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Phenotypic Analysis of Schizosaccharomyces Pombe Strains bearing Site-Directed Mutations in the Carboxy Terminal Domain of the Largest Subunit of RNA Polymerase II

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

PHENOTYPIC ANALYSIS OF *SCHIZOSACCHAROMYCES POMBE* STRAINS BEARING SITE-DIRECTED MUTATIONS IN THE CARBOXY TERMINAL DOMAIN OF THE LARGEST SUBUNIT OF RNA POLYMERASE II

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Abstract

The phosphorylation status of the largest sub-unit of RNA polymerase II (Rpb1p) is crucial to the control of transcription in eukaryotes. The domain subject to this phosphorylation is known as the carboxy terminal domain (CTD) and consists of multiple repeats (from 20 to 52 copies depending on the species in question) of the heptad sequence $Y_1S_2P_3T_4S_5P_6S_7$. Interestingly, differential phosphorylation of S_2, S_5, and S_7 residues is known to play an important role in the control of pre-mRNA processing. To determine the number of CTD repeats required for viability in *Schizosaccharomyces pombe*, truncated CTD constructs were integrated into the *S. pombe* genome through homologous recombination. I have observed that a minimum of eight heptad repeats is required for normal growth. While five heptad repeats is sufficient for viability, strains bearing these constructs exhibit impaired growth as well as morphological abnormalities. Next, in order to study the phenotypic effects of altered phosphorylation patterns, site-directed mutants were created in which alanine residues replaced serine (mimicking the non-phosphorylated state) or in which glutamate residues replaced serine (mimicking the phosphorylated state). Interestingly, alteration of the phosphorylation status of both S_5 and S_7 residues resulted in a variety of pleiotropic defects related to both cytokinesis and morphogenesis. Such a role for S_5 and S_7 phosphorylation is further supported by genetic analysis demonstrating synthetic genetic interactions between CTD site mutants and mutants affecting the function of the cell division machinery.

Keywords: RNA polymerase II, Carboxy Terminal Domain, Phosphorylation, Transcription, Fission Yeast

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Ade	Adenine
Bits	Binary Digits
Cdc	Cell Division Cycle
CDK	Cell Division Kinase
CTD	Carboxy Terminal Domain
DAPI	4’6,-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
EMM	Edinburgh Minimal Media
LatA	Latrunculin A
Leu	Leucine
Log	Logarithm
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pol	Polymerase
P-TEFb	Positive Transcription Elongation Factor b
RNAPII	RNA polymerase II
RT	Room Temperature
S2A	Serine-2 Alanine
S5A	Serine-5 Alanine
S5E	Serine-5 Glutamate
S7A	Serine-7 Alanine
S7E	Serine-7 Glutamate
Ser	Serine
SIN	Septation Initiation Network
TFIIH	Transcription Factor II H

TS	Temperature Sensitive
Ura	Uracil
UV	Ultraviolet
WWS	Wild-type, Wild-type, Serine-2 Alanine
WSS	Wild-type, Serine-2 Alanine, Serine-2 Alanine

Chapter 1: Introduction

1.1 Discovery and characterization of the carboxyl terminus of the largest subunit of RNA polymerase II

Transcription is the process by which an RNA copy of a gene sequence is produced and it is considered to be the first step in gene expression (Lee and Young 2000). Initially it was thought that, like in prokaryotes, one type of RNA polymerase could be responsible for all transcription in the eukaryotic cell. Further experiments disproved this idea. In the late 1960's, procedures and techniques were developed that allowed for the complete solubilisation of the RNA polymerase molecules (Roeder and Rutter 1969). Once solubilised the different RNA polymerases could be distinguished by chromatography or electrophoresis. Using these newly developed techniques, Roeder and Rutter (1969) were able to detect and partially purify three different DNA-dependent RNA polymerases from sea urchins and two from rat liver nuclei.

In addition to resolving the molecular weights and structural characteristics of the RNA polymerases, many researchers were interested in determining how they function in transcription. The first subunit to be allotted a functional role in transcription was the largest subunit of RNA polymerase II, now known as Rpb1. A mutation within the transcript region of this subunit, which was identified in *Drosophila melanogaster,* conferred resistance to amanitin binding (Greenleaf 1983). Amanitin is a toxin found in poisonous mushrooms that binds and inhibits the catalysis of DNA-dependent RNA polymerase II (Cochet-Meilhac, Nuret et al. 1974). The discoveries made by Greenleaf

implied that the largest subunit of RNA polymerase II has a role in DNA binding and RNA chain elongation.

Shortly after these discoveries, the complete purification of each RNA polymerase was accomplished and molecular masses were analysed. Of particular interest, the RNA polymerase II enzyme was found to exist in three distinct forms which were termed as IIO, IIA, and IIB. These were designated by the electrophoretic mobility of the largest subunit through polyacrylamide gels and the observation of three different protein bands (Schwartz and Roeder 1975). The differences in the molecular masses of the largest subunit were then explained by the ability of the largest subunit to be phosphorylated. This was done by growing HeLa cells in the presence of radio-labeled phosphate ($^{32}P_i$) along with casein kinase I or II, then detecting the amount of phosphorylation and the molecular masses of different subunits. The subunit that was found to be subjected to extensive phosphorylation was presumed to be the IIO subunit due to the similarity in molecular weights, the correlation with the highest polymerase activity, and its structural similarity to subunit IIA (Dahmus 1981).

Amino acid sequencing revealed the polypeptide, which could potentially undergo extensive phosphorylation, to be unique to subunits IIA and IIO (illustrated in Figure 1 by the blue and pink peptide sequence). The amino acid composition and lack of phosphorylation indicates that the IIB subunit is most likely a result of proteolysis of the unique peptide from the IIA subunit. This sequence of the peptide belonging to only subunit IIA and IIO is homologous to the C-terminal exon found in mouse the gene sequence of the largest subunit of RNA polymerase II (Corden, Cadena et al. 1985). This

domain consists of the highly conserved heptapeptide repeats of the consensus sequence $Y_1S_2P_3T_4S_5P_6S_7$. Further experiments confirmed that, through quantitative recovery of ^{32}P in the C-terminal peptide, this region was in fact the primary site of phosphorylation and that the IIO subunit was part of the transcriptionally active RNA polymerase II (Cadena and Dahmus 1987).

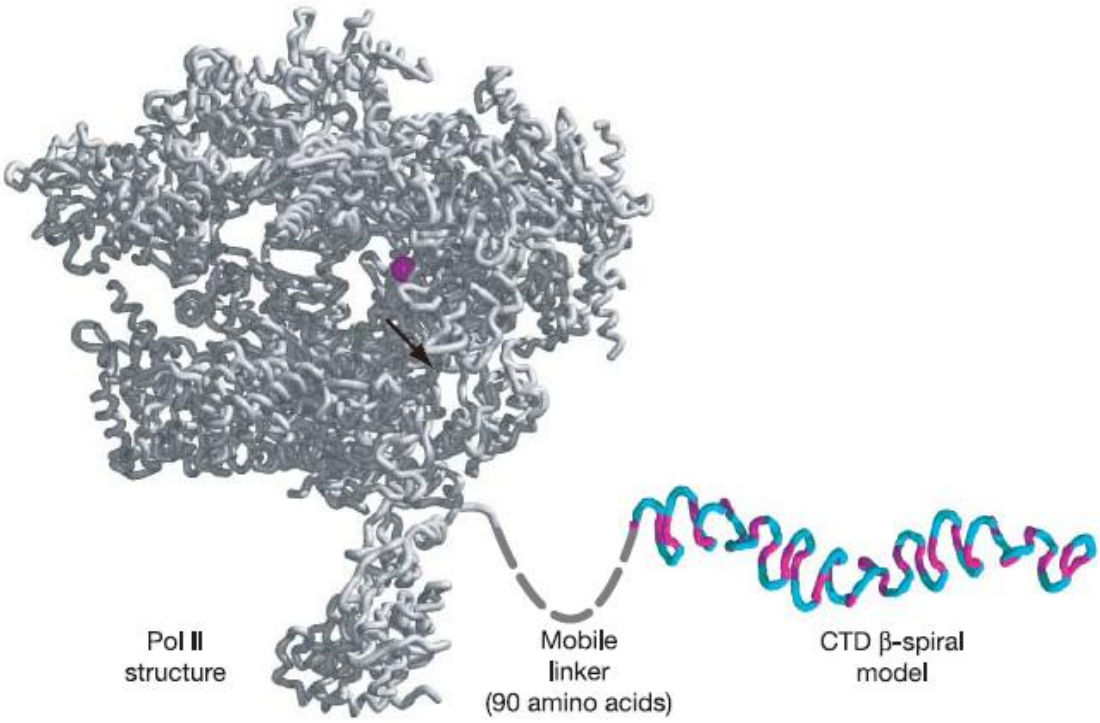

Figure 1. Structure of RNA polymerase II. The 12 subunit RNA polymerase II structure is represented in silver and grey along with the active site shown as a purple dot where the enzyme binds DNA as a substrate. The black arrow points to the opening where the RNA transcript is presumed to exit the molecule. The mobile linker (shown in grey) connects the CTD to the rpb1 subunit. The CTD consists of 26 repeats and is represented by alternating blue and pink regions. Serine-2 phosphorylated residues are central to each of the β-turns represented in blue. Pink indicates the extended regions of non-phosphorylated residues (modified from Meinhart *et al.,* 2004).

1.2 Identifying how the phosphorylation state of largest subunit of RNA pol II is regulated

Once the connection was made between the phosphorylation of the C-terminal domain (CTD) and a transcriptionally active enzyme, some thought that phosphorylation of the CTD was dependent upon transcription. The most obvious questions that needed to be answered were how the phosphorylation of this domain was accomplished and what factors control the modification of this subunit. Cadena and Dahmus (1987) examined whether the changes in phosphorylation state are dependent upon changes in the transcription of genes, or if the alterations are a consequence of activated nuclear factors or kinases under a condition/stimulus. To explore this idea, serum-stimulation of quiescent NIH 3T3 mouse fibroblasts were performed and changes in the phosphorylation level of RNA pol II were analyzed. There was a distinct increase in the amount of hyperphosphorylated IIO subunit upon serum stimulation compared to the amount of IIO subunit found in quiescent cells. Furthermore, this observation held true in transcription-inhibited cells upon serum stimulation, suggesting that the CTD modifications are a result of activated CTD kinases or inhibited phosphatases, and not directly due to alterations in the transcription pattern. Further experiments by (Dubois, Nguyen et al. 1994) show convincing evidence that MAP kinases, which function in a variety of signal transduction pathways, could phosphorylate the largest subunit of RNA pol II *in vivo*. These results strongly suggested that phosphorylation of the CTD of RNA pol II is accomplished by activated kinases as part of a signalling pathway and is independent of transcription. Lastly, Dahmus *et al.* (1987) propose that a change in a

growth condition that stimulates signalling pathways will result in the activation of CTD kinases that directly phosphorylate the RNA pol IIA subunit. The phosphorylation of this subunit could be a way to transcriptionally activate specific genes required for a response to the change in growth conditions.

1.3 CTD modifications during transcription

There has been evidence showing that the phosphorylation state of the CTD changes during the process of transcription. Early biochemical assays indicated that RNA polymerase II is in a hypophosphorylated IIA form during initiation while the elongating RNAPII enzyme is in the hyperphosphorylated II0 form (Cadena and Dahmus 1987). Furthermore, chromatin immunoprecipitation experiments indicate that phosphoserine-5 specific antibodies recognize RNAPII when at the promoter-proximal location whereas phosphoserine-2 specific antibodies bind RNAPII at distal promoter regions (Meinhart, Kamenski et al. 2005). More specifically, it has been discovered that initiation of transcription is accomplished when the cyclin-dependent kinase (CDK) sub-unit of TFIIH phosphorylates the fifth serine residue (Ser-5) of the CTD in the pre-initiation complex (Cramer, Srebrow et al. 2001). Following initiation, positive transcription elongation factor b (P-TEFb) promotes RNAPII transition from abortive to productive elongation (Lee and Greenleaf 1997). One of the subunits of P-TEFb, known as Cdk9, is the catalytic component of this complex and has specific kinase activity for the phosphorylation of serine-2 residues within the CTD (Peng, Marshall et al. 1998). Before and/or after termination the CTD becomes dephosphorylated by a number of different phosphatases

that work to recycle RNAPII back to the IIA form (Meinhart and Cramer 2004). These results explain how kinases and phosphatases modulate the CTD and are required for the progression of RNA pol II through the transcription cycle.

1.4 The function of the CTD for mRNA processing

The modulation of CTD phosphorylation during transcription correlates with the timely recruitment and binding of different factors that are required for RNA maturation and the packaging of mRNA. For example, the addition of the 5' guanosine-triphosphate cap is accomplished by capping enzymes that specifically bind to a ser-5 phosphorylated CTD. Furthermore, binding of capping enzymes is prevented from occurring when Ser-2 residues are phosphorylated (Ho and Shuman 1999). Others have found that splicing factors, Prp40 for example, assemble when the CTD is in its IIO form, but are inhibited from assembling when the CTD is in its IIA form (Morris and Greenleaf 2000). The CTD is also essential for 3'end processes such as the addition of the poly-A tail and for cleavage of the transcript. Yeast two hybrid assays have been a useful tool for identifying 3' end processing factors that interact with the CTD. Pcf11 is one of these factors and is required for cleavage and termination of transcription. Further analysis of Pcf11 has shown that this factor binds specifically to a CTD phosphorylated on serine-2 of the heptapeptide repeats (Licatalosi, Geiger et al. 2002). These results clearly demonstrate that there is a dependence upon the phosphorylation state of the CTD in order to coordinate the processes involved in mRNA biosynthesis. Figure 2 depicts a hypothetical image of the CTD acting as a binding scaffold for multiple interacting proteins.

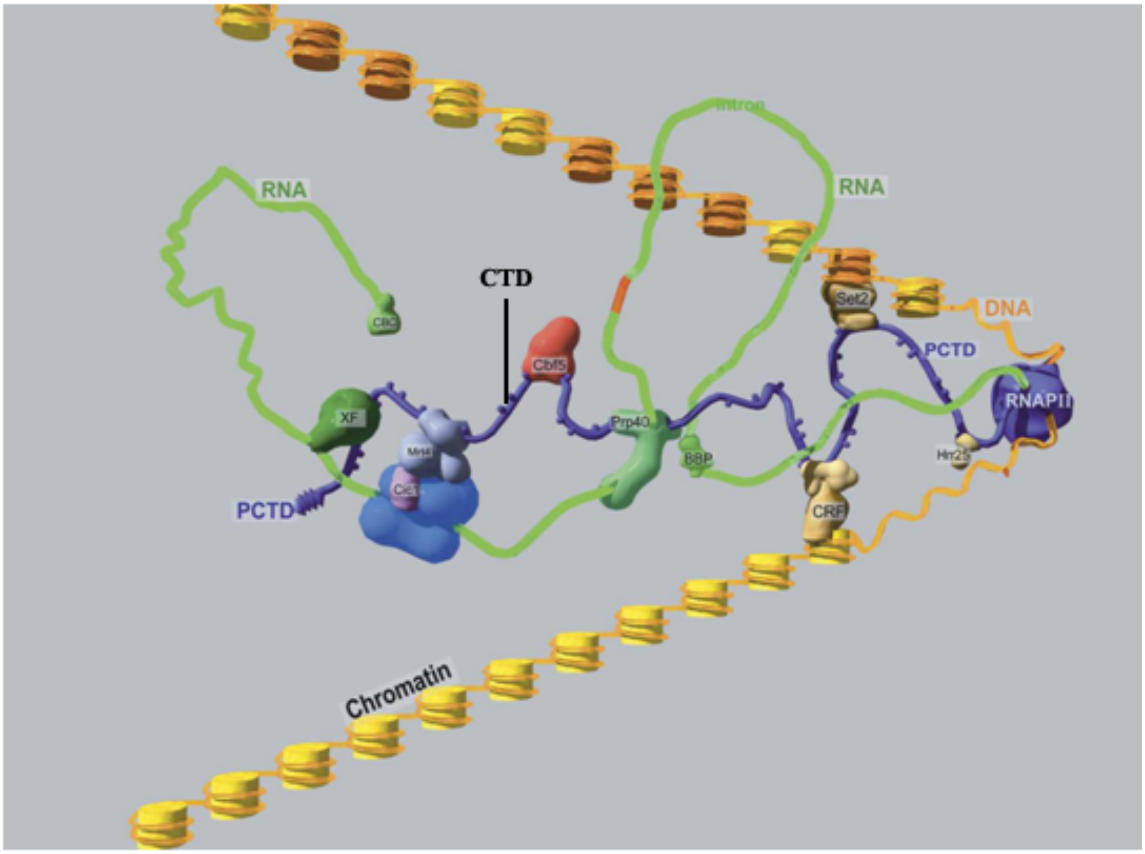

Figure 2. Hypothetical Model of an elongating RNA polymerase II and the binding of various proteins to the CTD. RNA polymerase II is represented in purple; DNA is orange and wrapped around yellow nucleosomal histones (darker yellow histones have been methylated by the enzyme Set2). The green RNA transcript is being sliced, capped, and processed by proteins that are bound to different regions of the CTD. (modified from Phatnani and Greenleaf, 2006)

1.5 The CTD code

In addition to its role in the processing and packaging of transcripts, some have also speculated that the CTD is crucial for the fine and intricate regulation of genetic networks that might be required for the development of increased complexity. Stiller and Hall (2002) hypothesized that: "…*the enhanced control over RNA polymerase II transcription conveyed by acquired CTD/protein interactions was an important step in the evolution of intricate patterns of gene expression that are a hallmark of large, developmentally complex eukaryotic organisms*." This hypothesis was supported by Stiller and Hall's (2002) phylogenetic analysis, showing how canonical CTD repeats are highly conserved in a group of eukaryotes all descended from a common ancestor and that these eukaryotes have more complex developmental processes than others lacking this highly conserved domain. In addition, the number of CTD repeats tends to increase with the complexity of the organism (15 repeats in Microsporidia compared to 52 in humans). The increase in number of repeats may be a means to further enhance the control over the expression of gene networks (Stiller and Hall 2002).

