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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Probing the Molecular Basis for the Functional Interactions Between the Histone
Acetyltransferase Gcn5 and Protein Phosphatase 2A

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

in

Biology

by

Shannon Lee Tomlinson

Committee in charge:

Professor Lorraine Pillus, Chair
Professor Tracy Johnson
Professor Jim Kadonaga

2012

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University of California, San Diego

2012

DEDICATION

I would like to thank Lorraine Pillus and all of the members of the Pillus lab that I have ever had the pleasure of working with. My development as a scientist and the results produced could not have been done without their input. I am truly grateful to all who were willing to lend an ear when I needed it and to offer advice.

I would like to thank my parents Mike and Cindy for their support and love. They taught me how to work hard for what I want and encouraged me to pursue my interests. Without them, I would not be the woman that I am today and I would not have accomplished this goal.

I would like to thank David Consalvo, the man who holds my heart, for his unwavering confidence in me. He has been a rock of support and I could not have completed this without him. His love and support mean more than I can say.

TABLE OF CONTENTS

Signature Page.....	iii
Dedication.....	iv
Table of Contents.....	v
List of Figures.....	vi
List of Tables.....	ix
Vita.....	x
Abstract of Thesis.....	xi
 Chapter 1. Introduction.....	 1
Chapter 2. Identification of the PP2A regulatory subunit <i>RTS1</i> as a suppressor of <i>gcn5Δ</i> phenotypes.....	 21
Chapter 3. Specific histone residues provide a functional link between <i>GCN5</i> and <i>RTS1</i>	 36
Chapter 4. Mutational analysis of histone lysine residues to dissect functional interactions between <i>GCN5</i> and <i>RTS1</i>	 62
Chapter 5. Potential models, discussion, and future directions.....	75
Chapter 6. Materials and methods.....	104
Bibliography.....	120

LIST OF FIGURES

Figure 1-1. The nucleosome is the basic unit of chromatin.....	3
Figure 1-2. Acetylation of histone tail lysine residues induces an open chromatin landscape.....	6
Figure 1-3. The Gcn5-containing SAGA and ADA complexes.....	10
Figure 1-4. Phosphatase complexes remove phosphate groups from histones and other substrates to allow dynamic modification...	14
Figure 1-5. The PP2A complex composition and its role in the cell.....	17
Figure 1-6. Dosage suppression analysis is a powerful tool.....	19
Figure 2-1. Overexpression of <i>RTS1</i> rescues the lethality of <i>gcn5Δ sas3^{ts}</i> ..	24
Figure 2-2. Overexpression of <i>RTS1</i> rescues the temperature sensitivity of <i>gcn5Δ</i>	27
Figure 2-3. Overexpression of <i>GCN5</i> does not rescue the temperature sensitivity of <i>rts1Δ</i>	30
Figure 2-4. Overexpression of <i>RTS1</i> does not rescue the sporulation defect of <i>gcn5Δ</i>	32
Figure 2-5. <i>RTS1</i> overexpression improves the DNA damage sensitivity of <i>gcn5Δ</i>	35
Figure 3-1. Identification of serine and threonine residues in the four core yeast histone sequences.....	38
Figure 3-2. Scheme of the “histone shuffle”.....	41
Figure 3-3. Suppression of <i>gcn5Δ</i> temperature sensitivity by <i>RTS1</i> overexpression is independent of H3 S10, H3 S28, H3 T45, and H2A S121 phosphorylation.....	45

Figure 3-4. Suppression of <i>gcn5Δ</i> temperature sensitivity by <i>RTS1</i> overexpression does not require the kinase activity of Snf1....	47
Figure 3-5. Suppression of <i>gcn5Δ</i> temperature sensitivity by <i>RTS1</i> overexpression is abrogated by mutating H2B T91 to alanine.	49
Figure 3-6. Mutation of H2B T91 to alanine improves the growth of <i>rts1Δ</i> at elevated temperature.....	52
Figure 3-7. Mutation of H2B T91 to alanine exacerbates the DNA damage sensitivity of <i>gcn5Δ</i> and inhibits the dosage-dependent suppression by <i>RTS1</i>	55
Figure 3-8. Mutation of H2B T91 to aspartate and glutamate to mimic constitutive phosphorylation results in lethality in <i>gcn5Δ</i>	58
Figure 3-9. Mutation of H4 T80 to alanine is lethal in <i>gcn5Δ</i> but not in <i>sas3Δ</i>	60
Figure 4-1. H2B K82 and H2B K88 are important for the response of <i>gcn5Δ</i> to heat stress in combination with H2B T91.....	66
Figure 4-2. Lateral domain residues H4 K77 and H4 K79 exacerbate <i>gcn5Δ</i> temperature sensitivity but do not inhibit rescue by <i>RTS1</i> overexpression.....	68
Figure 4-3. Suppression of <i>gcn5Δ</i> temperature sensitivity by <i>RTS1</i> overexpression does not require K11 or K16, the H2B acetylation targets of Gcn5.....	71
Figure 4-4. Mutation of the Gcn5 acetylation targets H2BK11 and H2BK16 to alanine does not rescue the temperature sensitivity of <i>rts1Δ</i> ...	73
Figure 5-1. The <i>gcn5Δ rts1Δ</i> double mutant is synthetically lethal and lethality is dependent on the catalytic activity of Gcn5.....	77

Figure 5-2. <i>GCN5</i> has genetic interactions with members of the PP2A complex.....	80
Figure 5-3. The <i>rts1Δ spt20Δ</i> double mutant is synthetically lethal, suggesting that <i>RTS1</i> functionally interacts with the SAGA complex.....	84
Figure 5-4. Model of potential Sch9 regulation in yeast.....	87
Figure 5-5. The <i>gcn5Δ sch9Δ</i> double mutant is synthetically lethal suggesting intersection with the TOR pathway.....	89
Figure 5-6. Proposed model of Gcn5 and PP2A-Rts1 interaction in regulating kinetochore function.....	93
Figure 5-7. Proposed model of <i>gcn5Δ</i> suppression by <i>RTS1</i> overexpression.	96
Figure 5-8. Important histone residues for the <i>GCN5-RTS1</i> interaction.....	100

LIST OF TABLES

Table 5-1. Summary of histone mutant phenotypes in <i>gcn5Δ</i>	103
Table 6-1. Strains used in this research.....	111
Table 6-2. Plasmids used in this research.....	114
Table 6-3. Primers used in this research.....	116
Table 6-4. Transformants used in this research.....	118

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ABSTRACT OF THE THESIS

Probing the Molecular Basis for the Functional Interactions Between the Histone
Acetyltransferase Gcn5 and Protein Phosphatase 2A

by

Shannon Lee Tomlinson

Master of Science in Biology

University of California, San Diego, 2012

Professor Lorraine Pillus, Chair

Histone acetyltransferases (HATs) are important regulators of chromatin structure and transcriptional activation. Gcn5 is a highly conserved HAT among eukaryotes and it functions in several protein complexes to regulate gene expression. Many types of histone modifications occur in complex patterns and contribute to epigenetic regulation of transcriptional activity. The complexes that

remove histone modifications also play an important role. Protein phosphatase complexes regulate protein activity by removing phosphate groups from target proteins. Phosphatases are important for cell cycle progression and are often central to signaling pathways. In this work, we investigated the functional interaction between Gcn5 and a regulatory subunit of the Protein Phosphatase 2A complex, Rts1 in *Saccharomyces cerevisiae*. Here we report that *RTS1* is a high-copy suppressor of the temperature sensitivity that results when Gcn5 is absent from the cell. Increased gene dosage of *RTS1* also modestly improves the DNA damage sensitivity of *gcn5Δ* mutants. Suppression of these phenotypes is abrogated when histone H2B T91 is mutated to alanine. Mutational analysis of histone residues along the lateral domain of the nucleosome reveals their importance in the suppressor relationship identified in this work. *GCN5* and *RTS1* have another functional interaction as they result in synthetic lethality when deleted in the cell. We conclude that the Gcn5-containing SAGA complex is involved as *rts1Δ* mutants are not viable when the SAGA complex is disrupted through mutation of genes encoding other non-catalytic components of the complex. Finally, we propose several models for the unique relationship between *GCN5* and *RTS1*.

