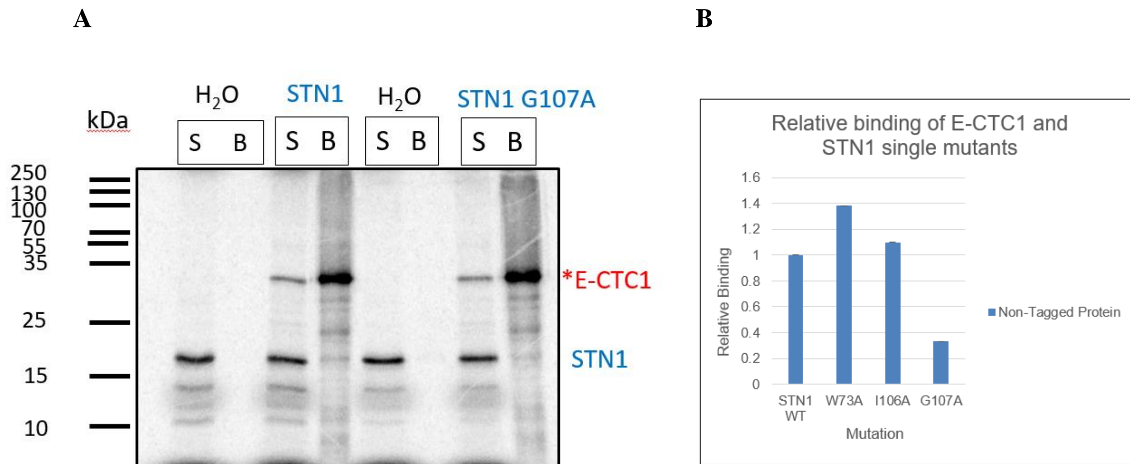
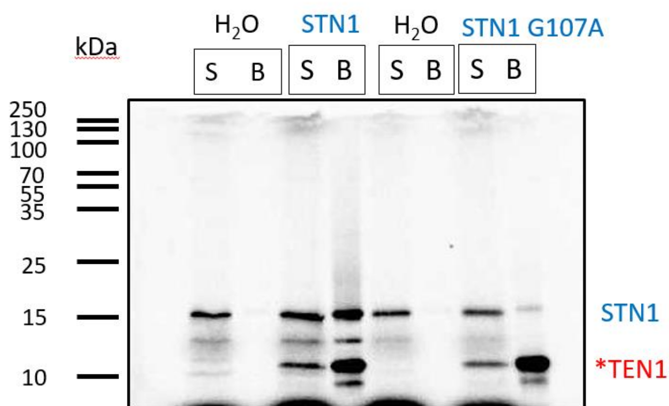


Figure 3-6: Testing of interaction between wild type E-CTC1 and STN1 single mutants (A) Result of Co-IP assay using T7 antibody in WT E-CTC1 pET28a(+) construct and STN1 pCITE4a wild type and G107A constructs. Supernatant (S) and Bead (B) fractions were analyzed and proteins separated in 12% SDS-PAGE. Water was used to establish beads-untagged protein background. Results were quantified through densitometric analysis with the program Quantity One. (B) Significant reduction of interaction occurred between wild type E-CTC1 and STN1 single mutant G107A.

To determine if STN1 single mutant G107A disrupts CTC1 binding but does not affect TEN1 binding, co-IP assays were conducted between wild type TEN1 and STN1 constructs (Fig. 3-7 A). Co-IP images were quantified in Quantity One using densitometric analysis, and results were recorded (Fig. 3-7 B). G107A STN1 single mutant showed a significant reduction in interaction with wild type TEN1. Mutant G107A's disruption of interaction with both wild type TEN1 and wild type E-CTC1 indicate a role in mediating interaction of STN1 with both proteins or a structural role within STN1.