Enhanced control over the expression of gene networks can be important when a cell needs to respond to a certain environmental condition, stimulus, or stress. CTD interacting proteins target the CTD and alter it into different configurations via the phosphorylation/dephosphorylation of serine residues. These interactions are ways that cellular signals get relayed to the transcriptional machinery in order to alter gene expression. For example, phosphorylation of the CTD has been tightly linked to the transcriptional regulation of genes involved in DNA damage response. A dependence

upon specific CTD phosphorylations under UV light exposure has been shown to cause alternative splicing that turns subsets of genes on or off (Munoz, Santangelo et al. 2009). Furthermore, through the use of splicing-sensitive microarrays, differentially expressed genes that are involved in DNA damage response and apoptosis have been identified (Munoz, Santangelo et al. 2009). Other studies have linked certain CTD phosphorylation patterns to blocking the recognition of RNA polymerase II to a particular promoter region. These examples suggest that the expression of different subsets of genes might involve specific CTD configurations or 'codes' (Egloff and Murphy 2008).

The complexity of this code can represent the information potential of the CTD to be modulated by upstream signalling molecules. Different 'codes' or configurations of each repeat can be created through reversible phosphorylation of each of the three serine residues, and through the isomerisation of each proline residue. The complexity of the CTD 'code' within an organism can be calculated by multiplying the number of phosphorylation states of each repeat (8), with the number of *cis-trans* isomerisation states (4), to the power of the number of CTD repeats found in that particular species (Egloff and Murphy 2008). For example, *S. pombe* typically has 29 CTD repeats giving 32^{29} possible CTD configurations. Figure 3 further illustrates how the potential complexity of the CTD code can be calculated.

Previous reports explain how signalling molecules, such as kinase complexes, can interact with both the CTD of Rpb1 and mRNA processing factors to regulate the expression of genes. Cyclin-dependent kinases are proteins which, when activated by a CDK-activating kinase or CAK, transmit signals within the cell that regulate many

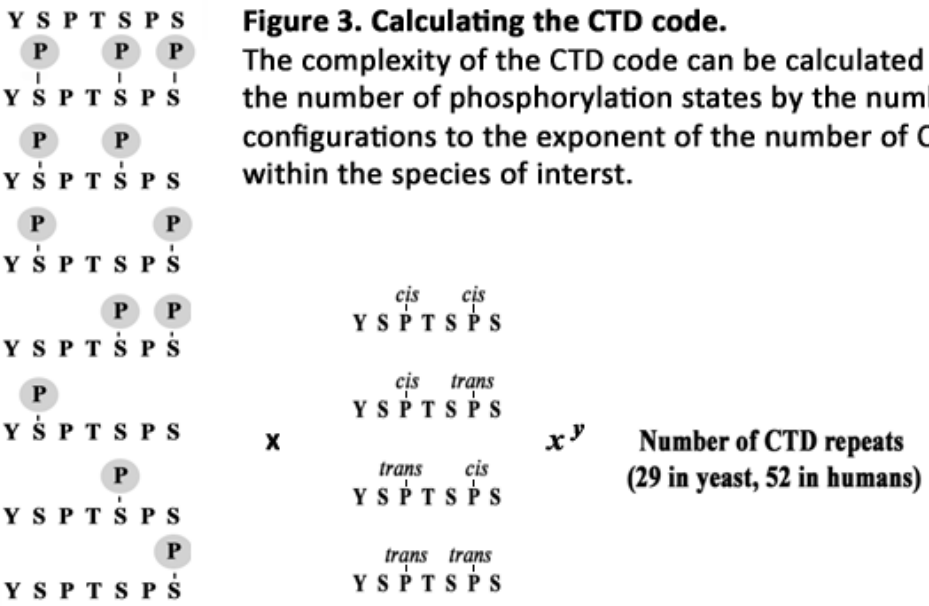

Figure 3. Calculating the CTD code.
The complexity of the CTD code can be calculated by multiplying the number of phosphorylation states by the number of cis-trans configurations to the exponent of the number of CTD repeats within the species of interst.

essential processes such as the cell cycle (Lee and Nurse 1987). CTD kinases have been identified that directly interact with the Rpb1 subunit and induce changes to the transcriptional machinery. A few CDK-cyclin complexes have been identified in yeast with the use of two-hybrid interaction screens. The kinase component that is part of a CDK-cyclin complex found in fission yeast, known as Cdk9, is activated to phosphorylate both the RNA pol II and the elongation factor Spt5 and also interacts with enzymes required for the 5' capping of mRNA (methyltransferase Pcm1 and triphosphatase Pct1) (Pei, Du et al. 2006). Without these interactions and addition of the 5' cap, transcript elongation cannot proceed. In addition, a functional redundancy for targeting the CTD of Rpb1 as a substrate was identified between Cdk9 and Mcs6, the kinase component of the TFIIH complex. Synthetic interactions were observed between these two kinases suggesting they perform similar functions; however, this functional redundancy is proposed to be a way to implement enhanced control over the expression of genes (Pei, Du et al. 2006). These results demonstrate how the CTD of RNA pol II is used to transmit signals from upstream kinase complexes that alter the function of the transcriptional machinery in order to control the expression of genes.

Many of the molecules identified in yeast that target the CTD have mammalian counterparts. For example, Cdc14 plays a role in the entry into the cell cycle for both yeast and mammals. This molecule functions as a phosphatase that specifically targets serine-5 within the CTD of RNA pol II. In mouse embryonic fibroblasts, Cdc14-null mutants displayed a significant increase in serine-5 of the CTD, early entry into S phase and mitosis, and a significant increase in multiple cell cycle regulators (Guillamot,

Manchado et al. 2011). Although the exact mechanism by which removal of phosphate from serine-5 affects gene expression is unknown, these results strongly suggest that the CTD is utilized to transmit signals for cell cycle progression to the transcriptional machinery within mammalian cells.

One of the best approaches to testing this hypothesis of a CTD 'code' is by creating defined mutations within the CTD that alter wild-type heptad repeats to different phosphorylation patterns or structural conformations and then analysing the consequences that these mutations have on both phenotype and gene expression profiles. The first study to take this approach used the budding yeast model *Saccharomyces cerevisiae* and gene expression analysis of interacting genetic networks within a CTD mutant containing five alanine residues (referred to as the 5A mutant) between each heptad repeat (Rogers, Guo et al. 2010). The 5A mutant had an enlarged growth phenotype compared to wild-type, along with increased DNA content, which was measured using flow cytometry. Upon microarray analysis, the 5A mutant had significant differences in the expression of a number of genes that could be categorized as having a role in chromosomal segregation, cell wall and membrane synthesis, and cell cycle and DNA repair. By using this approach the researcher can explain a particular phenotype by changes to the expression of gene networks that are a result of a particular CTD configuration.

1.6 Mutagenesis of the CTD of RNA polymerase II

Since the discovery of the CTD of RNA polymerase II, a number of different strategies have been developed to study the function of this domain *in vivo*. Typically,

these techniques involved substitution mutations of serine residues to alanine or serine to glutamate, mimicking a non-phosphorylated and hyperphosphorylated residue, respectively. In doing so, the function of different CTD phosphorylation patterns or configurations can be studied. (West and Corden 1995) cloned the 21 base-pair sequences that encode each heptad repeat by introducing an asymmetrical *AvaI* restriction site that flanks each repeat within the *rpb1* gene. Using this technique they cloned *rpb1*-CTD truncations and variants into yeast shuttle vectors, transformed them into *S. cerevisiae Δrpb1* strains, and assayed for growth and cell viability (West and Corden 1995). More recent studies have linked antibiotic resistance genes, such as *natMX*, to the *rpb1*-CTD constructs and cloned them into a vector that is designed to integrate into the host's genome at the *rpb1* locus through homologous recombination (Schwer and Shuman 2011). By integrating the *rpb1*-CTD constructs directly into the genome at the *rpb1* locus, the wild-type gene will be knocked out, and the mutated *rpb1* gene will be expressed under its native promoter. Lastly, a similar method of CTD construct integration has been used that does not require growth in the presence of an antibiotic. A cloning vector was created using a series of PCR reactions that used the wild-type genomic *rpb1* sequence as a template to incorporate a number of restriction enzyme sites for the cloning of CTD constructs along with a STOP codon to knock out the wild-type *rpb1* gene (Karagiannis and Balasubramanian 2007). This plasmid also contains the *ura4* gene so that the successfully transformed cells can be selected using media lacking uracil.

1.7 Using the fission yeast *Schizosaccharomyces pombe* as a model for genetic and phenotypic analysis

The fission yeast *Schizosaccharomyces pombe* is a simple, unicellular eukaryotic organism that makes for an excellent model for the study of the *rpb1*-CTD and the effects of site-directed mutations on cellular physiology. The complete genomic sequence of *S. pombe* was published over a decade ago and a significant number of these genes have been annotated (Wood, Gwilliam et al. 2002). Additionally, there are many molecular techniques that have been developed, making it very easy to genetically manipulate this model. Morphologically, *S. pombe* are cylindrical cells that undergo polar tip growth and divide by medial fission using an actomyosin contractile ring (Pelham and Chang 2002). Using fluorescence microscopy, which allows for the visualisation of nuclei and cell wall materials after staining, it is easy to identify abnormal phenotypes when determining the functions of genes through mutagenesis. In this way, many researchers have been able to identify a number of genes that are essential to the cell cycle and morphology (Verde, Mata et al. 1995). Lastly, many genes including *rpb1* are homologous to human genes; therefore we can gain a better understanding of how these genes may function within mammalian cells through the use of *S. pombe* as a model organism.

1.8 Research Hypothesis

Using the model eukaryote *Schizosaccharomyces pombe,* together with modern molecular techniques, I conducted a systematic study of site-directed mutations affecting CTD length and phosphorylation status. I hypothesized that defined alterations of the carboxy terminal domain of the largest subunit of RNA polymerase II would modulate the function of discrete cell signalling pathways. Within a eukaryotic cell, discrete pathways control many processes such as the regulation of cytokinesis, cell cycle control, and DNA damage response, to name a few. My prediction was that defined modulations of the phosphorylation state of RNA polymerase II would result in transcriptional changes of specific gene sub-sets (genes part of the same pathway or network) and I would be able to identify distinct phenotypes that are reflective of these changes. In addition, through genetic analysis, I hoped to identify genetic interactions between CTD site mutants and mutants affecting the function of gene networks. Ultimately, I hoped to provide further evidence that the CTD of the *rpb1* subunit of RNA polymerase II contains a programmable code that, when manipulated, could change the expression of specific genetic networks and result in discrete phenotypes.

Chapter 2: Materials and Methods

2.1 Strain, media, and growth conditions

Escherichia coli XL1-Blue cells were cultured in Luria-Bertannni Broth containing 100 µg/ml ampicillin at 36°C with shaking (200 rpm) when growing up cloning vectors. *S. pombe* strains (Table 2.1) were cultured in liquid YES media (Forsburg and Rhind 2006) to mid-log phase at 30°C with shaking (150 rpm). Genetic crosses were carried out using standard methods (Moreno, Klar et al. 1991). In cases where selection was required Edinburgh Minimal Media (Forsburg and Rhind 2006) was used by adding the required supplements (adenine, histidine, leucine, and/or uracil). SPAS Mating Media (Forsburg and Rhind 2006) was used for diploid sporulation prior to tetrad dissections and when mating was required.

2.2 Molecular Techniques

PCR methods: The primers used for Polymerase Chain Reaction (PCR) amplification were purchased through Integrated DNA Technologies© (Table 2.2). To confirm the integration of cloning vectors into the *S. pombe* genome, colony PCR reactions were performed. Genomic DNA template was extracted from *S. pombe* cells by mixing with 10 µl of 5% zymolyase in Phosphate Buffered Saline (PBS) pH 7.4 (Forsburg and Rhind 2006) and incubated at 36°C for 15 minutes. 5 µl of the extracted genomic DNA was used as the PCR template. Fermentas *Taq* DNA polymerase and *Taq* buffer were used for every reaction. Gel electrophoresis was performed with the PCR products for 1 hour

on a 1% agarose gel containing GelRed™ (Biotium, Inc.). The sizes of the PCR products were then observed under UV light (365 nm).

Table 2.1 *S. pombe* and *E. coli* strains used. Under relevant genotype the *E.coli* strain used is indicated and all other strains are *S. pombe.*

Stain Name	Relevant Genotype	Source
KH2	*rpb1-WTCTDx5::ura4+ ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH3	*rpb1-WTCTDx8::ura4+ ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH4	*rpb1-WTCTDx10::ura4+ ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH5	*rpb1-WTCTDx0::ura4+ ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH6	*rpb1-WTCTDx3::ura4+ ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH12	*rpb1-S5ACTDx12::ura4+ ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH13	*rpb1-S5ECTDx12::ura4+ ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH15	*rpb1-S7ECTDx12::ura4+ ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH16	*rpb1-WWSCTDx12::ura4+ ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH17	*rpb1-WSSCTDx12::ura4+ ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH18 (JK9)	*clp1::ura4 ura4-D18 leu+ h-*	This Study
KH19 (JK376)	*rpb1-S2ACTDx12::ura4 ura4-D18 leu1-32 h-*	This Study
KH21	*rpb1-S5A-CTDx12::ura4+ ura4-D18 leu1-*	This Study
KH24	*rpb1-WTCTDx8::ura4+ ura4-D18 leu1-*	This Study
KH25	*rpb1-WTCTDx10::ura4+ ura4-D18 leu1-*	This Study
KH26	*rpb1-WTCTDx5::ura4+ ura4-D18 leu1-*	This Study
KH27	*rpb1-S7ECTDx12::ura4+ ura4-D18 leu1-*	This Study
KH31	*rpb1-WWSCTDx12::ura4+ ura4-D18 leu1-*	This Study
KH32	*rpb1-WSSCTDx12::ura4+ ura4-D18 leu1-*	This Study
KH33 (derived from JK698)	*cdc12- 112 h+*	This Study
KH34 (derived from JK698)	*cdc12- 112 h-*	This Study
KH35 (derived from MBY154)	*cdc15-140 ade6-21x leu1-32 h+*	This Study
KH36 (derived from MBY154)	*cdc15-140 ade6-21x leu1-32 h-*	This Study

KH37 (derived from MBY2377)	*myo2-E1 ade6-21x leu1-32 ura4-D18 h+*	This Study
KH38 (derived from MBY2377)	*myo2-E1 ade6-21x leu1-32 ura4-D18 h-*	This Study
KH39	*rpb1-S7ACTDx12::ura4+/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	This Study
KH44	*rpb1-S5ACTDx12::ura4+ myo2-E1*	This Study
KH45	*rpb1-S7ACTDx12::ura4+*	This Study
KH51	*rpb1-S5ACTDx12::ura4+ cdc12-112*	This Study
KH52	*rpb1-S5ACTDx12::ura4+ cdc15-140 ade6-21x leu1-32 ura4-D18*	This Study
KH54	*rpb1-S5ACTDx12::ura4+ myo2-E1 ade6-21x leu1-32 ura4-D18*	This Study
KH55	*rpb1-S7ECTDx12::ura4+ cdc15-140 ade6-21x leu1-32 ura4-D18*	This Study
KH58	*rpb1-S7ACTDx12::ura4+ cdc15-140 ade6-21x leu1-32 ura4-D18*	This Study
KH61	*rpb1-S7ACTDx12::ura4+ cdc12-112*	This Study
JK9	*clp1::ura4 ura4-D18 leu+ h-*	JK Collection
JK366	*ura4-D18/ura4-D18 leu1-31/leu1-32 ade6-210/ade6-216 h-/h+*	JK Collection
JK375	*rpb1-CTDx12::ura4 ura4-D18 leu1-32 h-*	JK Collection
JK401	*rpb1-S2ACTDx12::ura4 ura4-D18*	JK Collection
JK402	*E.coli XL1-Blue*	JK Collection
JK484	*ura4-D18 leu1-32 ade6-210 his7-366 h –*	JK Collection
JK485	*ura4-D18 leu1-32 ade6-210 his7-366 h +*	JK Collection
JK698	*Cdc12-112*	JK Collection
MBY154	*cdc15-140 ade6-21x leu1-32 h-*	MBY Collection
MBY2377	*myo2-E1 leu1-32 ura4-D18 ade6-21x h+*	MBY Collection

Table 2.2 Primers. The following primers were used for Colony PCR.