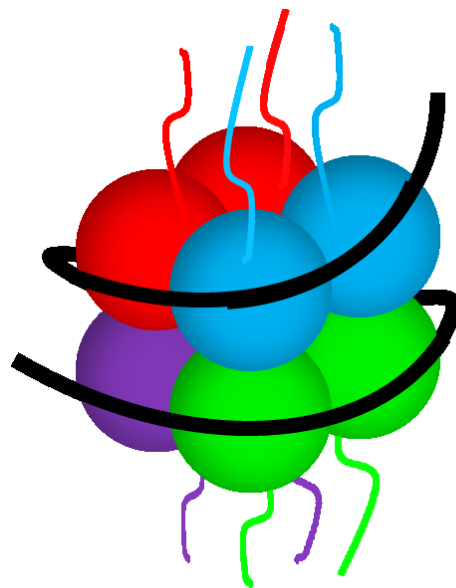
Chapter 1. Introduction

Histone modifications as a means of dynamic regulation. Post-translational modifications of histones have become a primary focus in research. In part this is because they contribute to for controlling the chromatin state as well as other cellular processes such as cell cycle progression, DNA damage signaling pathways, and transcriptional regulation.

In the nucleus, DNA is wrapped around core histone proteins to form the nucleosome: the basic unit of chromatin for the packaging of DNA. The nucleosome is comprised of a pair of each of the four highly conserved canonical histones (H2A, H2B, H3, and H4) to form an octamer (Figure 1-1). The octameric histone core is highly structured for the proper packaging and regulation of DNA. However, the N-terminal tails of all four histones and the H2A C-terminus are unstructured and protrude from the core where they are subject to many forms of post-translational modification such as acetylation, phosphorylation, methylation, ubiquitination, and SUMOylation [reviewed in (Kornberg and Lorch 1999)]. The multitude of available modifications can be on the same histone or on neighboring histones which allows for unique combinations (Rando 2012). These combinations are thought to provide a pattern by which protein complexes can perform complex spatio-temporal regulation [reviewed in (Millar and Grunstein 2006)].

Histone modifications are thought to serve a variety of functions as regulatory signals. For example, the physical state of the chromatin can be

Figure 1-1. The nucleosome is the basic unit of chromatin. The histone octamer is composed of two histone H3-H4 dimers and two histone H2A-H2B dimers. Each octamer has 147 base pairs of DNA wrapped around it which forms the nucleosome, the basic repeating unit of chromatin. The unstructured histone tails protrude from the histone core and receive the majority of post-translational modifications.

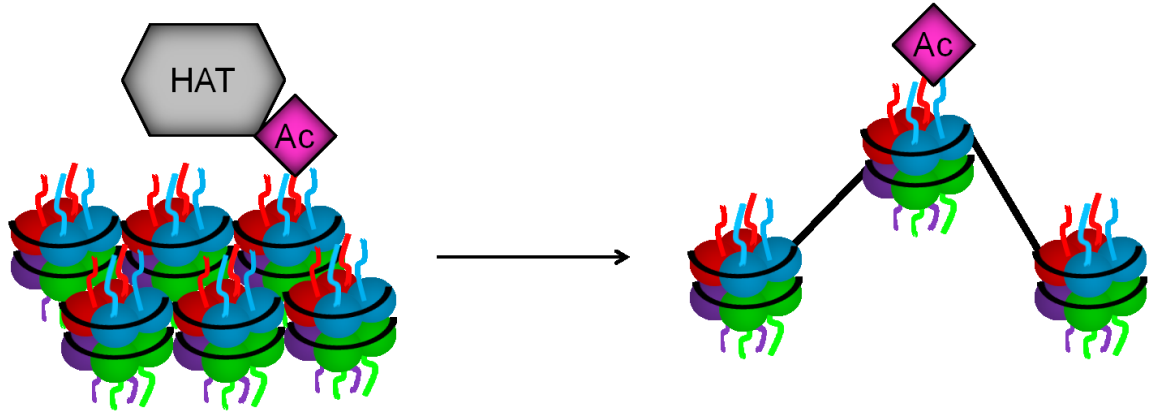


-  **Histone H2A**
-  **Histone H2B**
-  **Histone H3**
-  **Histone H4**

controlled by specific modifications to form a more open and therefore transcriptionally available landscape or it can form a more condensed barrier to transcription. Lysine acetylation is a well-characterized modification that has been established as a marker that delineates an “open” and less condensed chromatin landscape, in part by neutralizing the positive charge of the histones and disrupting the interaction between the histones and DNA (Figure 1-2) [reviewed in (Henikoff 2005)]. Lysine acetylation and deacetylation have also been shown to be important for progression through the cell cycle. Mutation of the four lysine residues on the tail of histone H4 to arginine mimics constitutive deacetylation and results in slow growth. Mutation of these four residues to glutamine, which mimics an acetylated state, results in a delay in G2-M progression in the cell cycle [reviewed in (Wade et al. 1997)]. This finding highlights the importance of dynamic histone acetylation in chromatin regulation.

Lysine acetylation is important not only for histone modification but is now emerging as a vital post-translational modification on non-histone proteins. Acetylation can serve as a signal in a regulatory network which allows for pathway regulation and it can even change the activation state of an enzyme. More acetylated proteins are being added to the growing acetylome which is even starting to rival the well established phosphoproteome which contains thousands of modified proteins [reviewed in (Norris et al. 2009)]. One example of how acetylation can contribute to controlling a kinase can be seen in the TOR pathway. S6 Kinase 1(S6K1) has an important role in the TOR pathway in the

Figure 1-2. Acetylation of histone tail lysine residues induces an open chromatin landscape. Histone acetyltransferases (HATs) transfer acetyl groups from Acetyl CoA to lysine residues on the histone tails. It is thought that neutralization of the positive charge on the lysine residue by attachment of an acetyl group loosens the interaction between the negatively charged DNA and the histones which facilitates the creation of open chromatin.



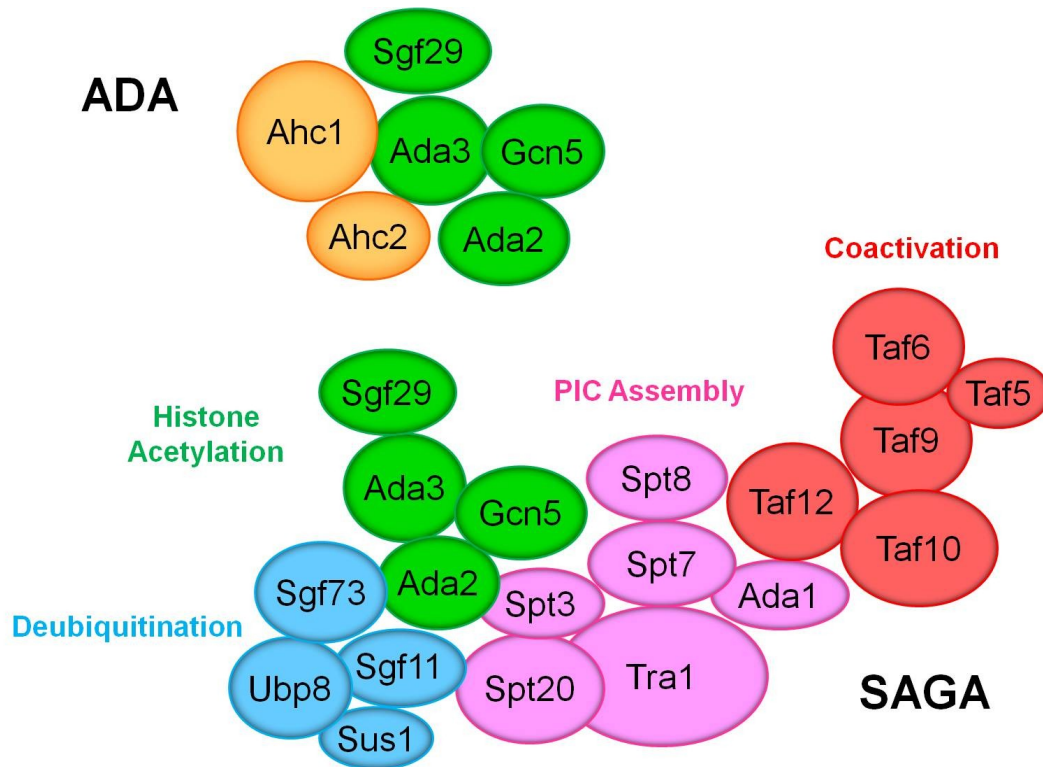
regulation of metabolism, cell growth, and protein synthesis. S6K1 is acetylated by p300, the mammalian homolog of the yeast Gcn5 histone acetyltransferase (Lo et al. 2001) , in response to growth factors (Fenton et al. 2010). Acetylation of substrates has been shown to be widely conserved, highlighting the importance of post-translational modifications for cellular processes.