A **B**



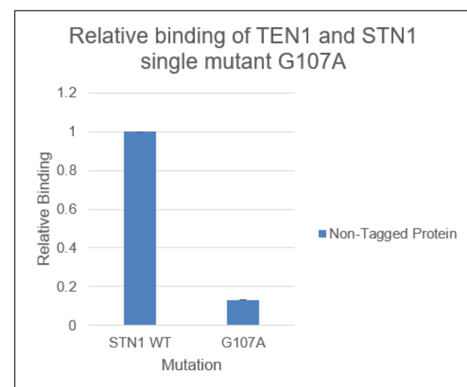


Figure 3-7: Testing of interaction between wild type TEN1 and STN1 single mutant G107A (A) Result of Co-IP assay using T7 antibody in WT TEN1 pET28a(+) construct and STN1 pCITE4a wild type and G107A constructs. Supernatant (S) and Bead (B) fractions were analyzed and proteins separated in 12% SDS-PAGE. Water was used to establish beads-untagged protein background. Results were quantified through densitometric analysis with the program Quantity One. (B) Significant reduction of interaction occurred between wild type TEN1 and STN1 single mutant G107A.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The CST complex plays a large role in preserving telomere integrity, and studies indicate that this trimeric complex may be involved in the change between the telomerase extendable and un-extendable states of the telomere (Teixeira et al, 2004). The current hypothesis for this conformational change highlights the interaction of CTC1 and STN1 with POT1a, a positive regulator of telomerase, and also with TEN1, a negative regulator of telomerase (Leehy et al, 2013; Miyake et al, 2009; Surovtseva et al, 2007). The model proposes a shift from the CST-complex in the closed conformation to the CSP-complex in the open conformation, leading to the switch from telomerase extendible to un-extendible states (Renfrew et al, 2014).

This study aims to determine the potential involvement of the CST complex in this conformational shift by determining the individual roles of these proteins in the complex through separation-of-function analysis. By disrupting interaction, but not structure, of the proteins in the CST complex, the role of the individual proteins in the trimeric complex can be determined, giving insight into the mechanism of interaction of the complex and its role in the states of telomerase-accessibility of the telomere.

The mutations used in this study were determined through conserved amino acid analysis and predicted protein structures. High conversation across orthologs indicates a residue may have a possible role in structure or function of the protein, due to the conserved function of orthologs across species. It was determined that the mutation of the conserved residue G107 disrupted interaction of STN1 with both CTC1 and TEN1, indicating involvement in mediating interaction with both proteins or a structural role. G114 remains a possible candidate for

mediating the interaction between STN1 and CTC1. After testing different combinations of the five conserved residues, the triple E-CTC1 mutant L791A/I793A/R721A showed a significant reduction in the interaction with STN1, but no significant change in the interaction with POT1a. The E-CTC1 L791A/I793A/R721A therefore separates the POT1a binding function of CTC1 from its binding to STN1.

Further directions of this project include verification of reduced interaction of E-CTC1 L791A/I793A/R721A with STN1 using a yeast two-hybrid assay. The constructs of STN1 and E-CTC1 used in the co-immunoprecipitation experiments have been ligated into cloning vectors pGBKT7 and pGADT7 (Clontech) and transformed into AH109 yeast cells (Clontech), with transformation success confirmed by selectively growing transformants on synthetic plates complete with dropouts. The transformed strains will then be used to test the strength of protein interaction between CTC1E and STN1 in two ways. Constructs will be grown on selective plates to qualitatively test for HIS3 expression. In addition, beta-galactosidase colorimetric assays will be conducted to quantitatively test strength of protein interactions. Further analyses of STN1 mutants will be executed to test for changes in protein interaction between STN1 mutants and CTC1 WT. This will allow a complete separate-of-function analysis for both proteins. Finally, transgenic *Arabidopsis* plants will be generated for separation-of-function analysis *in vivo* by specifically disrupting only one protein binding interface at a time. These plants will be used to conduct multiple tests of the biological consequences of disrupting interaction *in vivo.* Disruption of protein interaction in the CST complex could lead to disruption of the capping mechanism of the complex, leading to degradation or other results. Effects on telomere length will be assessed using Telomere Restriction Fragment (TRF) assays. Protein interaction could play an important role in recruitment and localization of the CST complex components on the telomere; chromatin-

immunoprecipitation (Ch-IP) will be used to study interaction of the protein components with each other and the DNA. Disrupting protein interaction may also affect the activity of telomerase, which can be assayed through Telomere Repeat Amplification Protocol (TRAP). Through these methods, the possible repercussions of disrupting protein interactions of the CST complex *in vivo* can be observed.

By analyzing the biological consequences of disrupting these protein interactions, specifically their effect on telomere integrity, this study will give insight to the individual roles of telomere-associated proteins *in vivo* and advance understanding of how telomeres provide genome stability. Telomere dysfunction occurs in a myriad of stem cell-related diseases as a result of defects in DNA and cell replication. Thus, the study of telomeres, specifically the CST complex, and telomerase dysfunction may provide important new insight into stem cell disease etiology.

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