Primer names	**Sequence**	**Purpose**
oJK82	5’- ACT GGC GCA TTT GAT ATT TA -3’	Confirm integration of pJK210 vector into rpb1 gene
MOH1207	5’- AAT TAA CCC TCA CTA AAG GG -3’	Confirm integration of pJK210 vector into rpb1 gene

Transformation of plasmid vectors into *S. pombe*: pJK210 cloning vectors were used to integrate CTD constructs into the *S. pombe* genome. The vectors were digested with *BamHI* to improve transformation efficiency then transformed into *S. pombe* using the lithium acetate protocol (Forsburg and Rhind 2006). Sonicated salmon sperm DNA purchased from Rockland (Gilbertsville, Pennsylvania) was used as a carrier for 3-10 µg of vector DNA into yeast cells. Transformed cells were selected by plating on EMM -ura.

Creation of truncated *rpb1*-CTD mutants: Wild-type *rpb1*-CTD truncated constructs were obtained from the Karagiannis lab collection. Each construct was digested with both *KpnI* and *SacI* restriction enzymes and cloned into the pJK210 vector containing the *ura4+* gene (selectable marker). DNA sequencing was done to confirm the number of *rpb1*-CTD heptapeptide repeats of each clone. Confirmed vectors were then digested with *BamHI* and transformed into the diploid *S. pombe* strain JK366 (ura4-D18/ura4-D18). The integration of the plasmid into the genome occurred via homologous recombination (Figure 2.1). *Ura4+* transformed cells were selected for and colony PCR was performed using primers MOH1207 and JK82 (Table 2.2) to confirm the correct integration of each vector. Restriction enzymes and DNA ligase were purchased from Fermentas and the manufacturer's protocols were followed (Double Digest™: http://www.fermentas.com/doubledigest/index.html).

Creation of site-directed *rpb1*-CTD mutants: Site-directed *rpb1-CTD* constructs were manually designed and purchased from Mr. Gene®. The amino acid substitution mutations of each construct are listed in Table 2.3. Each construct was digested out of

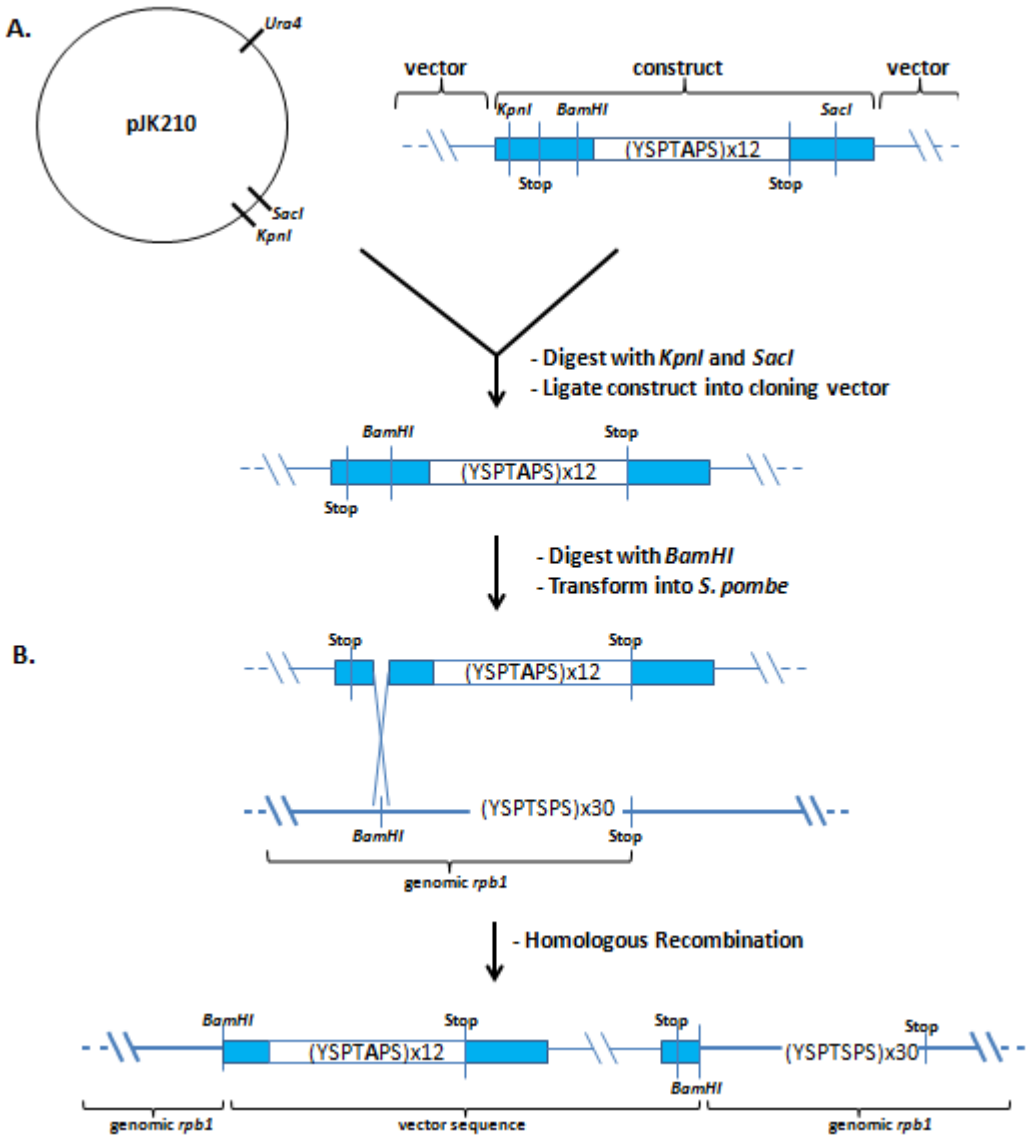

Figure 2.1 Integration of plasmid vector into *S. pombe* genome. A) Synthetic constructs digested from the screening vector and ligated into pJK210 cloning vector. B) Transformed vector integration into *S. pombe* genome at *BamHI* locus (modified from Karagiannis and Balasubramanian 2007).

Table 2.3 Site-directed Mutations. Names of site-directed CTD mutations along with the amino acid sequences of the heptapeptide repeats for each construct. The bold letter within each heptapeptide sequence represents the amino acid that has replaced the original serine residue.

Name of Mutation	**Amino Acid Sequence of CTD Repeats**
S5A	YSPT**A**PS x 12
S5E	YSPT**E**PS x 12
S7A	YSPTSP**A** x 12
S7E	YSPTSP**E** x 12
WWS	(YSPTSPS YSPTSPS Y**A**PTSPS) X 4
WSS	(YSPTSPS Y**A**PTSPS Y**A**PTSPS) X 4

the screening vector using the Fermentas restriction enzymes *KpnI* and *SacI* and cloned into pJK210 containing the *ura4+* gene used for selection. Double digestion and DNA sequencing (described below) was used to confirm the ligation of the correct construct into the cloning vector. The vector was then digested with *BamHI* and transformed into the diploid *S. pombe* strain JK366 (ura4-D18/ ura4-D18). Transformed cells were selected on agar plates lacking uracil and colony PCR was done using primers MOH1207 and JK82 (Table 2.2) to confirm the correct integration of each construct into the genome via homologous recombination.

DNA sequencing: The plasmid mini preparations were prepared by following the Robarts Sequencing Facility guidelines (http://www.robarts.ca/gateway.php?id=99). Sequencing was carried out at the facility and the results were analyzed and confirmed using GENtle software (University of Cologne, Version 1.9.4).

2.3 Genetic Techniques

Creation of double mutants: Haploid *S. pombe* strains containing site-directed CTD mutations were isolated from the tetrad dissections of sporulated diploid strains. Strains KH21, KH27, and KH45 were genetically crossed to each of the temperature sensitive strains JK698, MBY154, and MBY2377 (Table 2.1) using standard methods (Moreno, Klar et al. 1991) and grown on SPAS agar at room temperature (RT) for 48 hours. The spores produced from each cross were spread plated in serial dilutions on YES plates following the free spore analysis protocol (Forsburg and Rhind 2006) and grown at RT. Growth of the germinated spores from each cross was plated onto EMM –

ura grown at RT as well as YES plates grown at 36°C. Double mutants were identified as the strains containing the *ura4+* gene and having the temperature-sensitive phenotype at 36°C.

Tetrad Dissections: To analyze the viability of *rpb1-CTD* mutant strains, each heterozygous diploid strain was grown overnight at 30°C on SPAS media for sporulation. Individual tetrads were next isolated using a micromanipulator attached to Zeiss Axioskop 40 microscope and separated on YES media plates. By incubating the plates at 30°C for several hours the asci deteriorated and the individual spores were then separated using a micromanipulator needle. The four spores from each of the isolated tetrads were sorted in rows along the plate. Using this method, the two haploid spores that contain the integrated vector were left to grow separately from the wild-type spores over several days. These tetrad dissections were then replica-plated onto EMM-*ura* and haploid *rpb1-CTD* mutant strains were selected.

2.4 Fluorescence Microscopy

S. pombe strains of interest were grown to mid-log phase and fixed in ethanol (Forsburg and Rhind 2006) and stored in 15% glycerol and PBS pH 7.4 at 4°C. A mixture of 0.02 µg/µl 4'6,-diamidino-2-phenylindole (DAPI) and 1 µg/µl aniline blue were used to stain the cell's nucleus and cell wall/septa, respectively. Images were taken under fluorescent light using the Zeiss Axioskop 2 microscope along with ImageJ 1.41 software (National Institute of Health) and a Scion CFW Monochrome CCD Firewire Camera (Scion Corporation, Frederick Maryland).

2.5 Physiological Assays

Determination of Growth Rates: *S. pombe* strains were grown to mid-log phase in YES liquid media at 30°C with shaking (150 rpm). Each strain was then diluted to 0.1-0.2 optical density (OD) (wavelength 600 nm) and left to grow over 5 hours under the same conditions. OD measurements were taken every hour and doubling time calculations were performed using online software (Roth V. 2006 http://www.doubling-time.com/compute.php).

Latrunculin A treatment: *S. pombe* strains were grown in YES liquid media to mid-log phase overnight at 30°C with shaking (150 rpm). 2 mL of each cell culture was then treated with 0.3 µM Latrunculin A dissolved in DMSO (Karagiannis, Bimbo et al. 2005; Karagiannis and Balasubramanian 2007) and grown at 30°C for 5 hours with shaking (200 rpm). The treated cells were then fixed in ethanol, stained and observed under fluorescence microscopy. Latrunculin A was purchased from Enzo Life Sciences International Inc. (Plymouth Meeting, Pennsylvania).

Chapter 3: Results

3.1 Heptapeptide repeat number affects growth and morphology

3.1.1 Tetrad dissections reveal the minimum number of wild-type CTD repeats required for viability in *S. pombe*

The overall length and number of repeats of the CTD contributes significantly to the complexity of the CTD 'code'. Theoretically, having more CTD repeats increases the capacity to acquire more information from signaling molecules and CTD interacting proteins. This theory has been supported by the observation that eukaryotic organisms with more complex developmental processes generally have CTDs that are longer and contain more heptapeptide repeats (Stiller and Hall 2002). It is also interesting to note how many CTD repeats are actually required to support viability in an organism. Although in *S. pombe* a CTD of 29 repeats is conserved, only a small proportion of these repeats are actually required for this species to grow and divide. This suggests that the majority of the repeats are needed for other important but non-essential cellular functions.

To identify the minimum number of CTD repeats required for viability in *S. pombe*, truncated CTD mutants were created having zero, three, five, eight, and ten wild-type heptapeptide repeats within the CTD of one of the *rpb1* alleles of a diploid cell. Each strain was transferred to SPAS media (Forsburg and Rhind 2006) to induce sporulation of the diploid cells. Upon sporulating, four spores form within an ascus sack (known as a tetrad). Two of the four spores will contain the mutated *rpb1* allele, while the other two spores will contain the wild-type *rpb1* gene with a full length CTD

sequence. The separation of the four spores from a tetrad on YES agar plates via tetrad dissections allowed for the growth of haploid spores to be observed in order to assess viability for each truncated CTD strain (See Figure 3.1).

As shown in Figure 3.1, cell death resulted in CTD truncated mutants containing zero and three wild-type repeats following tetrad dissections as only the two spores having the wild-type *rpb1* gene were able to grow from each tetrad. The strain that possessed only five CTD repeats was able to grow from a few of the tetrad dissections; however, the colonies produced from the spores that contain the CTD mutation appeared much smaller than the colony growths from the spores that were wild-type (*rpb1-5xCTD*, figure 3.1). This observation suggested a possible impact on the growth rate of these cells. Figure 3.1 also showed that strains having eight or more wild-type CTD repeats showed complete viability and appeared to be growing at normal rates. Based on the observations that were made following tetrad dissections, it was determined that a minimum of five wild-type CTD repeats was required for cell viability but may have resulted in growth restrictions.