The yeast histone acetyltransferase Gcn5 and its complexes. Gcn5 is a histone acetyltransferase (Lo et al. 2001) that is a member of the GNAT family. The GNAT (Gcn5-related N-acetyltransferase) superfamily has been characterized based on similarity in the structural motifs present in each member. Gcn5 is the most well-characterized HAT, both structurally and functionally, and it has been widely studied in yeast. On its own, Gcn5 is unable to acetylate nucleosomal histones but instead requires incorporation into a complex to perform this function. The predominant lysine targets of Gcn5 are on the tails of histones H3 and, to a much lesser extent, H2B (Grant et al. 1997). Gcn5 is implicated in both global acetylation of histones across the genome as well as at specifically targeted promoters such as the *HIS3* and *PHO5* genes [reviewed in (Sterner and Berger 2000)]. Microarray analysis of *gcn5* mutants has shown that Gcn5 plays an important role in transcriptional activation (Durant and Pugh 2006) and even has connections to chromatin remodeling activity (Gregory et al. 1998).

Gcn5 is the catalytic subunit of at least three known complexes in yeast, named SAGA, ADA, and SLIK/SALSA (Pray-Grant et al. 2002) [reviewed in

(Lee et al. 2011; Samara and Wolberger 2011)]. SAGA is a large complex composed of at least 19 subunits, with different clusters of proteins grouped into functional modules (Figure 1-3) [reviewed in (Lee et al. 2011)]. SAGA functions *in vivo* as a transcriptional activator and is principally responsible for activating transcription of highly regulated genes involved in stress responses such as heat, starvation, and DNA damage repair [reviewed in (Baker and Grant 2007)]. SAGA is also implicated in the export of transcribed mRNA and the HAT activity of Gcn5 is thought to facilitate nucleosome eviction which aids in the processivity of RNA polymerase II (Govind et al. 2007). The ADA complex is much smaller than the SAGA complex and consists of the same subunits in the SAGA HAT module as well as several structural subunits necessary for complex integrity (Figure 1-3). The ADA complex retains the ability to acetylate nucleosomes but little is yet known about its specific roles in the cell. Recently, it was discovered that Gcn5 is physically present at the centromere and might be important for kinetochore assembly. This function discovered by the genetic dissection of the interaction between Gcn5 and kinetochore subunits is most likely mediated by the ADA complex (Vernarecci et al. 2008). The combination of subunits in the SLIK/SALSA complex is not as well known as the other two complexes. However, SLIK/SALSA does contain a mixture of Spt, Ada, and other proteins. It also uniquely contains Rtg2, a protein that functions primarily in the yeast retrograde response pathway in sensing mitochondrial dysfunction (Pray-Grant et al. 2002). These three complexes all contain Gcn5, highlighting its significance in contributing to a variety of cellular processes.

Figure 1-3. The Gcn5-containing SAGA and ADA complexes. Diagram of the SAGA and ADA complexes. SAGA has many member proteins which can be divided into modules based on their primary function. In addition to transcription related functions such as coactivation and assembly of the preinitiation complex (PIC) that recruits RNA Pol II, SAGA also has a module that deubiquitinates histone H2B. Gcn5 is the catalytic subunit of the module implicated in histone acetylation. The SLIK/SALSA complex contains many of the same Spt and Ada proteins that are contained in the SAGA complex with the addition of a unique subunit Rtg2 and a truncated version of Spt7. The ADA complex is primarily composed of the same members in the SAGA HAT module, with the addition of at least two unique structural subunits Ahc1 and Ahc2. Cartoon of complexes is modified from (Lee et al. 2011)



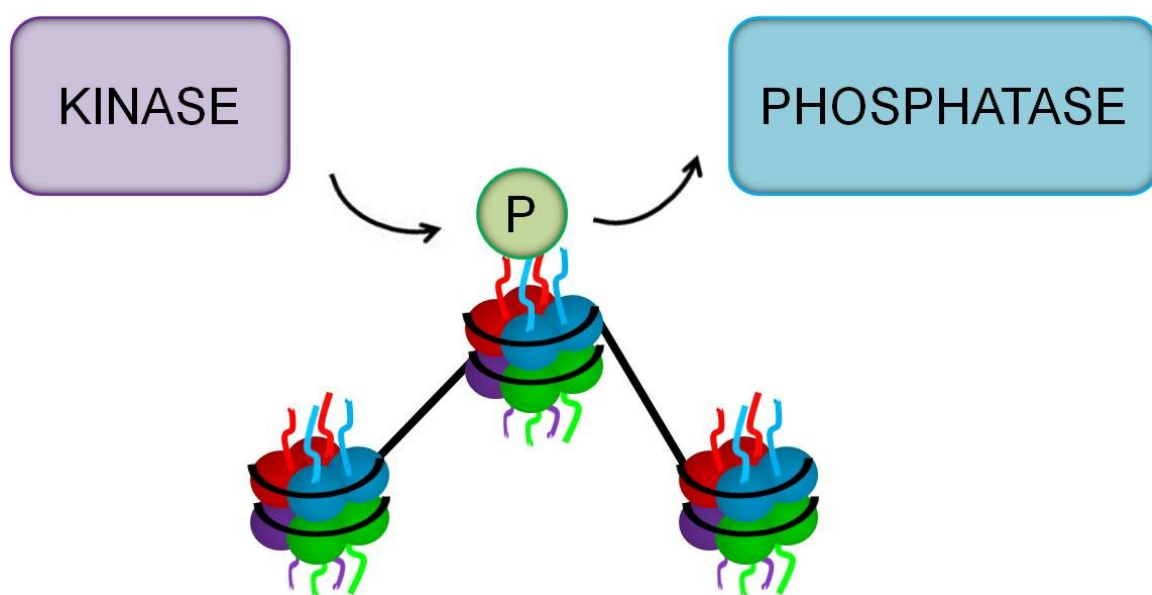
Cross-talk between phosphorylation and acetylation is important for chromatin regulation. Phosphorylation is perhaps the most abundant post-translational modification in yeast with over 4,000 phosphorylation events affecting at least 1,325 different proteins (Ptacek et al. 2005). Phosphorylation of serine and threonine residues by protein kinases is widely used to control basic pathways in the cell through signal cascades, and this serves many regulatory functions including mitotic progression of the cell cycle [reviewed in (Cohen 2000)]. Histone phosphorylation in yeast has been less well investigated than other modifications but it has already been found to contribute to the cell cycle, DNA damage repair, and transcription [reviewed in (Banerjee and Chakravarti 2011)]. For example, phosphorylation of H2A-S122 has been shown to be important for mediating repair of DNA damage (Moore et al. 2007). H3 S10 is a highly conserved phosphorylation site that is implicated in cell cycle progression and chromosome condensation (Hsu et al. 2000). Crosstalk between phosphorylation and acetylation has recently been established in yeast and these modifications functionally link the different chromatin modifying complexes together (Lo et al. 2000). For example, the SAGA complex preferentially binds and acetylates histones that have already been phosphorylated at H3 S10 and phosphorylation is important for optimal recruitment [reviewed in (Baker and Grant 2007)]. This is just one example of how differential modification of histones can work in combination to regulate cellular processes.

The PP2A complex and its function in removing phosphate groups.

The addition of chemical groups to histones or other substrates by acetylases, kinases, and methylases has been well characterized but determining which protein complexes remove these marks remains a challenge. The addition and removal of modifications are not static processes, rather they are incredibly dynamic and the timing can be important. In particular, phosphatase complexes that remove the multitude of phosphate modifications (Figure 1-4) have great importance since phosphorylation plays such a key role in cell cycle progression, cytoskeleton structure, signaling pathways, and many other processes [reviewed in (Shi 2009)].

The PP2A phosphatase complex is highly conserved among eukaryotes and is composed of a structural subunit, a catalytic subunit, and a regulatory subunit that confers substrate specificity (Figure 1-5) [reviewed in (Janssens and Goris 2001)]. The catalytic subunits, Pph21 and Pph22, appear functionally redundant in yeast. The regulatory subunit, however, has at least two different distinct forms in yeast (Cdc55 and Rts1). In mammals there are four families of regulatory subunits: B, B' (homolog of Rts1), B'', and B'''. Each of these families has multiple isoforms and splice variants allowing for greater variety and complexity [reviewed in (Shi 2009)]. So far, only Cdc55 and Rts1 have been identified as regulatory subunits in yeast. The function of the PP2A complex changes based on which regulatory subunit is incorporated into the holoenzyme.

Figure 1-4. Phosphatase complexes remove phosphate groups from histones and other substrates to promote dynamic modification. The attachment of a phosphate group to a substrate group by a kinase is important for signaling pathways and can control the state of an enzyme. The removal of this modification by phosphatase complexes is critical for proper regulation of signaling pathways.



The PP2A-Cdc55 form of the complex has well-defined roles in both mitosis (Harvey et al. 2011) and meiosis (Bizzari and Marston 2011) but the PP2A-Rts1 form has not yet been well studied. What is known is that PP2A-Rts1 regulates the spindle position checkpoint (Chan and Amon 2009), is involved in stress response (Evangelista et al. 1996), is required for proper G1 cyclin levels (Artiles et al. 2009), and protects centromeric cohesion during meiosis (Yu and Koshland 2007). Recent data indicate Rts1 is involved in these processes, although its exact role is unclear. In addition, Rts1 is localized to both the nucleus and cytoplasm throughout the cell cycle, but is found at the bud neck only during the early stages of cytokinesis and never in the late stages (Figure 1-5) (Gentry and Hallberg 2002). Thus, PP2A performs many cellular functions and is required for normal cell growth.

Suppression analysis is a means of determining functional interaction. Classically, a suppressor is a mutant that restores another mutant phenotype to wild-type functioning. When gene overexpression restores the wild type phenotype of a mutant strain it is called dosage suppression (Figure 1-6). It is generally thought that the proteins encoded by the two genes function in parallel pathways or have a physical interaction with one another (Hawley and Walker 2003). Suppression screens are widely used in yeast for determining functional interactions between genes and are very powerful tools. The abundance of plasmid libraries and the ease with which yeast can be

Figure 1-5. The PP2A complex composition and its role in the cell. PP2A is composed of a structural subunit (Tpd3), a catalytic subunit (Pph21 or Pph22), and a regulatory subunit (Rts1 or Cdc55). The form of the complex that contains Rts1 has vital roles at both the kinetochore and the bud neck during chromosome segregation in mitosis.

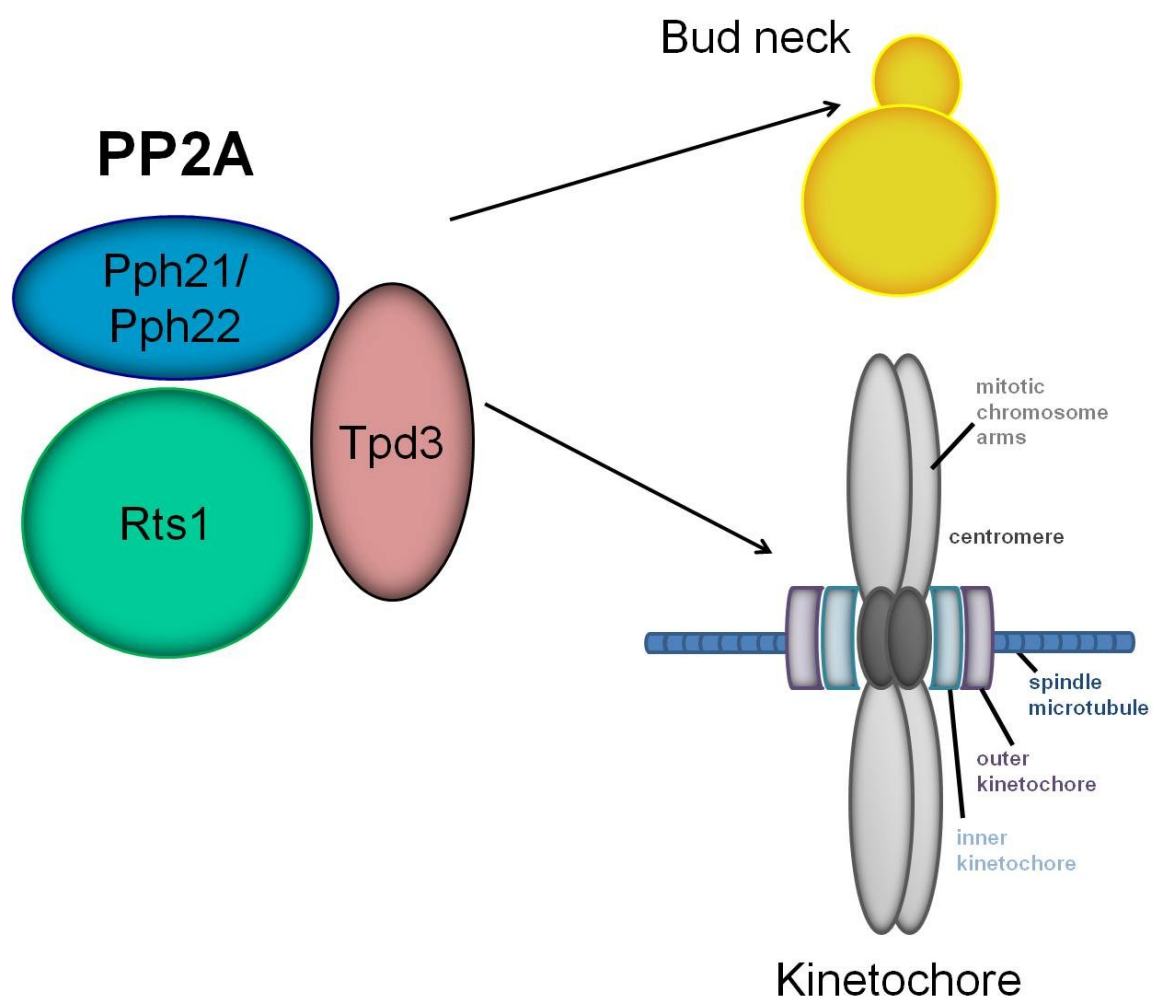
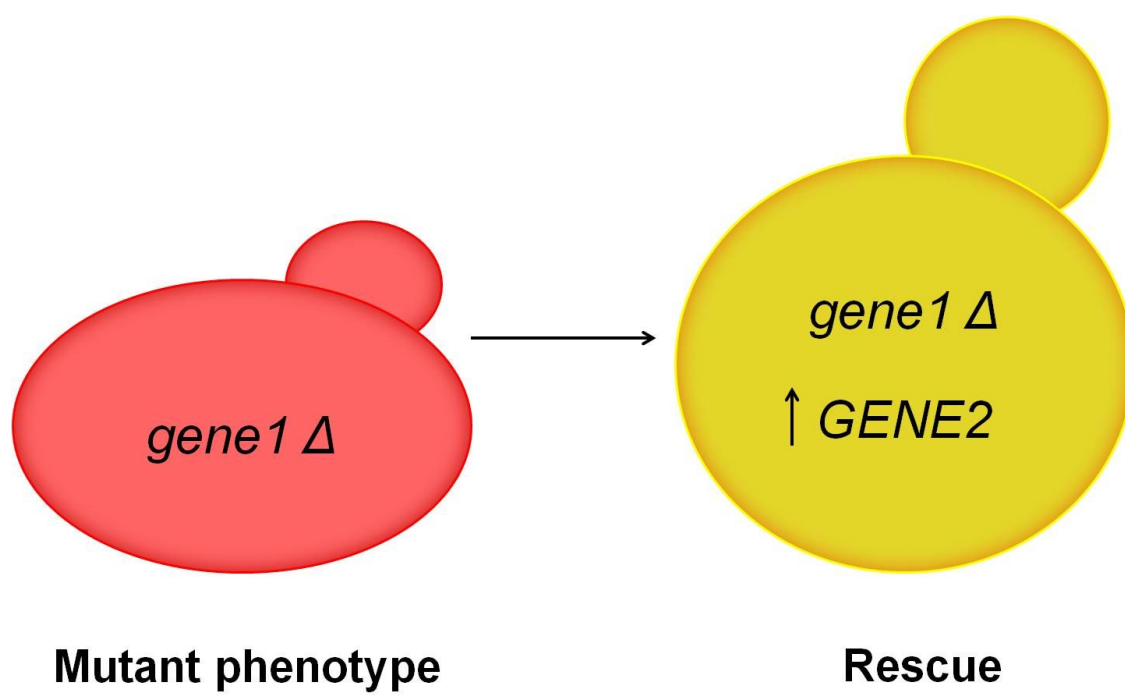