3.1.2 Truncated CTD mutants containing five wild-type repeats have significantly slower growth rates than wild-type

Since the *rpb1-5xWTCTD* appeared to be growing slower than normal on tetrad dissection plates (Figure 3.1), the doubling time of this strain was analyzed and compared to both wild-type and other truncated CTD mutants. As shown in Figure 3.2, liquid cultures of cells having only five wild-type CTD repeats took almost an hour longer to double in density compared to wild-type (3.4 hr versus 2.5 hr). In addition, the

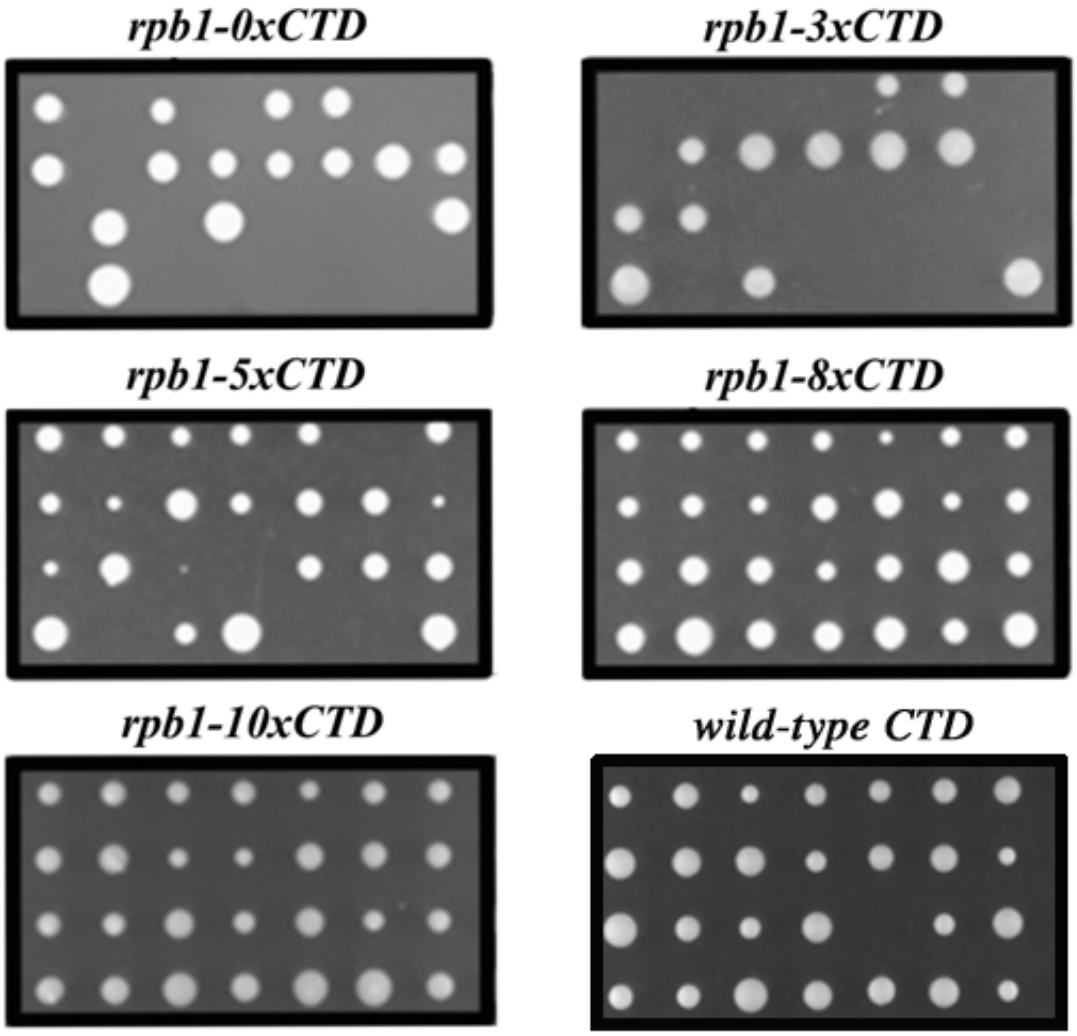

Figure 3.1. A minimum of five wild-type CTD repeats is required for viability. Diploid strains of indicated genotypes were sporulated on SPAS media (Forsburg and Rhind 2006) and tetrad dissections were performed, separating four spores from each ascus sack into columns on YES plates. The dissected tetrads were grown for several days at 30°C before imaging.

growth rates of *S. pombe* strains having eight, ten and twelve CTD repeats were not significantly different when compared to wild-type (Figure 3.2).

3.1.3 Morphological abnormalities and cell separation defects are caused by truncated CTDs with only five repeats while cells with eight CTD repeats appear normal

Once the *rpb1-5xWTCTD* strain was confirmed to be growing significantly slower than wild-type, a phenotypic analysis was performed to try to identify any causes for restricted growth. The phenotype of each truncated CTD strain was observed using fluorescence microscopy after the cells were stained with DAPI/aniline blue. *Rpb1-5xWTCTD* appeared to have multiple division septa and nuclei within a single cell, characteristic of cell separation defects and failed cytokinesis (Figure 3.3A). All other viable truncated CTD strains appeared to have normal phenotypes that resembled wild-type (Figure 3.3B-D). The phenotypic and growth rate analysis of the truncated CTD mutants suggests that there is not enough capacity in a CTD containing five wild-type heptad repeats to properly regulate the function of many essential cellular processes such as cell cycle control and cytokinesis.

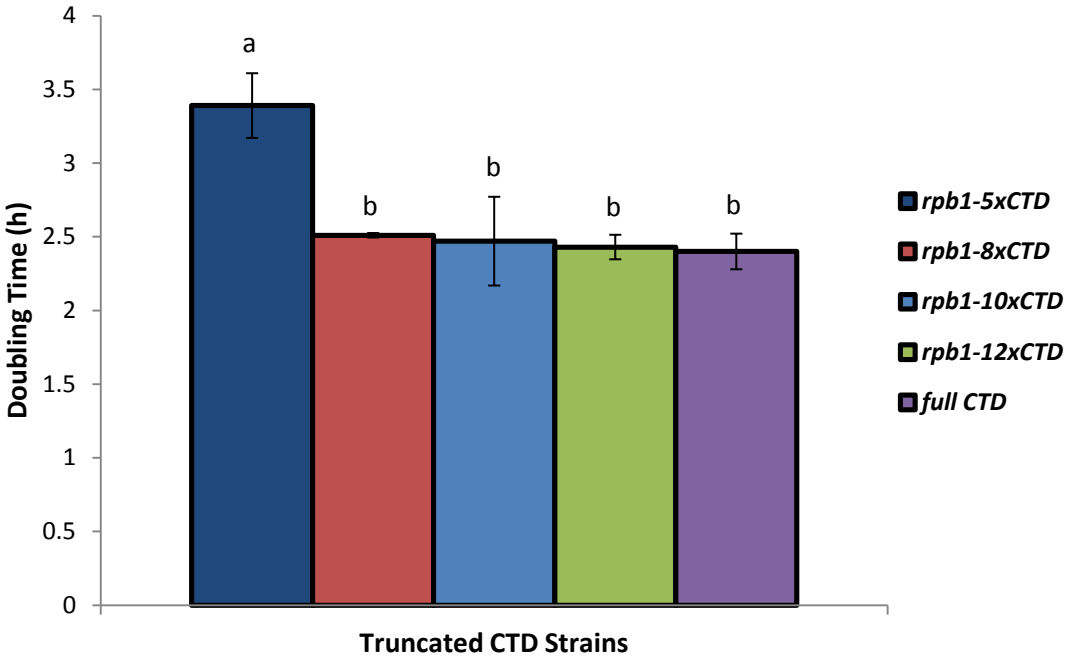

Figure 3.2. *Rpb1-WTCTDx5* grows slower than wild-type. Optical density (OD) measurements were taken every hour for five hours of logarithmically growing cells of indicated genotype and average doubling time was calculated over three trials. The data represent the average (±SD) across three trials. Different lower case letters denote significant differences (one-way ANOVA, followed by Tukey test, $p<0.001$).

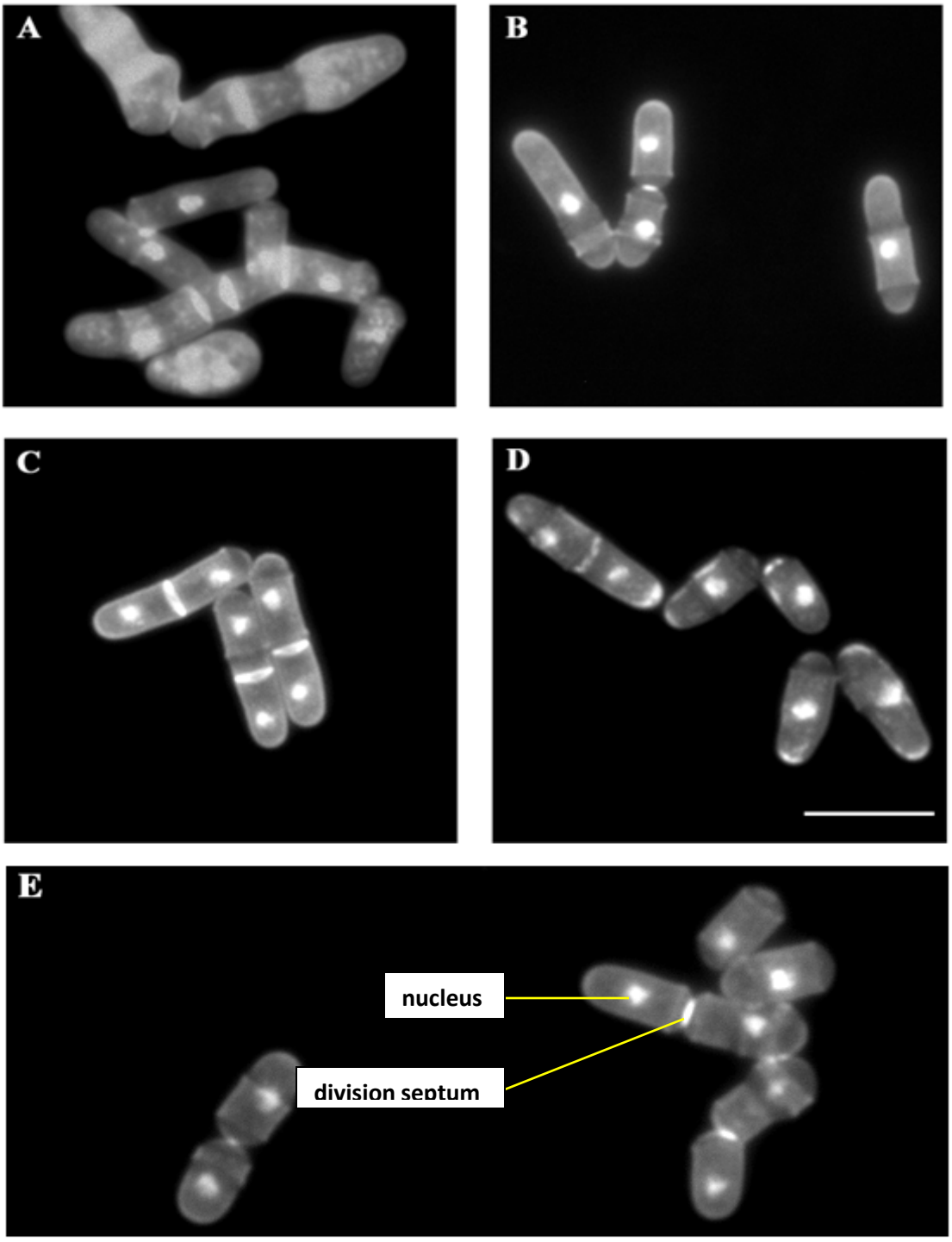

Figure 3.3. Five wild-type CTD repeats in *S. pombe* cause morphological abnormalities and cell separation defects. A) *rpb1-5xCTD* stained with DAPI/aniline blue showing multiple division septa and nuclei present. B) *rpb1-8xCTD*, C) *rpb1-10xCTD,* D) *rpb1-12xCTD* images show normal phenotypes when compared to wild-type in E). Bar, 10 µm.

3.2 Site-directed CTD mutations affecting serine phosphorylation can cause lethality, poor growth and/or morphological abnormalities

3.2.1 *S5A* and *S7E* mutations result in poor growth of dissected spores while the *S5E* mutation is lethal to *S. pombe*

Recent research has shown that the CTD of the *rpb1* subunit of RNA polymerase II is able to alter the expression of different subsets of genes through the modulation of serine phosphorylation within the domain repeats (Ostapenko and Solomon 2003). Protein kinases and phosphatases have been identified that interact with the CTD resulting in changes to the regulation of genetic pathways. These observations suggest that there is a CTD 'code' and by modifying this 'code' the expression of certain networks of genes can be affected. In order to test this hypothesis, site-directed CTD mutants were created that had defined mutations that changed serine-5 and serine-7 residues within each repeat to either alanine (*S5A* and *S7A*) or glutamate (*S5E* and *S7E*). Alanine is similar to serine in structure but cannot be phosphorylated; therefore, this mutation represents a non-phosphorylated form at that position. Serine to glutamate mutations will mimic a hyperphosphorylated position due to the chemical and structural similarities of glutamate to phosphoserine.

Once the site-directed CTD mutants were created, tetrad dissections were performed to test if the mutations were viable or lethal to the cell. Each heterozygous diploid CTD mutant was grown on YES agar at 30°C before being transferred to SPAS plates. Cells were left to sporulate on SPAS overnight and the tetrads produced were dissected on YES plates then grown for several days at 30°C. The growth of the tetrad

dissections were analyzed and it was determined that *S5A* and *S7E* mutations caused poor growth and the *S5E* mutation resulted in lethality. In Figure 3.4, the *S7A* mutation did not appear to have caused any growth restrictions and appeared completely viable from tetrad dissections. Although one of the spores failed to germinate in the *rpb1-12xS7ACTD* (as seen in Figure 3.4, fourth column, third row under *rpb1-12xS7ACTD*), this observation occurs frequently with wild-type *S. pombe* tetrad dissections as well and is probably not a cause of the CTD mutation (refer to Figure 3.1 for wild-type tetrad dissection).

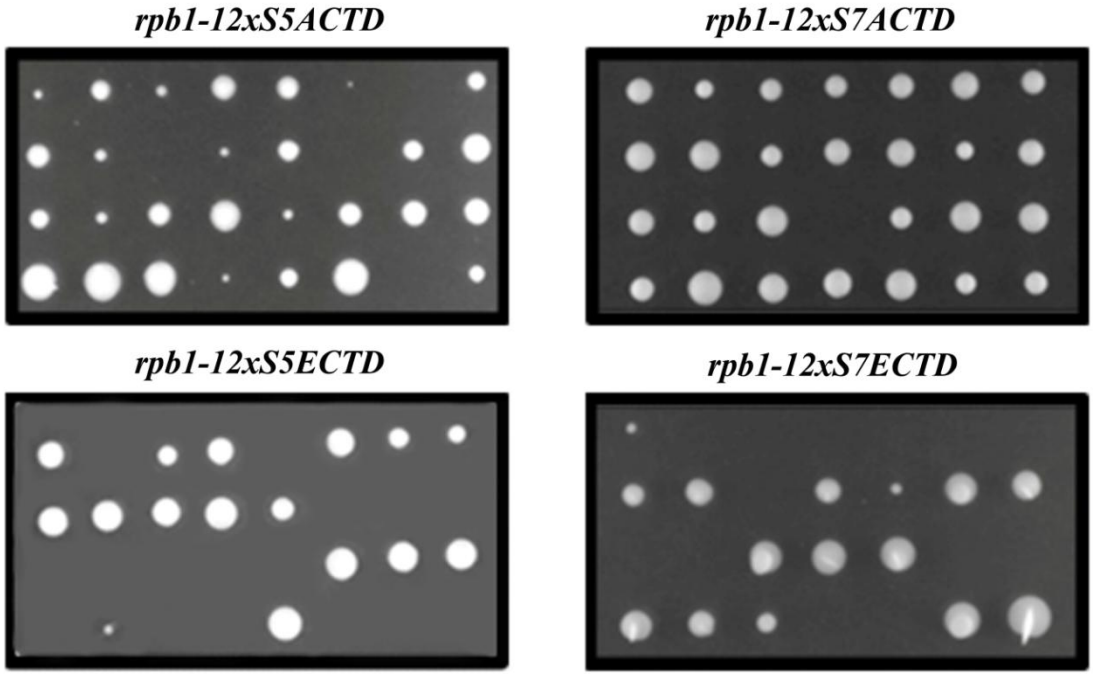

Figure 3.4. Site-directed CTD mutation with Serine-5 to glutamate is lethal while serine-5 to alanine and serine-7 to glutamate causes poor growth. Tetrads of diploid strains of indicated genotype were dissected in columns on YES plates and grown at 30°C over several days.

3.2.2 The *S5A* and *S7E* CTD mutations cause morphological abnormalities and the formation of multiple division septa

As an initial step to try to identify the consequences of these defined CTD mutations, each of the CTD mutant strains were grown at 30°C in YES to mid-log phase then fixed and stained with DAPI/aniline blue for phenotypic analysis. When imaged, morphological abnormalities were observed in the *S5A* as well as the *S7E* CTD mutants. The cells containing the *S5A* mutation appeared to result in the formation of multiple irregular septa and, in some cases, had undergone nuclear division without septum formation (Figure 3.5). Figure 3.5 also shows that these cells appear to be significantly larger than wild-type. The *S7E* appeared elongated, slightly curved and had multiple division septa (Figure 3.5). The *S7A* mutant resembled wild-type cells with no apparent abnormalities when grown at 30°C.