Figure 1-6. Dosage suppression analysis is a powerful tool. Mutant phenotypes associated with the loss of function of one gene can be restored to almost wild-type by overexpression of another gene. This establishes a functional relationship between the two genes and can imply involvement in parallel or overlapping pathways in the cell.



transformed with plasmids make it easy to screen thousands of genes for dosage suppression capability for any mutant background of interest (Rine 1991). The focus of this research and the subsequent chapters came as a result of a dosage screen for the suppression of *gcn5Δ sas3^{ts}* conditional lethality. In Chapter 2, *RTS1* is reported as a suppressor of this lethality and it was subsequently identified as specific to *GCN5*. Deletion of both *GCN5* and *RTS1* provided another functional link between the two, as this was also discovered to be a lethal combination. *RTS1* overexpression suppresses the temperature sensitivity and susceptibility to DNA damage of *gcn5Δ* and this was found to involve specific histone residues (Chapters 3 and 4). The interaction between *GCN5* and *RTS1* most likely involves the SAGA complex and the PP2A complex as there were found to be even more functional interactions between the subunits of each complex (Chapter 5). The interactions elucidated in this work contribute to our understanding of how crosstalk between histone modifications can contribute to cellular processes.

Chapter 2. Identification of the PP2A regulatory subunit *RTS1* as a suppressor of *gcn5Δ* phenotypes

Introduction. The HAT Gcn5 is the catalytic subunit of the SAGA, ADA, and SLIK/SALSA complexes and has vital implications in global histone H3 acetylation [reviewed in (Sternier and Berger 2000)], transcriptional activation (Durant and Pugh 2006), chromatin remodeling activity (Gregory et al. 1998), and regulation of the response to stressors such as heat, nutrient starvation, and DNA damage [reviewed in (Baker and Grant 2007)]. Disruption of *GCN5* results in sensitivity to heat stress and DNA damage [reviewed in (Burgess and Zhang 2010)], as well as a defect in sporulation (Burgess et al. 1999), highlighting its importance in genome integrity. However, Gcn5 does not work alone and has functional overlap with other chromatin modifiers. One of Gcn5's functional partners is Sas3, a member of the MYST family of HATs that has a role in transcriptional regulation at the silent mating-type loci [reviewed in (Lafon et al. 2007)]. Gcn5 and Sas3 were found to overlap in their recruitment to a large number of actively transcribed genes, and both HATs preferentially acetylate H3K14 (Rosales et al. 2007). In addition, the *gcn5Δ sas3Δ* double mutant is synthetically lethal, correlated with the global loss of H3 acetylation due to disruption of HAT activity (Howe et al. 2001).

Suppression analysis is an important and powerful tool for finding functional connections in the yeast genome. This chapter begins with a dosage suppressor screen utilizing a temperature sensitive allele of *SAS3* which was first

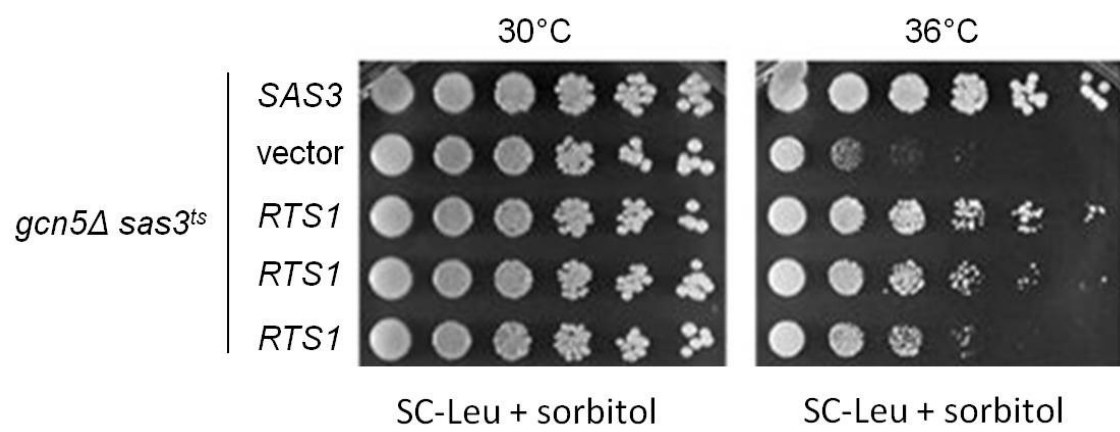
described by Howe et al. (Howe et al. 2001). The temperature sensitive allele of SAS3 has a cysteine residue at position 357 mutated to tyrosine (C357Y) and a proline residue at position 375 mutated to alanine (P375A). The temperature sensitive allele of SAS3 (denoted *sas3^{ts}*) grows at the permissive temperature of 30°C but dies at the restrictive temperature of 37°C. The dosage suppressor screen was conducted in order to find candidate genes that, when overexpressed, suppress the conditional lethality of *gcn5 sas3^{ts}* (Lafon, unpublished data).

***RTS1* identified as a dosage suppressor of *gcn5Δ sas3^{ts}* lethality.** In order to find genes that functionally interact with *GCN5* and *SAS3*, a dosage suppressor screen was performed by Anne Lafon, a former postdoctoral fellow in the lab. She optimized conditions for transforming the *gcn5Δ sas3^{ts}* background using a *LEU2*-marked plasmid library. Transformants were plated on selective media lacking leucine and grown at the restrictive temperature of 35°C to identify candidate suppressors of the lethality. The plasmids were extracted and sequenced to determine which gene was conferring suppression when overexpressed. In cases where more than one gene was present on the insert, deletion analysis was performed. There were seven total suppressors but *RTS1* was recovered the most frequently as a high-copy suppressor of *gcn5Δ sas3^{ts}* conditional lethality (Figure 2-1).

RTS1 is one of the two functionally distinct regulatory subunits of the PP2A complex and it was first discovered as a high-copy suppressor of a

Figure 2-1. Overexpression of *RTS1* rescues the lethality of *gcn5Δ sas3^{ts}*.

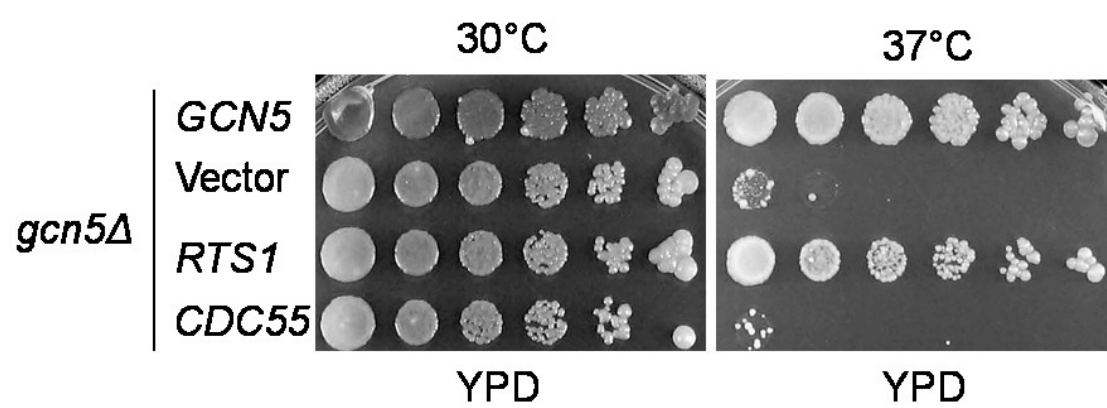
The candidate suppressors from the dosage screen were subjected to growth analysis by dilution assay to determine the strength of the suppression. The *gcn5Δ sas3^{ts}* strain (LPY11437) was transformed with wild type *SAS3* (pLP0645) and vector alone (pLP135) for comparison. The strains were plated on SC-Leu for selection with sorbitol added to decrease the background of the screen and optimize viability at the restrictive temperature.



temperature sensitive allele of *ROX3* (Evangelista et al. 1996), a gene that encodes a subunit of the mediator and RNA polymerase II holoenzyme (Gustafsson et al. 1997). *RTS1* has also been identified as a high-copy suppressor of a temperature sensitive allele of *HSP60*, a gene that encodes a mitochondrial chaperonin that functions in the re-folding of proteins after heat shock (Shu and Hallberg 1995). In addition, increased dosage of *RTS1* has been found to restore transcription of heat shock genes mediated by transcription factor Hsf1, possibly by remodeling of the cell wall (Imazu and Sakurai 2005). It is possible that suppression of *gcn5Δ sas3^{ts}* conditional lethality by *RTS1* could be mediated through histone modification and transcriptional regulation or by the global stress response pathway.