3.2.3 The *S7A* CTD mutation causes curved morphology at increased temperatures

Originally, when each CTD mutant was grown at 30°C before being fixed and stained, the *S7A* mutation did not appear to have any morphological consequences and resembled the wild-type phenotype. During further phenotypic analysis, each CTD mutant was grown to mid-log phase at a range of temperatures from 26°C - 36°C in YES media before being fixed and stained for imaging. The *S7A* mutation caused a curve-shape cell morphology at a temperature of 36°C (Figure 3.6). All other CTD mutants did not show changes in phenotype with increased temperatures.

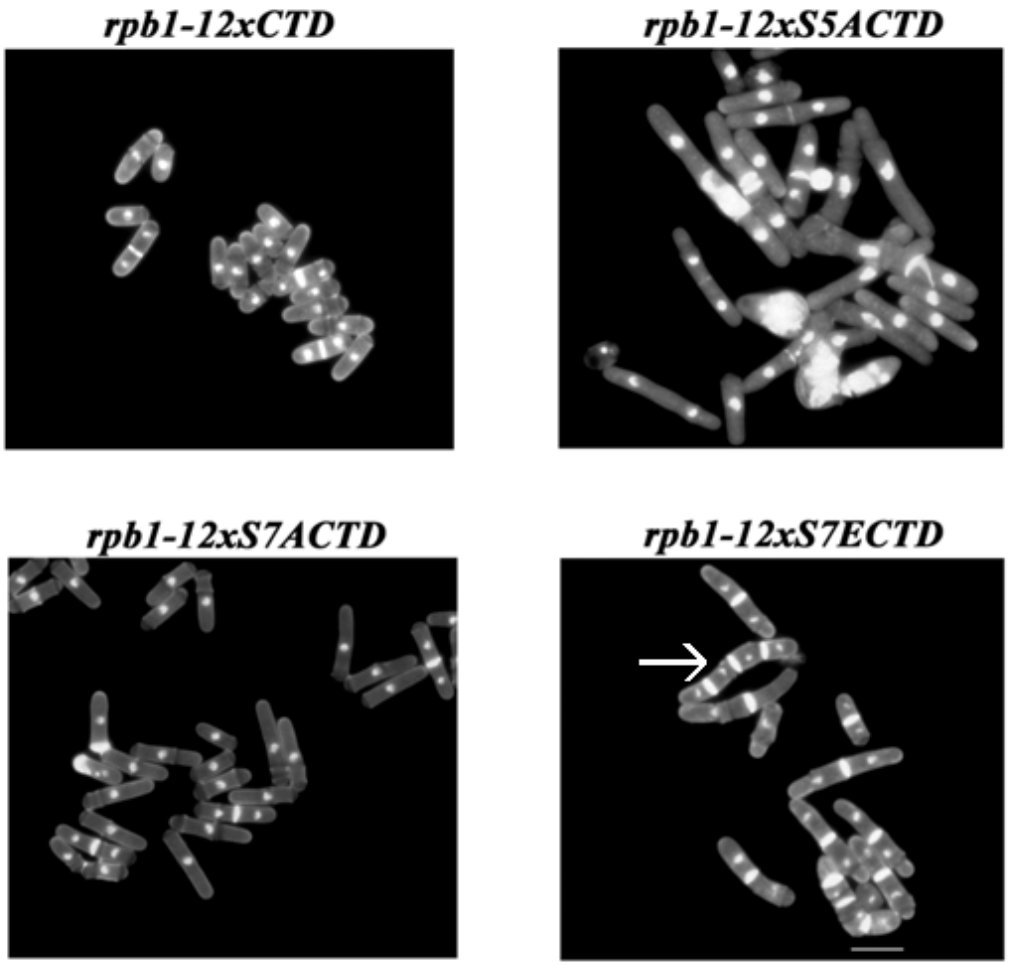

Figure 3.5. *S5A* and *S7E* CTD mutants cause morphological abnormalities. Cells of indicated genotype were grown to mid-log phase at 30°C in YES media before being fixed in ethanol and stained with DAPI/ aniline blue. Stained cells were then imaged using fluorescence microscopy. *Rpb1-12xS7ECTD* abnormal phenotype is pointed to with white arrow. Bar, 10 µm.

3.2.4 *S5E* CTD mutation cause highly branched cell morphology before leading to cell death

Tetrad dissections indicated that the *S5E* CTD mutation was lethal to the cell due to the observation that only two viable *ura-* spores were able to grow from each tetrad of the diploid heterozygous strain. Further analysis of the growth of these tetrads was done to try to identify if there was a lethal phenotype of the *S5E* CTD mutant or if the *ura+* spores failed to germinate altogether. Bright field microscopy was used to analyze the phenotypic consequences of this lethal mutation (Figure 3.7). The *S5E* mutants had appeared as either highly branched and elongated cells or they failed to grow after germination.

From the analysis of the site-directed CTD mutants it was clear that distinct phenotypes can be associated with specific modifications to the structure of the CTD. These results suggest that the CTD 'code' can be modulated to affect certain genetic pathways that lead to the distinct phenotypes that are being observed. Furthermore, the fact that multiple distinct phenotypes were identified in each of the CTD mutants that were tested, supports the theory that the CTD 'code' can be very complex and that one out of many CTD configuration states can lead to a specific phenotype.

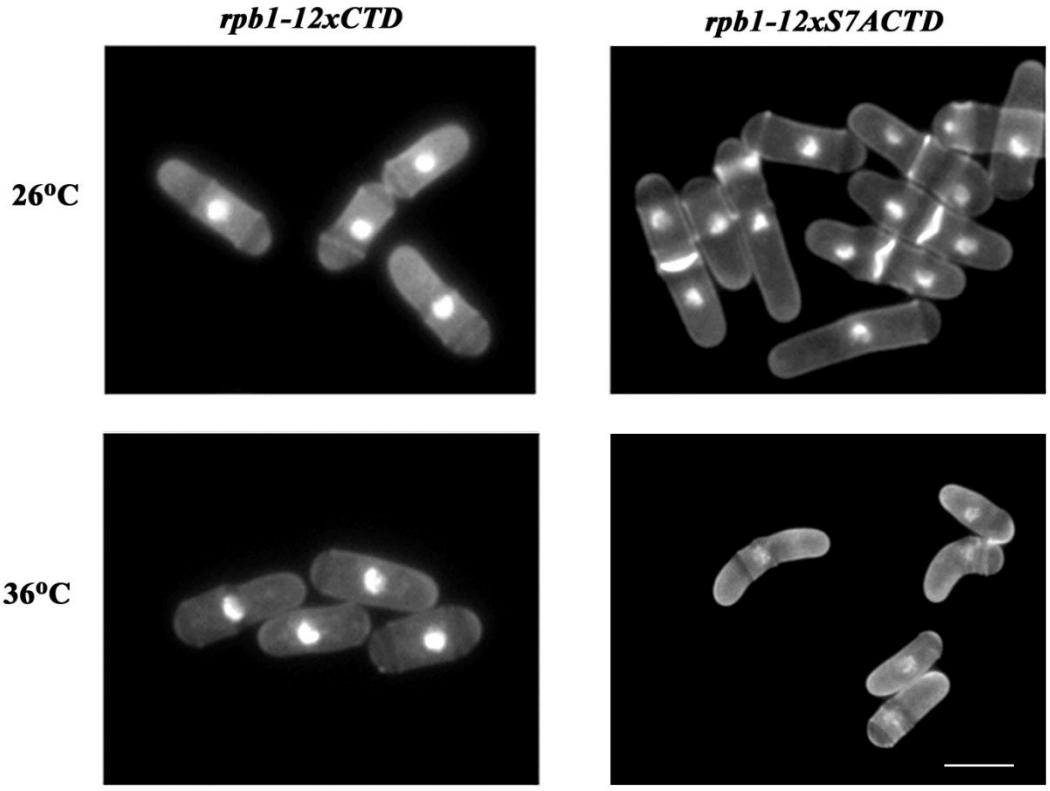

Figure 3.6. Temperature sensitive phenotype of *S7A* CTD mutant. Cells of were grown to mid-log phase in YES media at indicated temperatures (26°C or 36°C) and fixed in ethanol. Cells were stained with DAPI/aniline blue and fluorescence microscopy was used for imaging. *S7A* temperature-sensitive phenotype can be seen at 36°C but is not present at room temperature. Bar, 10 µm.

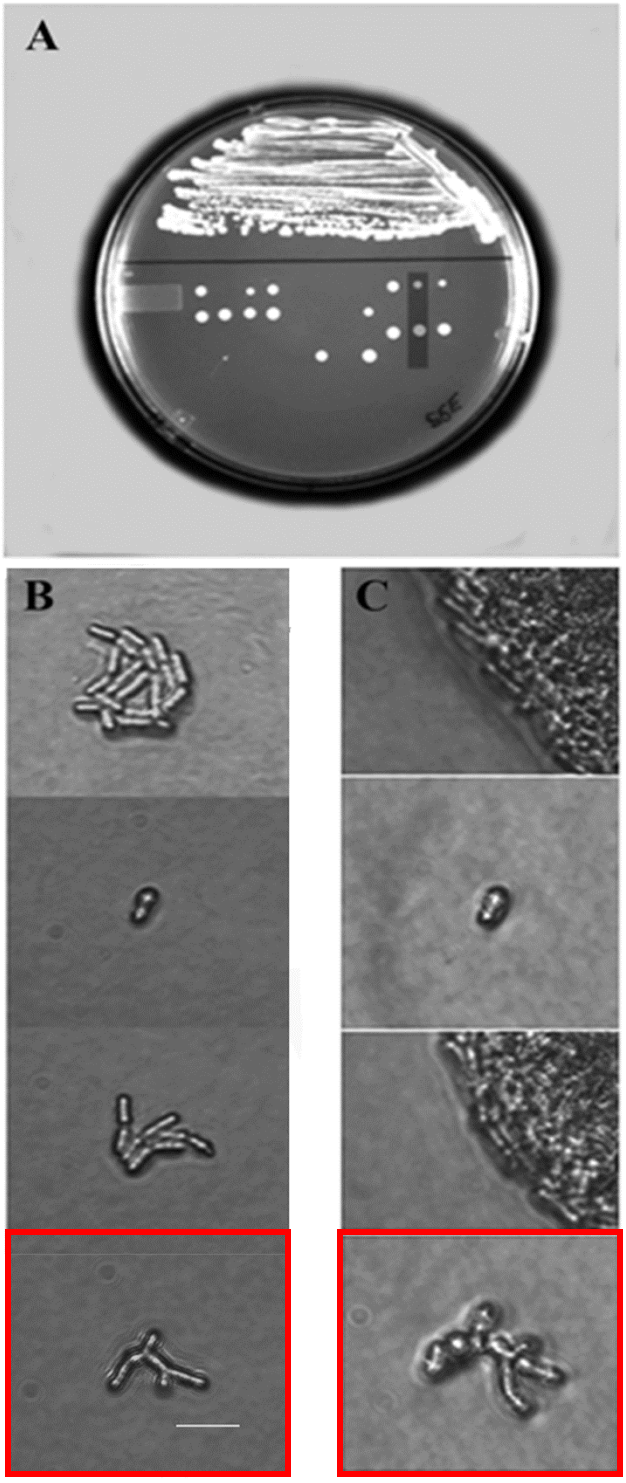

Figure 3.7. *S5E* CTD mutation causes highly branched lethal phenotype. A) Tetrad dissections of *rpb1-12xS5ECTD* on YES agar plate. Shaded area on the petri plate in A indicates the tetrad dissection used for imaging in B and C. B) 24 hours growth of spores dissected. Magnification, 20X. C) 48 hours growth of dissected spores. Magnification, 20X. Highly branched phenotype is outlined with red box. Bar, 20 µm.

3.3 Different ratios of *wild-type: S2A* mutations in CTD repeats changes the cell's capability to regulate cytokinesis under Latrunculin A treatment

The chemical Latrunculin A (LatA), which is a toxin purified from the red sea sponge, has been shown to bind actin monomers and inhibit actin polymerization (Coue, Brenner et al. 1987). The proper functioning of the actomyosin-based contractile ring (actomyosin ring) is essential for eukaryotic cells to divide into two daughter cells. Treating cells with low doses of Latrunculin A (0.2 - 0.5 µM) results in mild perturbation of the cell division machinery and disrupts the integrity of the actomyosin ring (Mishra, Karagiannis et al. 2004). Wild-type cells can overcome this mild perturbation through maintenance of the actomyosin ring structure and by delaying cell cycle progression until cytokinesis can be completed (Mishra, Karagiannis et al. 2004). Because of this, LatA assays are a useful tool to screen for mutants that are defective in the regulation of cytokinesis and in the cytokinesis checkpoint response; which controls progression into the next mitosis.

In a previous study (Karagiannis and Balasubramanian 2007), phosphorylation of serine-2 was shown to play a role in the regulation of cytokinesis. The *rpb1-12xS2ACTD* mutant was shown to be hyper-sensitive to low doses of LatA. Under LatA treatment (Karagiannis and Balasubramanian 2007) the *rpb1-12xS2ACTD* mutant was unable to complete cytokinesis, which resulted in multinucleated cells with fragmented division septa. Wild-type strains are able to successfully complete cytokinesis under the same conditions by continuous activation of the Septation Initiation Network (SIN). The activation of this network re-establishes the integrity of the actomyosin ring and allows

for proper constriction to occur. To test how many serine residues are required for the proper regulation of cytokinesis, two strains that contained different ratios of serine-2 to alanine (*S2A*) substitution mutations were created and LatA assays were performed.

One of the *S2A* mutants was created having 12 CTD repeats where every third repeat has the mutation. The other mutant that was tested had 12 CTD repeats with every second and third repeat having the *S2A* substitution (refer to Table 2.3, WWS and WSS constructs). These two strains along with the *rpb1-12xS2ACTD* mutant and a wild-type strain were treated with 0.3 µM LatA for five hours and then stained with DAPI/aniline blue for phenotypic analysis. The observations made from phenotypic analysis and the data from the cell counts clearly shows how the sensitivity to LatA increases with the number of *S2A* mutations within the CTD (Figure 3.8 and 3.9, respectively). It appears that the more CTD repeats having the serine-2 residue contributes to better control over the regulation of cytokinesis. These results may also suggest that having a full length CTD is required for normal control over the regulation of this genetic network.