Suppression of *gcn5Δ sas3^{ts}* by *RTS1* overexpression is due to a specific interaction between *GCN5* and *RTS1*. Since *RTS1* was found to suppress the conditional lethality of *gcn5Δ sas3^{ts}*, it was necessary to dissect the functional interactions to determine if the suppression was specific to *gcn5Δ* or to *sas3Δ*. As previously mentioned, Gcn5 is involved in stress response and its absence results in temperature sensitivity at 37°C. Overexpression of *RTS1* was found to specifically suppress the temperature sensitivity of *gcn5Δ* (Figure 2-2). Furthermore, this suppression is specific to the Rts1 regulatory subunit of the PP2A complex as overexpression of the other regulatory subunit, Cdc55, did not rescue the temperature sensitivity defect (Figure 2-2). Since *RTS1*

Figure 2-2. Overexpression of *RTS1* rescues the temperature sensitivity of *gcn5Δ*. The *gcn5Δ* strain (LPY13435) was transformed with 2μ plasmids bearing wild type *GCN5* (pLP1524), vector control (pLP135), *RTS1* (pLP2196), and *CDC55* (pLP2330) and plated on YPD at the permissive temperature (30°C) and the restrictive temperature (37°C). Only *RTS1* overexpression rescued the growth at high temperature compared to wild type.



overexpression clearly suppresses the temperature sensitivity of *gcn5Δ*, it was logical to see if this relationship worked in reverse. Deletion of *RTS1* in yeast also results in temperature sensitivity at 37°C (Shu et al. 1997). However, overexpression of *GCN5* in the *rts1Δ* strain failed to rescue the temperature sensitivity (Figure 2-3). It appears that the suppressor relationship between the two genes works only in one direction.

Increased dosage of *RTS1* does not suppress the sporulation defect of *gcn5Δ*. Thermosensitivity is only one of the established defects of *gcn5Δ*; this mutant is also sensitive to DNA damage and other stressors [reviewed in (Burgess and Zhang 2010)] and has a sporulation defect (Burgess et al. 1999). Since *RTS1* overexpression rescues the temperature sensitivity of *gcn5Δ*, the ability of *RTS1* to potentially rescue other *gcn5Δ* phenotypes was tested. In order to evaluate the sporulation defect, diploid strains that were homozygous wild type (WT/WT), heterozygous *gcn5Δ* (WT/*gcn5Δ*), and homozygous *gcn5Δ* (*gcn5Δ/gcn5Δ*) were transformed with 2μ plasmids containing a vector control, *GCN5*, and *RTS1*. Over 200 cells were evaluated for the presence of tetrads as evidence of sporulation. It was found that *RTS1* overexpression did not rescue the sporulation defect of *gcn5Δ* (Figure 2-4).

***RTS1* overexpression improves *gcn5Δ* DNA damage phenotypes.** The dosage effect of *RTS1* was further tested to see its effects on the sensitivity of *gcn5Δ* to DNA damage. A *gcn5Δ* strain was transformed with a 2μ vector control plasmid and a 2μ *RTS1* plasmid and compared to wild type transformants. The

Figure 2-3. Overexpression of *GCN5* does not rescue the temperature sensitivity of *rts1Δ*. The *rts1Δ* strain (LPY14653) was transformed with 2μ plasmids containing vector control (pLP136), *GCN5* (pLP1641), and *RTS1* (pLP2462) and plated on SC-Ura at the permissive temperature of 30°C and the restrictive temperature of 37°C.

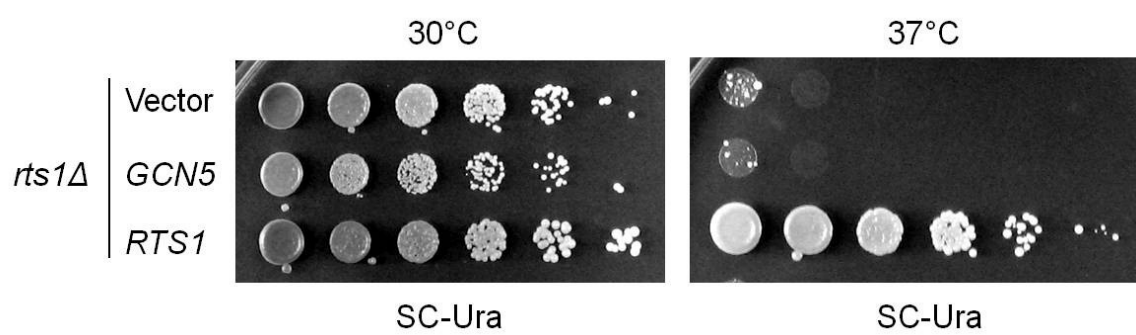
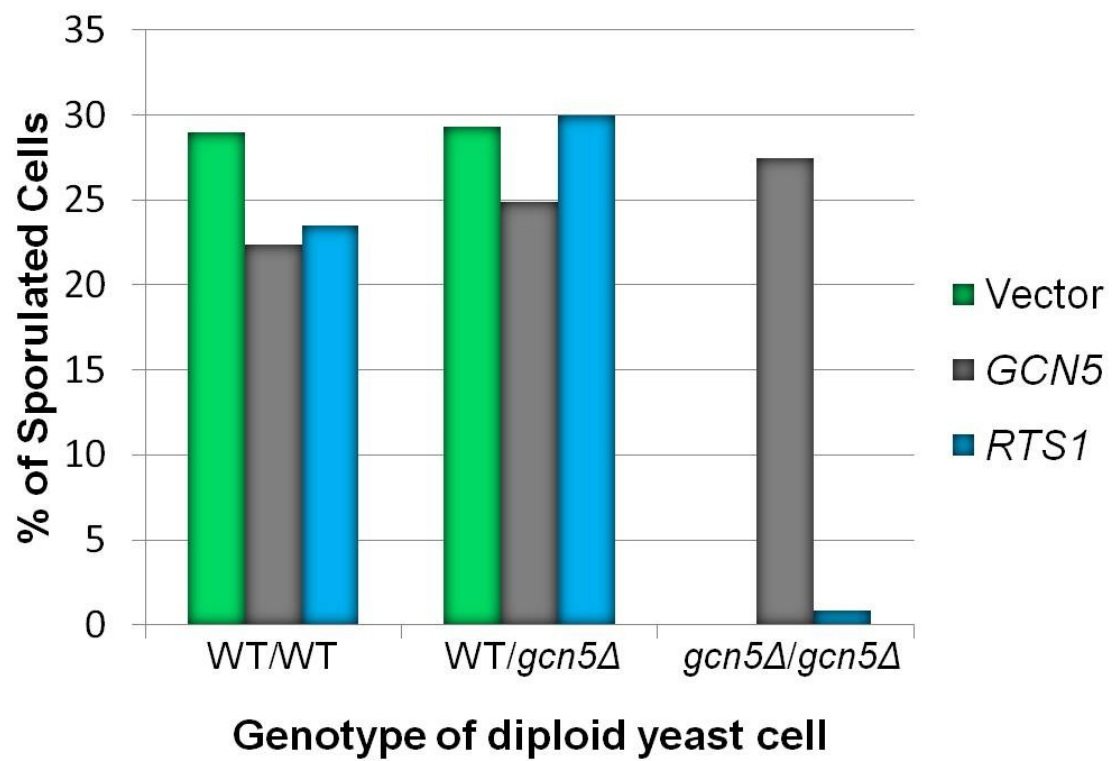