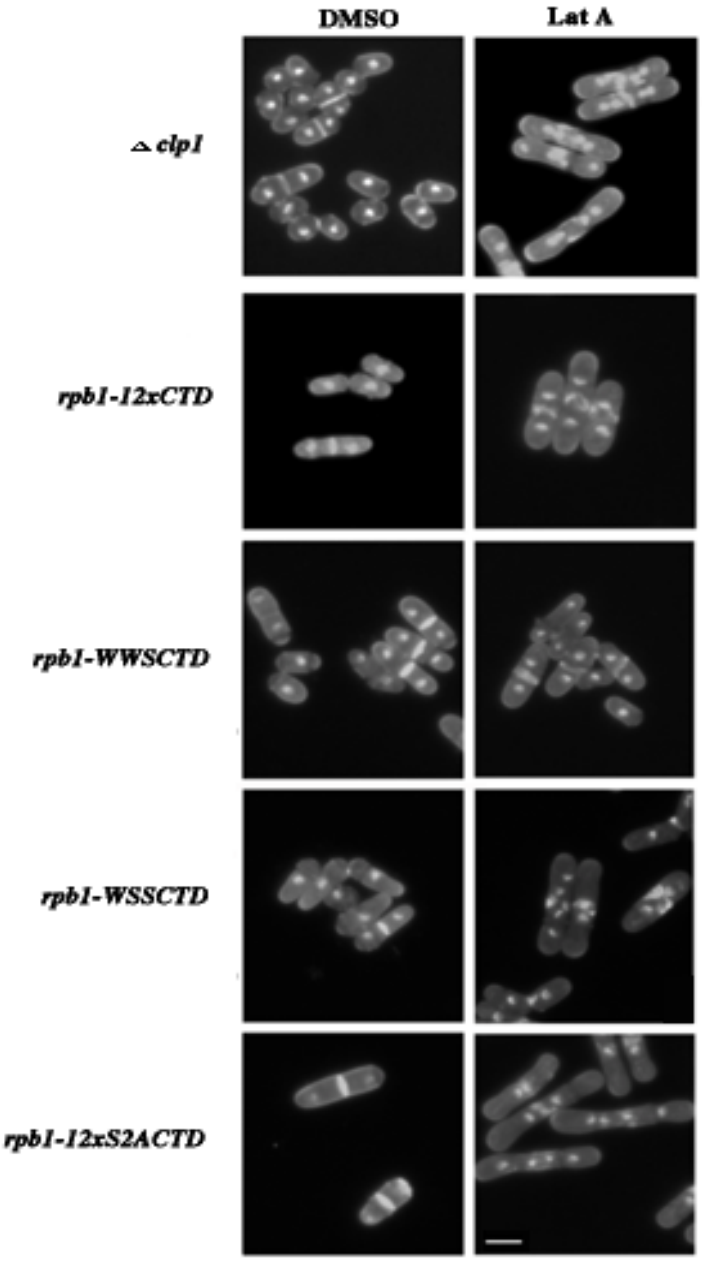

Figure 3.8. Different ratios of *wild-type: S2A* mutations in CTD heptapeptide repeats changes the threshold of Latrunculin A sensitivity and capability to regulate cytokinesis. Strains of indicated genotype were grown to mid log phase in YES liquid media at 30°C then treated with 0.3uM Latrunculin A (Lat A) or DMSO (solvent control) and grown for five hours before being fixed then stained with DAPI and aniline blue. *ΔClp1* is known to be sensitive to Lat A and was used as a control. Wild-type control is represented by *rpb1-12xCTD*. *Rpb1-12xS2ACTD* and *rpb1-12xWSSCTD* result in tetra-nucleate cells with non-functional septa under Lat A treatment. *Rpb1-12xWWSCTD* shows a higher threshold of Lat A sensitivity by being able to re-establish actomyosin ring in a greater number of cells. Bar, 10 µm.

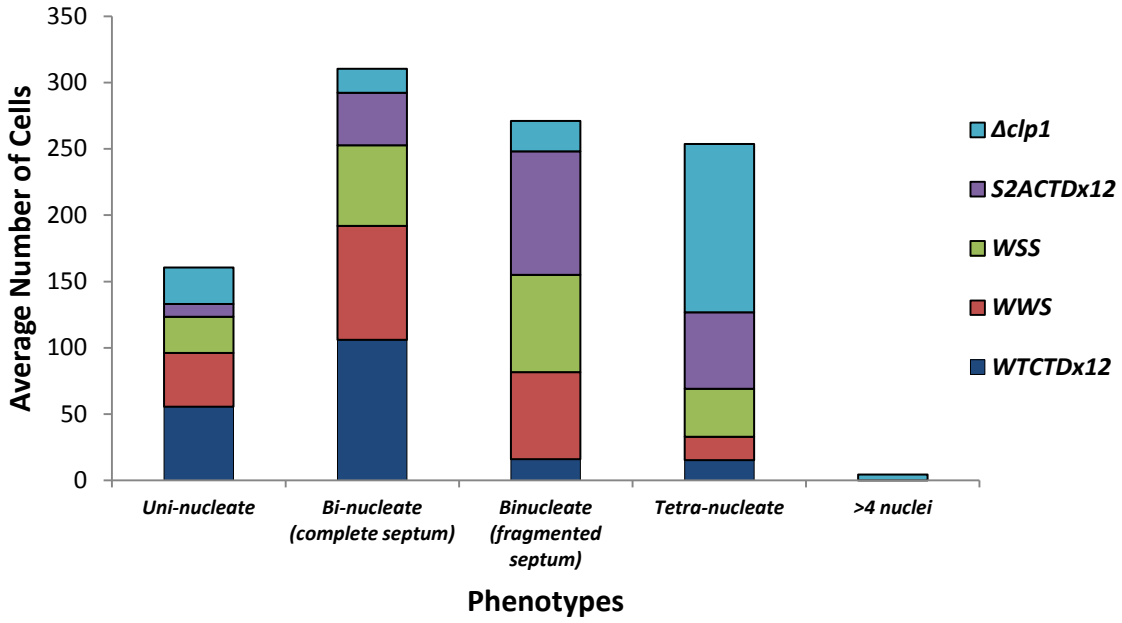

Figure 3.9. **Latrunculin A Cell Counts.** Each of the strains (genotypes indicated above) were grown to mid-log phase and treated with 0.3 µM Latrunculin A for five hours at 30°C. The cells were then fixed, and stained with DAPI and aniline blue to observe the nuclei and cell wall/septum, respectively. 200 cells were randomly counted in three different trials and the averages were categorized into different phenotypes as shown on the x-axis. Uni-nucleate and bi-nucleate with functional septum categories indicate the cells that successfully complete cytokinesis and are not sensitive to Lat A. Bi-nucleate non-functional septum, tetra-nucleate, and >4 nuclei categories are the cells that have failed cytokinesis and did not form complete septa.

3.4 *S5A, S7A*, and *S7E* CTD mutations show interactions with temperature sensitive mutations in genes required for cell division.

If alterations in CTD phosphorylation modulate the functions of different networks of genes, then one should be able to identify genetic interactions between CTD mutants and other conditional mutants that affect different cellular processes. Screening for genetic interactions between CTD mutants and temperature-sensitive mutants is an excellent way to test this hypothesis. By doing so, phenotypic analysis can be used to identify increases or decreases in the restrictive temperature of these conditional mutants when combined with CTD mutations. When observing the double mutants, if the temperature-sensitive phenotype is not observed at the normal restrictive temperature of the single mutant, the strain with a particular CTD modification has rescued the conditional mutant that it was mated with. Furthermore, if the restrictive temperature is decreased, the CTD configuration has compromised the function of that pathway or process. Both of these results would suggest that the CTD phosphorylation pattern or 'code' plays a role in modulating the function of genetic pathways.

3.4.1 *S5A* and *S7E* mutants lower the restrictive temperature of conditional mutants

Based on the phenotypic observations of the site-directed CTD mutants, it appeared that altering the phosphorylation status of serine-5 and serine-7 could play a potential role in regulating the function of genes required for cell division. In attempts to further support this idea, the *S5A, S7A*, and *S7E* CTD mutants were crossed with strains containing temperature-sensitive mutations in the genes required for cytokinesis;

cdc12-112, cdc15-140, and *myo2-E1*. By analyzing the phenotypes and restrictive temperatures of the double mutants that were created it was possible to identify genetic interactions that had occurred.

Once CTD mutant strains were crossed with temperature-sensitive mutant strains, the double mutants were identified as the cells having the *ura4* gene and also showing a temperature-sensitive phenotype when grown at 36°C. Each double mutant was grown at room temperature to mid-log phase then exposed to a range of temperatures (26-36°C) for five hours before being stained with DAPI and aniline blue. Fluorescent images indicate that the *S5A* and *S7E* mutations interact with the *cdc15-140* mutation and decrease the restrictive temperature in the double mutants (Figure 3.10). In addition to these observations, the *S5A* was also found to lower the restrictive temperature of the *cdc12-112* and *myo2-E1* mutants (Figure 3.11 and 3.12, respectively), suggesting a possible role for serine-5 phosphorylation for the regulation of multiple genes involved in cytokinesis. The *S7A* modification did not appear to have any interaction with either *cdc12-112* or *cdc15-140* mutations as the restrictive temperatures of these double mutants remained the same as the single temperature sensitive mutants.

3.4.2 *S7E cdc12-112, S7E myo2-E1, S7A myo2-E1* double mutants are synthetically lethal at room temperature

Tetrad dissections were used as a tool to analyze the segregation of mutant alleles during the mating of CTD and temperature-sensitive mutants. Selection plating and growth at 36°C confirmed the segregation and location of double mutants on the

tetrad dissection plates. The tetrad dissections that resulted from *S7E cdc12-112, S7E myo2-E1, and S7A myo2-E1* crosses showed incomplete growth of all dissected spores, suggesting possible lethal interactions. Upon further growth analysis (via selection plating and growth at 36°C), all of the spores that did not grow were confirmed to be the double mutants that resulted from each cross. Bright field images were taken of the double mutants on the dissection plates to identify the phenotypes that resulted from the synthetic lethal interactions (figure 3.13). The *S7A myo2-E1* mutant appear elongated with slightly curved morphology (first column Figure 3.13). Interestingly, the *S7E cdc12-112* cells grew to excessive lengths and were also branched with curve morphologies (second column Figure 3.13). The third column in Figure 3.13 shows the *S7E myo2-E1* double mutant having an elongated morphology and in one image the cell appears to have broken open (second image from the top of third column).

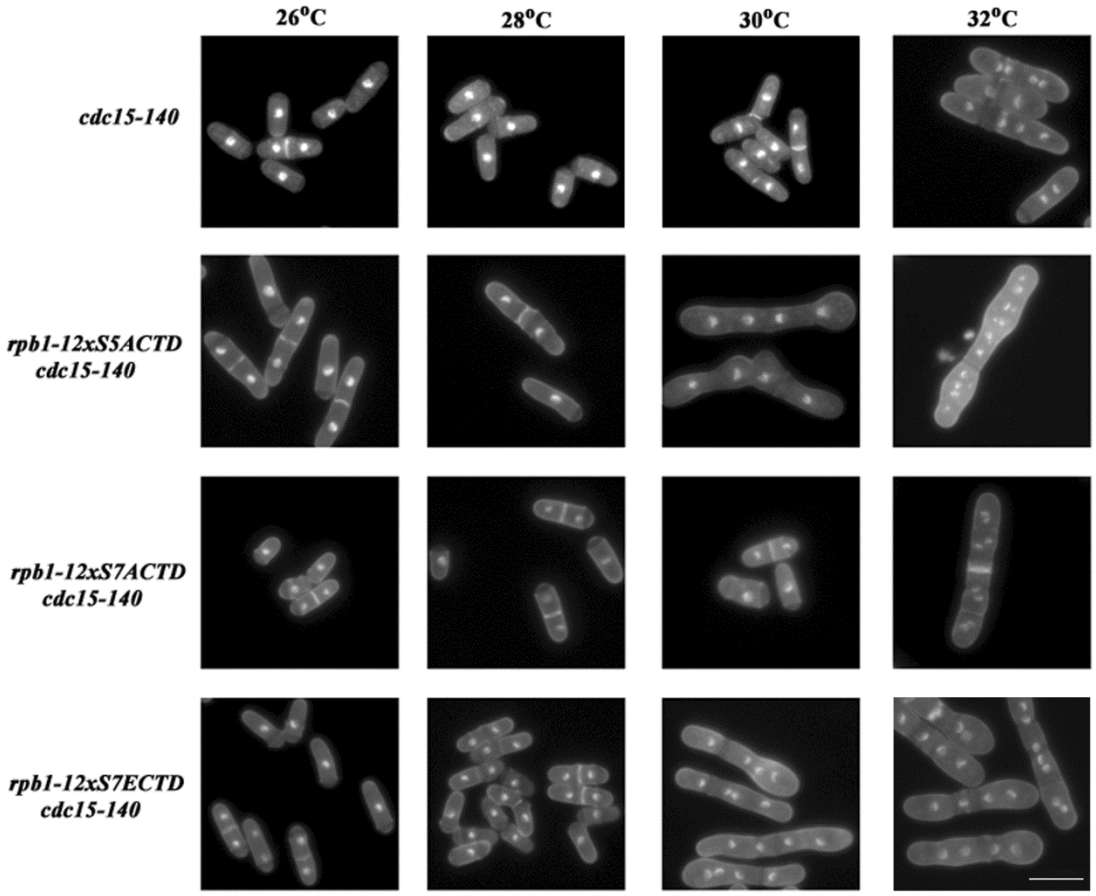

Figure 3.10. *S5A* and *S7E* lower the restrictive temperature of *cdc15-140*. Each strain (genotypes shown above) was grown to mid-log phase at room temperature before being shifted to the indicated temperatures for five hours. These cells were then fixed and stained with DAPI/aniline blue for fluorescent imaging. Bar, 10 µm.

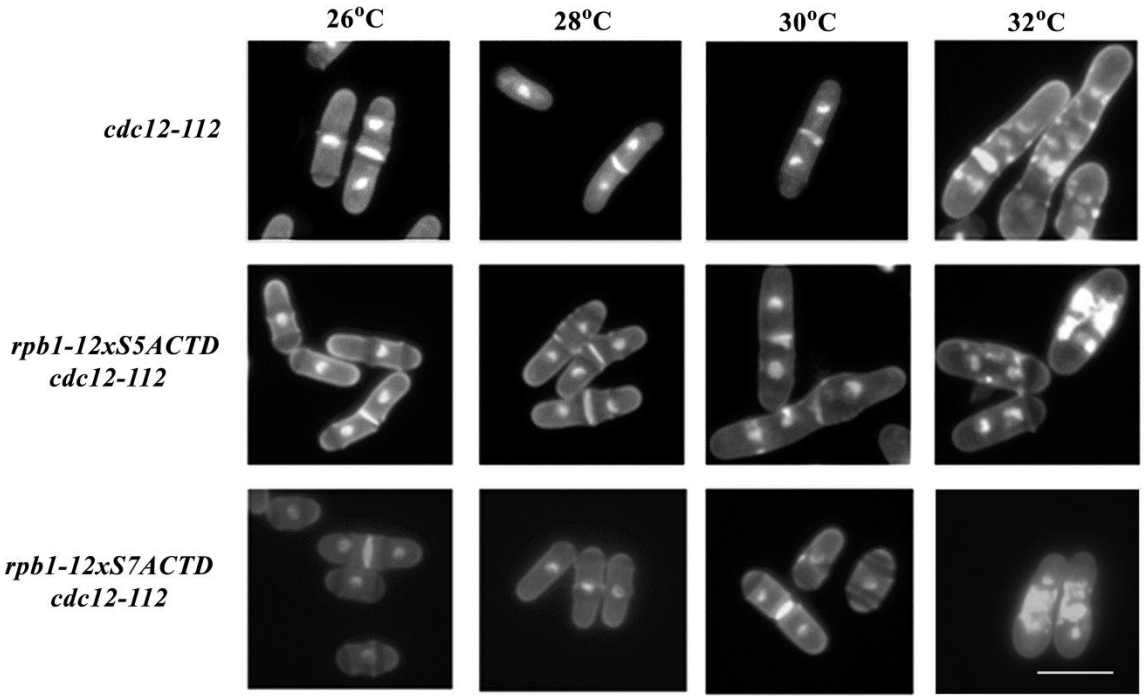

Figure 3.11. *S5A* decreases the restrictive temperature of *cdc12-112*. Strains of indicated genotype were grown to mid-log phase at room temperature before being shifted to a range of temperatures (shown above) for five hours. These cells were then fixed and stained with DAPI/aniline blue for fluorescent imaging. Bar, 10 µm.

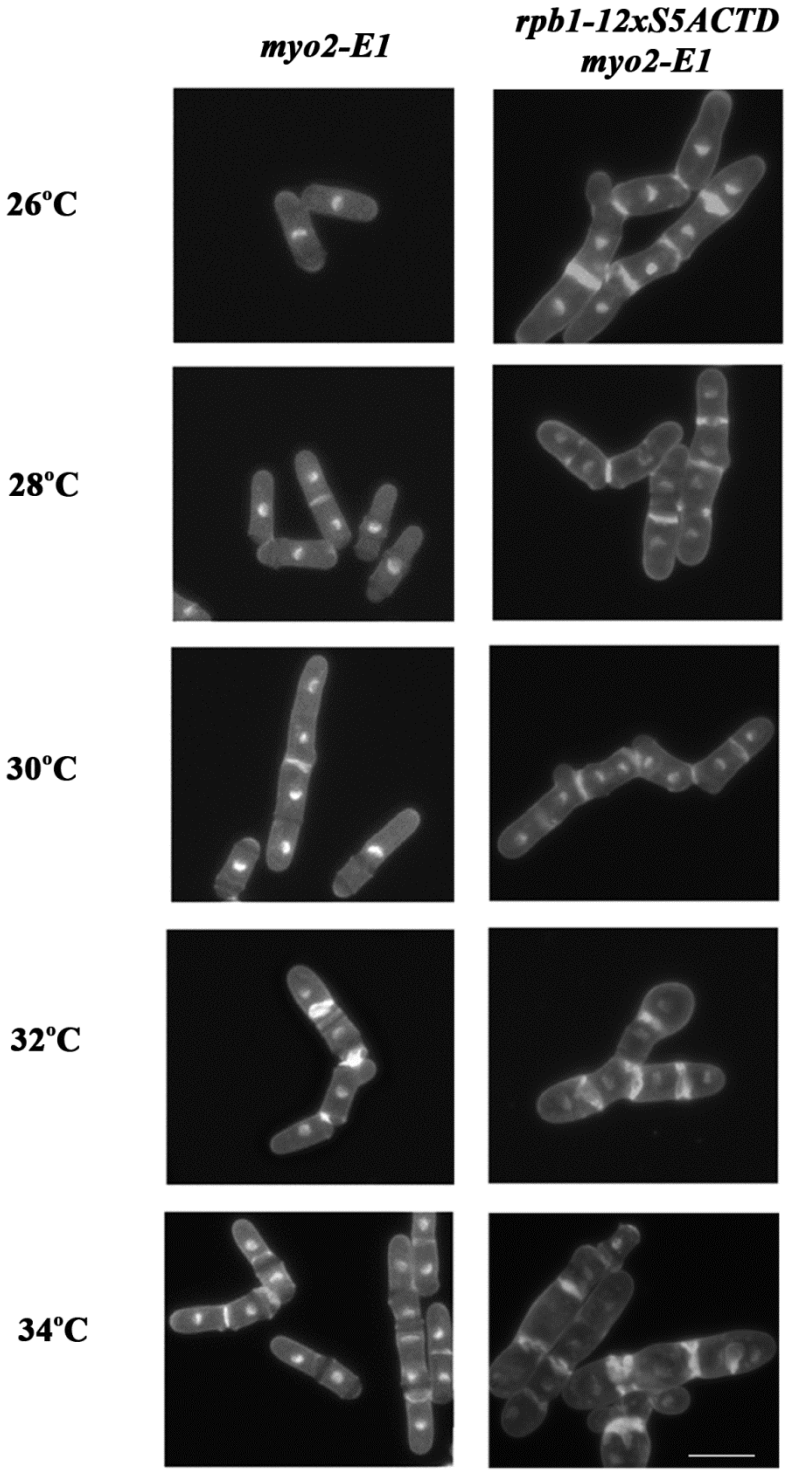

Figure 3.12. *S5A* causes *myo2-E1* ts phenotype at room temperature. *Myo2-E1* single mutant and *rpb1-12xS5ACTD myo2-E1* double mutant strains were grown to mid-log phase at room temperature before being shifted to a range of temperatures (shown above) for five hours and stained using DAPI and aniline blue. Bar, 10 µm.

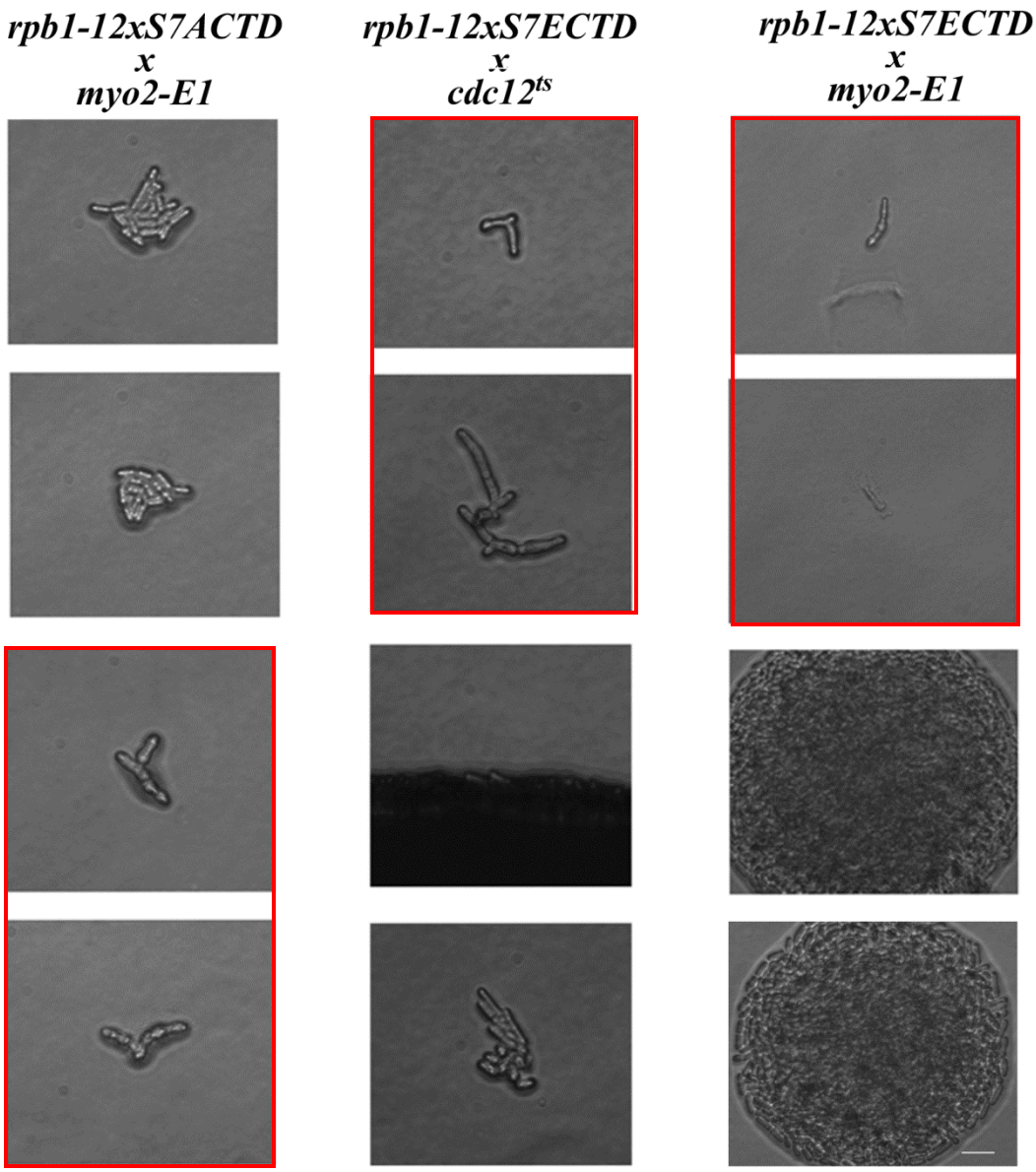

Figure 3.13. Synthetically lethal interactions result in the double mutants *S7A myo2-E1, S7E cdc12-112, and S7E myo2-E1*. Genetic crosses were performed using strains of indicated genotypes and the tetrads of each cross were dissected on YES plates. The growth from each of the four spores from a single tetrad is shown in a column. Colonies that grew were confirmed to be wild type since they did not have the *ura+* selectable marker (no growth on EMM –*ura*) and did not show temperature sensitive phenotypes when grown at 36°C (data not shown). Images outlined in red indicate double mutant lethal phenotypes. All images are taken at 20X magnification. Bar, 20 µm.

Chapter 4: Discussion

The main purpose of this study was to examine the phenotypic effects of modulating the structure and phosphorylation of the carboxy terminal domain of RNA polymerase II. This was done in part to investigate the possibility of a definable "CTD code" that has the capacity to modulate the functions of discrete genetic pathways. In order to test this hypothesis the main objectives were to first, determine the minimum number of repeats required for viability, then to perform phenotypic analysis of site-directed CTD mutants with altered CTD phosphorylation status, and lastly to identify genetic interactions between CTD mutants and other temperature-sensitive mutants.

4.1 Evolutionary Significance of the Length of the CTD

The CTD of the *rpb1* subunit of RNAPII consists of a very long tail-like structure made up of multiple heptapeptide repeats arranged in tandem. This unique domain is highly conserved across all eukaryotic organisms and has been hypothesized to be evolutionarily important for large, developmentally complex organisms. This notion was supported by the observation that there was strong stabilizing selection for the canonical repeat CTD structure in a group of more developmentally complex eukaryotes compared to other eukaryotic species (Stiller and Hall 2002). In addition, within this CTD-clade of eukaryotes, the number of CTD repeats was found to increase with the complexity of the organism. This may suggest that a CTD with a larger capacity can accommodate more sophisticated ways to control gene expression, possible through alternative splicing of introns (Stiller and Hall 2002).

There is no correlation between the number of genes and the complexity of species, suggesting that the differences could be accounted for by the number of ways gene expression is controlled. Regulation of gene expression can be controlled at many different levels including the transcription of a gene, the packaging of mRNA, transport of mRNA to the cytosol, translation into proteins, and by protein-protein interaction (Villard 2004). In addition, at the level of mRNA processing, the number of introns within a genome along with alternative splicing mechanisms can allow for the number of gene products to drastically exceed the total number of genes (Black 2003). A cell having a greater number of gene products would also have more protein products that could interact with one another and form additional complex intracellular networks. Recent attempts to estimate the number of protein interacting networks within eukaryotic genomes have found significant correlation with the complexity of organisms (Stumpf, Thorne et al. 2008). Since the CTD plays a role in the differential splicing of introns for gene regulation (Munoz, Santangelo et al. 2009), one might hypothesize that a longer CTD could facilitate additional interactions with proteins involved in splicing mechanisms and potentially offer more variability in splicing and the biosynthesis of gene products. If a longer CTD with more repeats can offer additional ways to regulate gene expression or increase the number of gene products so that cellular functions are enhanced, this may increase the fitness of an individual organism and thus there would be a strong selective advantage for the maintenance of CTD length.

In this study it was determined that five wild-type CTD repeats are required for viability in *Schizosaccharomyces pombe* and eight repeats are needed for normal growth

rate and phenotype. Normally there are 29 CTD repeats that are highly conserved in *S. pombe* suggesting that, although only a small proportion of the repeats are required for viability, a longer and more extensive CTD could play a role in other specialized functions. In theory, a CTD having more repeats will have a larger capacity to obtain information from upstream signaling molecules. A longer CTD could also facilitate additional protein interactions and could potentially play a more dynamic role in cellular functions.

Growth rate experiments and phenotypic analysis of the *rpb1-5xCTD* mutant suggests that the timing of cell cycle progression is delayed and the cells are unable to divide properly. One possible explanation for this is that a CTD with only five repeats does not possess enough capacity to properly regulate these processes. The phenotypic analysis of the *rpb1-8xCTD* mutant demonstrates that eight CTD repeats is sufficient to support basic cellular functions (i.e. cell cycle control, cell division) under normal growth conditions. These results illustrate how the CTD is indispensible in an organism and suggest that eight repeats could be needed to maintain the structural integrity of this domain for essential CTD-protein interactions.

4.2 Phenotypic Consequences of Defined CTD Modifications

In addition to the importance of the overall length and number of CTD repeats, the results from phenotypic analysis shown in this study illustrate how altering serine phosphorylation within each repeat can impact the function of certain pathways (specific pathways remain to be identified). With the use of site-directed mutagenesis

and homologous recombination, it was possible to create *S. pombe* strains that have synthetic CTD constructs with defined mutations integrated directly into the host's genome and expressed under the native promoter. These defined mutations within each synthetic construct are ways in which the structure of the CTD can be manipulated by CTD-interacting kinases and phosphatases that target serine residues. A specific modification of the structure could, in theory, result in functional alterations of the transcriptional machinery that would change the expression of subsets of genes. The subsets of genes being differentially expressed may function in a particular genetic network; for example, one that is required to elicit a response to an environmental stimulus or growth condition. If this were the case then one would predict that each of these distinct CTD mutants would produce distinct phenotypes that are a consequence of different alterations in gene expression. Accordingly, specific phenotypes were seen in CTD mutant's *rpb1-12xS5ACTD, rpb1-12xS7ACTD,* and *rpb1-12xS7ECTD*. These mutants appeared enlarged with deformed division septa, curved shape at an increase in temperature, and elongated with multiple division septa, respectively.

4.3 Identifying CTD function in Specific Genetic Pathways

After analyzing the distinctive phenotypes of CTD mutants identified in this study, genetic analyses were performed. Further evidence for a CTD 'code' was supported by the data obtained from screening for genetic interactions between CTD mutants and other conditional mutants. If a particular CTD configuration can alter the function of a certain genetic network then it should be possible to observe genetic interactions between a CTD mutation and a temperature sensitive mutation in a protein

that is part of that network. This approach was shown to be successful in identifying genetic interactions between a CTD mutant and *ts* mutants involved in the regulation of cytokinesis. The *rpb1-12xS2ACTD* strain was found to suppress the lethal phenotype of a *cdc16-116 ts* mutation. Additionally, the same CTD mutant was determined to have a synthetic lethal interaction with *cdc14-118* (Karagiannis and Balasubramanian 2007).

Since the discrete phenotypes observed in the site-directed mutants showed different problems with cell division, these mutants were crossed to other strains having temperature-sensitive mutations in genes required for the functioning of different aspects of the cell division machinery. Differential interactions were observed in the double mutants analyzed in this study. *S5A, S7A,* and *S7E* CTD mutations were shown to differentially interact with *ts* mutants' *cdc12-112, cdc15-140,* and *myo2-E1*. The results from the genetic analysis presented here suggest that certain CTD configurations or 'codes' do not just alter transcription on a global level within the cell, but also have the capability to modulate the function of discrete pathways.

4.4 The CTD 'code' and the regulation of the Septation Initiation Network

There has been some speculation that there could be redundancies within the CTD code. In other words, more than one specific CTD configuration may affect the function of the transcription machinery in the same way. This would imply that certain CTD alterations are not needed and that a cell could have the same ability to regulate the function of a particular genetic pathway with or without certain modifications. This notion is supported by the results obtained from Latrunculin A (Lat A) treatment

experiments of *S2A* CTD mutants (serine-2 to alanine substitution mutations). The phosphorylation of serine-2 within the CTD by a cyclin dependent kinase complex has been shown to promote cytokinesis through the positive regulation of the Septation Initiation Network (SIN) (Karagiannis and Balasubramanian 2007). The continuous signaling of this network is necessary for the reestablishment of the actomyosin ring during low doses of Latrunculin A treatment. Unlike wild-type strains, the *rpb1-12xS2ACTD* mutant was not able to reestablish the ring under Latrunculin A treatment, resulting in cells with multiple nuclei and non-functional division septa. The next question that needed to be considered was whether or not the phosphorylation of all serine-2 residues are required for SIN signaling, or if a cell can properly regulate this network with only a proportion of these residues. In other words, are serine-2 modifications redundant for the regulation of the Septation Initiation Network?

The results shown in this thesis indicate a requirement for all CTD ser-2 residues in order to have full control over SIN signaling. When replacing four out of the twelve ser-2 residues with alanine (*rpb1-12xWWSCTD*), the cell's ability to regulate SIN signaling was compromised and fewer cells were able to successfully complete cytokinesis when compared to wild-type (*rpb1-12xCTD*). The control over SIN signaling was further compromised in the *rpb1-12xWSSCTD* when compared to *rpb1-12xWWSCTD* cells under Lat A treatment. This suggests that complete control over SIN signaling requires all ser-2 CTD residues and that the proper regulation of this network cannot rely only on the phosphorylation of a proportion of wild-type CTD repeats containing ser-2. These results do not support the hypothesis of there being redundancies within the CTD 'code'

since each of the *S2A* CTD mutants modulates the function of the SIN network differently.

4.5 The Structural Function of the CTD

Within this study I have shown how defined CTD modifications result in discrete phenotypes and also how changes in the expression of gene networks could be a plausible explanation for the cause of these phenotypes (suggested by the observation of genetic interactions between *ts* mutants). There still remains the question as to how structural modifications to the CTD alter the function of the transcriptional machinery in a way to change the expression of only certain gene subsets. There has been extensive research showing how the CTD phosphorylation status changes during transcription. It has also been thoroughly described how this domain acts as a binding scaffold for a number of different nuclear factors that function in the progression of RNA pol II during transcription and the processing of mRNA (Phatnani and Greenleaf 2006). Furthermore, the binding and functioning of these nuclear factors is known to be regulated by the activity of CTD kinases and phosphatases that target specific serine residues within the CTD. Since these processes are generally required during the transcription of every protein coding gene, in order to provide evidence of a CTD code it would be beneficial to identify how certain CTD modifications result in gene-specific transcriptional changes.