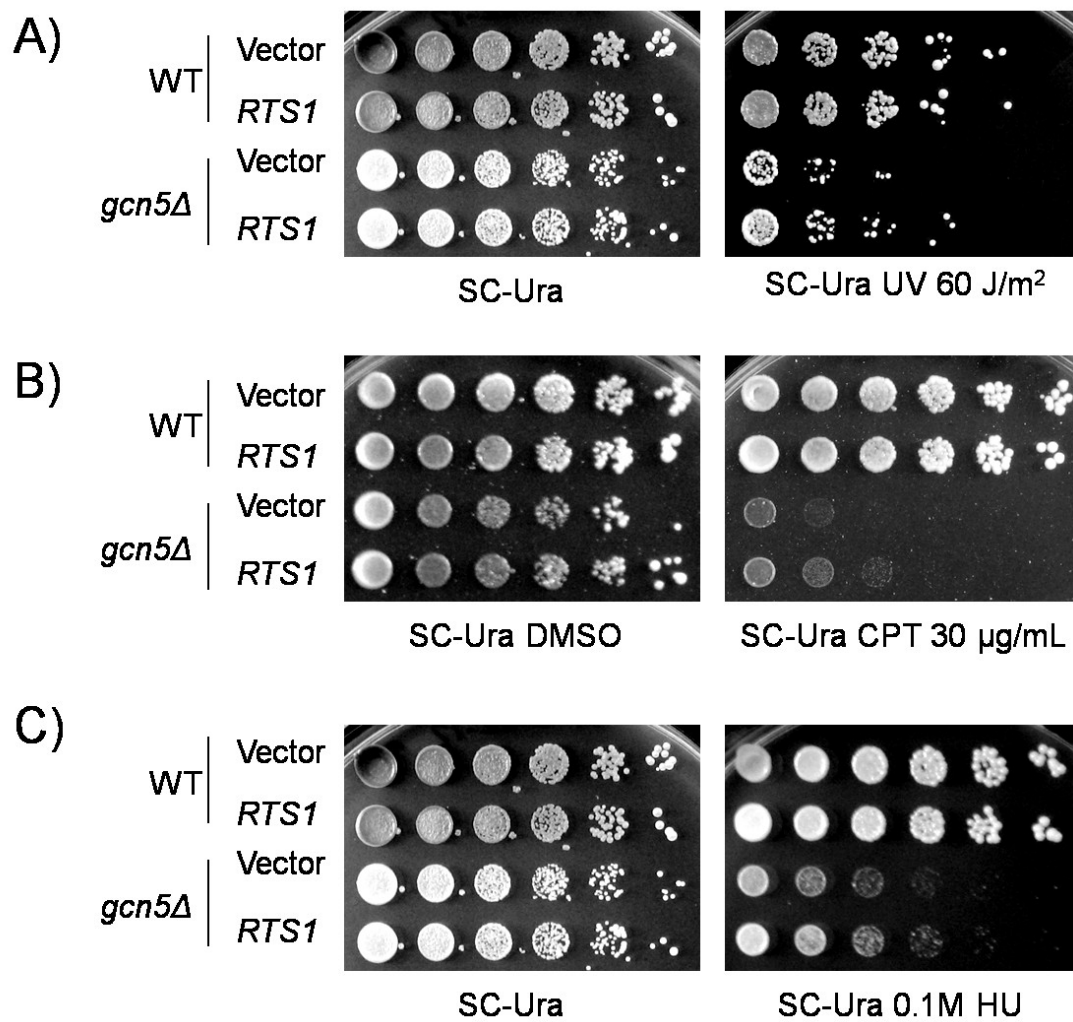
Figure 2-4. Overexpression of *RTS1* does not rescue the sporulation defect of *gcn5Δ*. Diploid cells homozygous for wild type genes (LPY1552), heterozygous for *gcn5Δ* (LPY15460), or homozygous for *gcn5Δ* (LPY15180) were transformed with 2μ plasmids containing vector control (pLP135), *GCN5* (pLP1524), and *RTS1* (pLP2197). More than 200 cells were observed and the number of tetrads was recorded. The experiment was repeated twice independently and the average for each condition between the two experiments is shown here. *RTS1* overexpression was unable to rescue the sporulation defect and only the addition of *GCN5* to the homozygous *gcn5Δ* diploid restored the ability of the cells to undergo sporulation.



cells were grown to select for the plasmid and then assessed for growth by dilution assay on a range of plates with DNA damaging agents such as camptothecin (CPT), which causes double-stranded breaks (DSBs) by inhibiting topoisomerase I (Top1) [reviewed in (Pommier et al. 2003)], hydroxyurea (HU), which causes DSBs in a replication-dependent manner by inhibiting ribonucleotide reductase (Slater 1973), and ultra-violet (UV) radiation, which crosslinks DNA and makes thymine dimers. An increased dosage of *RTS1* in *gcn5Δ* cells improved the growth when subjected to CPT, HU, and UV (Figure 2-5). It thus appears that *RTS1* might function in multiple stress response pathways to which *GCN5* contributes. Consideration of the potential mechanism for this suppression is discussed in Chapter 5.

Acknowledgements. The experiments in Figures 2-1 and 2-2 were performed by Anne Lafon. The data generated in Figure 2-4 are an average of two independent experiments, one of which was performed by Bryce Mendelsohn.

Figure 2-5. *RTS1* overexpression improves the DNA damage sensitivity of *gcn5Δ*. A wild type strain (LPY5) and a *gcn5Δ* strain (LPY10182) were transformed with a 2μ plasmid bearing *RTS1* (pLP2462) and a 2μ vector control (pLP136) and were analyzed by 5-fold dilution assay. A) The *gcn5Δ* mutant shows increased resistance to UV damage at 60 J/m² with *RTS1* overexpression. B) *gcn5Δ* shows increased resistance to 30 μg/mL CPT buffered in pH 7.5 phosphate buffer with *RTS1* overexpression. The cells show no sensitivity to the DMSO solvent control. C) The *gcn5Δ* mutant shows modestly increased resistance to 0.1 M HU with *RTS1* overexpression compared to vector control.



Chapter 3. Specific histone residues provide a functional link between *GCN5* and *RTS1*

Introduction. Post-translational histone modifications can serve as signals in chromatin regulation pathways [reviewed in (Fischle et al. 2003)]. Histone phosphorylation in particular has roles in the cell cycle, DNA damage repair, and transcription [reviewed in (Banerjee and Chakravarti 2011)]. In mice and humans, proteomic studies have led to the identification of a multitude of post-translationally modified residues. These results have been curated into an online database called PhosphoSite Plus (Hornbeck et al. 2012). However, of all of the serine and threonine residues that are part of the four canonical histone sequences, only a handful that are phosphorylated have been well-studied in yeast (Figure 3-1). Since *GCN5* and *RTS1* have a functional relationship and they are both implicated in dynamic histone modification, it followed that the suppression of *gcn5Δ* temperature sensitivity by *RTS1* might be mediated through specific histone residues. The PP2A complex removes phosphate groups from serine and threonine residues, therefore, dephosphorylation of a specific serine or threonine residue might be the mechanism by which *RTS1* overexpression suppresses *gcn5Δ* temperature sensitivity. Changing the dynamics of histone modifications could alter transcriptional activation or the recruitment of other protein complexes to the nucleosome.

Mutational analysis is often used to determine whether or not amino acid residues are modified or play some important role in regulation. A valuable

Figure 3-1. Identification of serine and threonine residues in the four core yeast histone sequences. The residues that have been identified as phosphorylated in yeast are highlighted in yellow. There are a total of 74 serine and threonine residues contained in the sequences of the four histones. The serine and threonine residues that have not been well-studied or determined to be phosphorylated in yeast are highlighted in blue. The important residues discovered in this work, H2B T91 and H4 T80, are highlighted in purple. Proteomic studies in mice and humans have revealed that there are more modified residues, including the residues homologous to T91 and T80, than what is indicated in this figure but have not yet been confirmed in yeast.