Biochemical and structural studies have determined CTD specificities of numerous different cyclin-dependent kinases and phosphatases (Meinhart, Kamenski et al. 2005). The activity of all of these identified enzymes is not required during the

transcription of every gene. Moreover, the upregulation of certain genes required for a stress response has been found to be dependent upon the specific CTD kinases. Upon UV irradiation of budding yeast cells, expression of genes required for DNA damage response required the phosphorylation of serine-2 within the CTD repeats in a manner dependent on Ctk1p, a component of the CTDK-I complex (Ostapenko and Solomon 2003). These results demonstrate that a specific CTD-kinase interaction can modify the CTD in a way that affects the expression of only certain gene subsets.

As described earlier, the mechanism by which CTD phosphorylation regulates the expression of DNA damage response genes may be explained via alternative splicing and the inhibition of transcript elongation. The choice of splice sites within the transcript region can be used as a means to turn genes on or off. The importance of alternative splicing as a mechanism for regulating gene expression is supported by the observation that the expression of roughly 65% of human genes are affected this way (Kim, Magen et al. 2007). With the use of splicing-sensitive microarrays, researchers are able to detect changes in the splicing locations within the same gene. The phosphorylation of the CTD upon UV irradiation was found to correlate with the alternative splicing and the differential expression of genes involved in the DNA damage response (Munoz, Santangelo et al. 2009). In addition, CTD mutants that mimic the same phosphorylation pattern duplicated the effect that UV irradiation had on the alternative splicing of these genes (Munoz, Santangelo et al. 2009). A suggested explanation for how CTD phosphorylation affects alternative splicing is by changing the structure of the domain in a way that regulates the binding and timely recruitment of splicing enzymes during

transcription. This mechanism would ultimately be controlled by the interplay between CTD-interacting kinases and phosphatases and the upstream signaling pathways that activate them.

Lastly, if the changes in the phosphorylation pattern of the CTD function only in controlling the progression of RNA pol II through transcription, then there should be a fairly heterogeneous mixture of phospho-CTDs at any given instance within the cell. If the modulation of CTD phosphorylation is used as a mechanism to respond to environmental conditions or stresses through regulating the expression of certain genes, then the cells under that condition should possess a larger quantity of distinct CTD configurations at a point in time. Western blots have shown that a particular CTD phosphorylation state is more highly represented after UV irradiation and DNA damage compared to the CTDs in unexposed cells (Munoz, Santangelo et al. 2009). The observation of there being a discrete collection of CTD configurations at a particular instance under cellular stress provides support that the CTD could play a role in the differential expression of genes in response to different environmental conditions.

4.6 Discrepancies in the Analysis of CTD Mutants

There has been significant interest in the study of a CTD 'code' and many researchers have taken up the task of deciphering this code; however, not all published reports have been in agreement. There have been discrepancies when analyzing the phenotypes of different CTD mutants. These include determining the minimal number of CTD repeats that are required for cell viability in *S. pombe* and also the identification

of certain site-directed mutants as lethal or viable. For example, in a recent study the *rpb1-S5ACTD* mutant was identified as being lethal when introduced into *S. pombe* (Schwer and Shuman 2011), whereas the results in this study indicate that the *rpb1-S5ACTD* CTD mutant is viable. In addition, the Schwer and Shuman report states that the truncated CTD containing eight wild-type repeats was lethal and *S. pombe* strains having 10-13 repeats grew slower than wild-type. These discrepancies may be a result of different methodologies that were used when analyzing these site-directed CTD mutants. While both this study and the study by Schwer and Shuman (2011) involve the integration of CTD constructs directly into the host's genome, selection and growth conditions for the stably integrated CTD mutants differ. The CTD constructs that were integrated in this study contained the *ura4* gene and could be selected for by growth on media lacking uracil. Selected strains could then be analyzed for viability using tetrad dissections and growth under normal conditions. The CTD cassettes integrated by Schwer and Shuman contained an antibiotic resistance gene and involved growth of *S. pombe* in the presence of an antibiotic. Where there was no growth, the integrated CTD cassette was identified as being lethal. Considering CTD configurations have been shown to be altered under changes to the cell's environment and under different stresses, growing these site-directed CTD mutants in the presence of an antibiotic may account for the differences between the results being observed.

4.7 Applying Mathematical Theory of Communication: Calculation of the Informational Capacity of the CTD

In conclusion, it is interesting to speculate on the informational capacity of the CTD and the implications of this analysis on genetic regulation in eukaryotes. In the same way that a signal is sent through a phone line and is transmitted into an audio message that is acquired by the receiver, the CTD can be thought of as a receiver of messages from upstream signaling molecules. The message received by the CTD gets transmitted to the transcriptional machinery and would reach the certain targeted genes as its final destination. Although highly speculative, if we consider the CTD as a communicator of information then we can calculate the informational potential of the domain based on its potential complexity. As previously outlined, each CTD repeat has eight potential phosphorylation states and four *cis-trans* configurations, resulting in 32 possible configurations for each repeat. Therefore, 32 to the power of the number of CTD repeats found in a particular species (32^{29} in yeast) would determine the total number of potential configurations of the CTD. If we consider the CTD as a molecule that receives a message, these numbers can be applied to mathematical models in order to calculate the informational potential of the CTD.

In 1948, Claude Shannon hypothesized a way to measure information as a quantity, H (or entropy) using logarithms. In the case where every message (or CTD code) has an equal chance of appearing, he devised the formula, $H = L \cdot \log_2 M$, where L is the number of characters in the message, and M is the number of symbols (CTD configurations) used to write the message. There are a number of different units that

can be used to measure information including binary digits or *bits* (0 or 1's), and decimal digits (0-9). The base of the logarithm will depend on which of these units of measure are used (2 if *bits* are chosen, or 10 for decimal digits). To calculate the information potential for each CTD repeat we would take the logarithm of the total number of possible configurations and use the units of measure for the logarithm base ($\log_2$ if using *bits*). Using this approach we can calculate the information potential of the full *S. pombe* CTD that has 30 repeats as; $H = 30 \cdot \log_2 32$ (Karagiannis 2012). Therefore, the wild type *S. pombe* CTD could potentially encode 150 *bits* of information.

Applying these calculations to truncated CTD mutants, approximately 40 *bits* ($8 \cdot \log_2 32$) bits of information could be generated from cells that have the minimum number of CTD repeats needed for normal growth and morphology. These basic calculations represent the differences in potential informational capacity of the truncated CTD mutants, which may account for the distinctions in viability and phenotypes being observed. In other words, without having enough information capacity to properly transmit the messages to the transcriptional machinery; vital cellular functions may not be accomplished.

4.8 Implications for Decoding the CTD

The study of how this unique carboxy terminal domain functions in the process of transcription is crucial for the knowledge of how a cell responds to its environment through the transcriptional regulation of gene networks. Transcriptional regulation has been regarded as the most important step for control over gene expression. Numerous

human diseases, including haemoglobinopathies, leukemia, and epilepsy, are a result of the improper regulation of gene expression at the transcriptional level (Villard 2004).

In future research, with the use of yeast models, it may be of value to create a library of CTD mutants and determine the phenotypes and gene expression profiles for each. If we know how each CTD configuration specifically alters the expression of different gene networks, which possibly results in a discrete phenotype, we could gain a better understanding of how the modulation of the CTD within a cell controls many different processes. Although much research still needs to be done to understand how these mechanisms function, the tools and technologies needed to pursue such experiments are already available to us today.

Literature Cited

Black, D. L. (2003). "Mechanisms of Alternative Pre-messenger RNA Splicing." Annual Review of Biochemistry **72**: 291-336.

Cadena, D. L. and M. E. Dahmus (1987). "Messenger-RNA Synthesis in Mammalian-Cells is Catalyzed by the Phosphorylated Form of RNA Polymerase-II." Journal of Biological Chemistry **262**(26): 12468-12474.

Cochet-Meilhac, M., P. Nuret, et al. (1974). "Animal DNA-dependent RNA polymerases. 12. Determination of the cellular number of RNA polymerase B molecules." Biochim Biophys Acta **353**(2): 185-192.

Corden, J. L., D. L. Cadena, et al. (1985). "A Unique Structure at the Carboxyl Terminus of the Largest Subunit of Eukaryotic Rna Polymerase-Ii." Proceedings of the National Academy of Sciences of the United States of America **82**(23): 7934-7938.

Coue, M., S. L. Brenner, et al. (1987). "Inhibition of Actin Polymerization by Latrunculin-A." FEBS Letters **213**(2): 316-318.

Cramer, P., A. Srebrow, et al. (2001). "Coordination between transcription and pre-mRNA processing." FEBS Letters **498**(2-3): 179-182.

Dahmus, M. E. (1981). "Phosphorylation of Eukaryotic DNA-Dependent RNA-Polymerase - Identification of Calf Thymus RNA-Polymerase Subunits Phosphorylated by 2 Purified Protein-Kinases, Correlation with In vivo Sites of Phosphorylation in Hela-Cell RNA Polymerase-II." Journal of Biological Chemistry **256**(7): 3332-3339.

Dubois, M. F., V. T. Nguyen, et al. (1994). "Enhanced phosphorylation of the C-terminal domain of RNA polymerase II upon serum stimulation of quiescent cells: possible involvement of MAP kinases." EMBO J **13**(20): 4787-4797.

Egloff, S. and S. Murphy (2008). "Cracking the RNA polymerase II CTD code." Trends in Genetics **24**(6): 280-288.

Forsburg, S. L. and N. Rhind (2006). "Basic methods for fission yeast." Yeast **23**(3): 173-183.

Greenleaf, A. L. (1983). "Amanitin-resistant RNA polymerase II mutations are in the enzyme's largest subunit." Journal of Biological Chemistry **258**(22): 13403-13406.

Guillamot, M., E. Manchado, et al. (2011). "Cdc14b regulates mammalian RNA polymerase II and represses cell cycle transcription." Sci Rep **1**: 189.

Ho, C. K. and S. Shuman (1999). "Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme." Molecular Cell **3**(3): 405-411.

Karagiannis, J. and M. K. Balasubramanian (2007). "A Cyclin-Dependent Kinase that Promotes Cytokinesis through Modulating Phosphorylation of the Carboxy Terminal Domain of the RNA Pol II Rpb1p Sub-Unit." PLoS One **2**(5).

Karagiannis, J., A. Bimbo, et al. (2005). "The nuclear kinase Lsk1p positively regulates the septation initiation network and promotes the successful completion of cytokinesis in response to perturbation of the actomyosin ring in *Schizosaccharomyces pombe*." Molecular Biology of the Cell **16**(1): 358-371.

Kim, E., A. Magen, et al. (2007). "Different levels of alternative splicing among eukaryotes." Nucleic Acids Res **35**(1): 125-131.

Lee, J. M. and A. L. Greenleaf (1997). "Modulation of RNA polymerase II elongation efficiency by C-terminal heptapeptide repeat domain kinase I." Journal of Biological Chemistry **272**(17): 10990-10993.

Lee, M. G. and P. Nurse (1987). "Complementation Used to Clone a Human Homolog of the Fission Yeast-Cell Cycle Control Gene Cdc2." Nature **327**(6117): 31-35.

Lee, T. I. and R. A. Young (2000). "Transcription of eukaryotic protein-coding genes." Annual Review of Genetics **34**: 77-137.

Licatalosi, D. D., G. Geiger, et al. (2002). "Functional interaction of yeast pre-mRNA 3 ' end processing factors with RNA polymerase II." Molecular Cell **9**(5): 1101-1111.

Meinhart, A. and P. Cramer (2004). "Recognition of RNA polymerase II carboxy-terminal domain by 3 '-RNA-processing factors." Nature **430**(6996): 223-226.

Meinhart, A., T. Kamenski, et al. (2005). "A structural perspective of CTD function." Genes & Development **19**(12): 1401-1415.

Mishra, M., J. Karagiannis, et al. (2004). "The Clp1p/Flp1p phosphatase ensures completion of cytokinesis in response to minor perturbation of the cell division machinery in *Schizosaccharomyces pombe*." Journal of Cell Science **117**(17): 3897-3910.

Moreno, S., A. Klar, et al. (1991). "Molecular Genetic-Analysis of Fission Yeast *Schizosaccharomyces pombe*." Methods in Enzymology **194**: 795-823.

Morris, D. P. and A. L. Greenleaf (2000). "The splicing factor, Prp40, binds the phosphorylated carboxy-terminal domain of RNA polymerase II." Journal of Biological Chemistry **275**(51): 39935-39943.

Munoz, M. J., M. S. P. Santangelo, et al. (2009). "DNA Damage Regulates Alternative Splicing through Inhibition of RNA Polymerase II Elongation." Cell **137**(4): 708-720.

Ostapenko, D. and M. J. Solomon (2003). "Budding yeast CTDK-I is required for DNA damage-induced transcription." Eukaryotic Cell **2**(2): 274-283.

Pei, Y., H. Du, et al. (2006). "Cyclin-dependent kinase 9 (Cdk9) of fission yeast is activated by the CDK-activating kinase Csk1, overlaps functionally with the TFIIH-associated kinase Mcs6, and associates with the mRNA cap methyltransferase Pcm1 *in vivo*." Mol Cell Biol **26**(3): 777-788.

Pelham, R. J. and F. Chang (2002). "Actin dynamics in the contractile ring during cytokinesis in fission yeast." Nature **419**(6902): 82-86.

Peng, J. M., N. F. Marshall, et al. (1998). "Identification of a cyclin subunit required for the function of Drosophila P-TEFb." Journal of Biological Chemistry **273**(22): 13855-13860.

Phatnani, H. P. and A. L. Greenleaf (2006). "Phosphorylation and functions of the RNA polymerase IICTD." Genes & Development **20**(21): 2922-2936.

Roeder, R. G. and W. J. Rutter (1969). "Multiple Forms of DNA-Dependent RNA Polymerase in Eukaryotic Organisms." Nature **224**(5216): 234-&.

Rogers, C., Z. H. Guo, et al. (2010). "Connecting Mutations of the RNA Polymerase II C-Terminal Domain to Complex Phenotypic Changes Using Combined Gene Expression and Network Analyses." PLoS One **5**(6).

Schwartz, L. B. and R. G. Roeder (1975). "Purification and Subunit Structure of Deoxyribonucleic Acid-Dependent Ribonucleic-Acid Polymerase II from Mouse Plasmacytoma, Mopc 315." Journal of Biological Chemistry **250**(9): 3221-3228.

Schwer, B. and S. Shuman (2011). "Deciphering the RNA Polymerase II CTD Code in Fission Yeast." Molecular Cell **43**(2): 311-318.

Stiller, J. W. and B. D. Hall (2002). "Evolution of the RNA polymerase IIC-terminal domain." Proceedings of the National Academy of Sciences of the United States of America **99**(9): 6091-6096.

Stumpf, M. P., T. Thorne, et al. (2008). "Estimating the size of the human interactome." Proc Natl Acad Sci U S A **105**(19): 6959-6964.

Verde, F., J. Mata, et al. (1995). "Fission Yeast-Cell Morphogenesis - Identification of New Genes and Analysis of Their Role during the Cell-Cycle." Journal of Cell Biology **131**(6): 1529-1538.

Villard, J. (2004). "Transcription regulation and human diseases." Swiss Med Wkly **134**(39-40): 571-579.

West, M. L. and J. L. Corden (1995). "Construction and Analysis of Yeast RNA Polymerase II Ctd Deletion and Substitution Mutations." Genetics **140**(4): 1223-1233.

Wood, V., R. Gwilliam, et al. (2002). "The genome sequence of *Schizosaccharomyces pombe*." Nature **415**(6874): 871-880.

Curriculum Vitae

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Education

2010 – 2012	Masters of Science Department of Biology, University of Western Ontario
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Scholarships

2010 – 2012	Western Graduate Research Scholarship (WGRS)
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Related Work Experience

2010 – 2012	Graduate Teaching Assistant *Department of Biology – University of Western Ontario*
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