H4

1- **S**GRGKGGKGLCKGGAKRHRKILRDNIQGI**T**KPAIRRLARRGGVKKRI**S**G
 49- LIYEEVRAVLK**S**FL**S**EVIRD**S**V**T**Y**T**EHAKRK**T**V**T**SILDVVVALKRQGR**T**
 97- LYGFGG

H2A

1- **S**GGKGGKAG**S**AAKA**S**Q**S**RR**S**AKAGL**T**FPVGRVHRLLRRGNVQRIG**S**GAP
 49- VYI**T**AVLEVLA**S**AEI**S**ELAGNAARDNKK**T**RIIPRHLQLAIRNDDELNKL
 97- LGNV**T**IAQGGVLPNIHQNLILPKK**S**AKA**T**KA**S**QEI

H2B

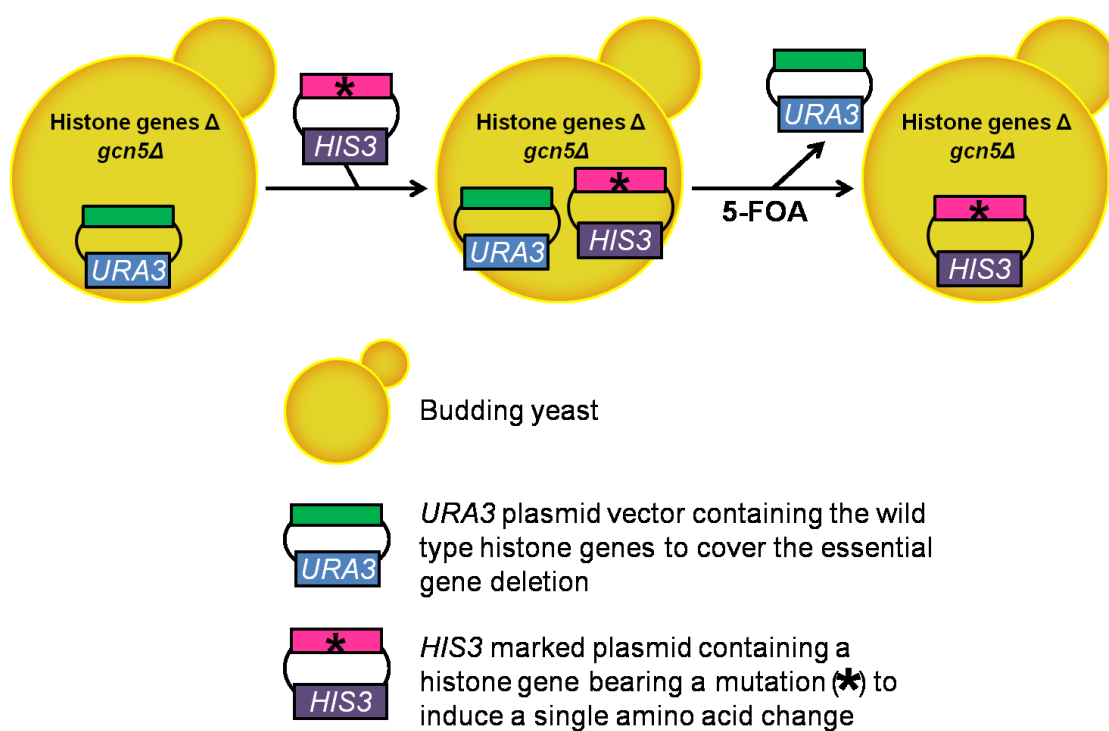
1- **S**AKAEKKPA**S**KAPAEKKKPA**S**AKK**T**S**T**S**T**DGKKR**S**KARK**E****T**Y**S**S**S**YIYKVL
 49- KQ**T**HPD**T**GI**S**QK**S**M**S**I**S**ILN**S**FFVNDIFFER**I**A**T**E**S**KLAAV**N**KK**S****T**I**S**ARE
 97- IQ**T**AVRLIILPGELAKHAV**S**EG**T**RAV**T**KY**S**S**S****T**QA

H3

1- AR**T**KQ**T**ARK**S****T**GGKAPRKQLA**S**KAAARK**S**AP**S****T**GGVKKPHRYKPG**T**V**A**L
 49- REIRRFQK**S****T**ELIIRKILPFQRLVREIAQDFK**T**DLRFQ**S****S**AI**G**ALQ**E****S**V
 97- EAVLV**S**LFED**T**NLA**S**IHA**K**RV**T**IQKKDIK**L**ARR**L**RGER**S**

plasmid library was created in which each amino acid residue in each of the four canonical histones was systematically mutated to alanine. Thus, each plasmid bears a mutation that results in a single amino acid change out of the entire histone sequence. This plasmid collection is called the scanning histone mutagenesis with alanine (Kawashima et al. 2010) library (Nakanishi et al. 2008). The SHIMA library was utilized extensively in this research to find histone residues that are necessary for suppression of *gcn5Δ* temperature sensitivity by *RTS1* overexpression. To accomplish this, a screen of all 74 serine and threonine residues in the core histones mutated to alanine was performed. Each mutation-bearing plasmid was transformed into a *gcn5Δ* yeast strain with either the H2A-H2B genes deleted or the H3-H4 genes deleted from the genome. Since the histone genes are essential for viability, the starting strain contained a *URA3*-marked plasmid bearing the wild type histone genes. After transformation with the SHIMA plasmid, yeast strains were plated on media containing 5-fluoroorotic acid (5-FOA), a compound which the product of the *URA3* gene, orotidine 5-phosphate decarboxylase, converts into 5-fluorouracil, a toxic substance to the cell. Plating on 5-FOA therefore selects against cells retaining the wild type *URA3* plasmid and selects for the yeast that have lost that plasmid (Boeke et al. 1984), resulting in complete dependence on the mutant SHIMA plasmid (Figure 3-2). This process of transformation and selection on 5-FOA was deemed the “histone shuffle” and was performed for each plasmid bearing a mutant serine or threonine residue.

Figure 3-2. Scheme of the “histone shuffle.” Yeast strains with *GCN5* deleted, along with *HTA-HTB* or *HHT-HHF* deletions were transformed with the SHIMA library plasmid and then plated on 5-FOA to select against the wild type histone *URA3* plasmid.



Suppression of *gcn5Δ* temperature sensitivity by *RTS1*

overexpression does not require H3 S10, H3 S28, H3 T45, or H2A S121

phosphorylation. It seemed logical to start the screen of the serine and threonine histone residues with residues that are already known to be phosphorylated. H3 S10 is a highly conserved phosphorylation site that is implicated in cell cycle progression and chromosome condensation (Hsu et al. 2000). In *Drosophila*, phosphorylation of H3 S10 changes drastically upon heat shock and is important for the stress response (Nowak and Corces 2000). The SAGA complex preferentially binds and acetylates histones that have already been phosphorylated at H3 S10 and phosphorylation is important for optimal recruitment [reviewed in (Baker and Grant 2007)]. H3 S28 phosphorylation is not as well understood as H3 S10, but it appears to be linked to H3 S10 phosphorylation in some organisms [reviewed in (Banerjee and Chakravarti 2011)]. H3 T45 phosphorylation has been shown to be important for DNA replication in yeast (Baker et al. 2010). H2A S122* (Sherman et al. 1985) has been shown to be important for mediating repair of DNA damage in yeast (Moore et al. 2007). In the fission yeast *Schizosaccharomyces pombe*, H2A S121 phosphorylation by the Bub1 kinase is required for the recruitment of shugoshin proteins which interact with the PP2A-Rts1 complex to protect centromeric cohesion (Kawashima et al. 2010), a process that is conserved in budding yeast (Haase et al. 2012). Despite their roles in other pathways in the cell, H3

*There is some discrepancy in the literature for the nomenclature of histones. The N-terminal methionine residue is removed from many proteins and therefore is not included in the numerical order of amino acid residues (Sherman et al. 1985).

S10, H3 S28, H3 T45, and H2A S121 are not necessary for the suppression of *gcn5Δ* temperature sensitivity by *RTS1* overexpression (Figure 3-3). Each of these histone residues was mutated to alanine and tested to see if overexpression of *RTS1* could still rescue the temperature sensitivity of *gcn5Δ*. However, none of these mutations influenced the growth of *gcn5Δ* with increased dosage of *RTS1*, and thus phosphorylation of these residues is not required for the mechanism of suppression.

Snf1 is the reported kinase that phosphorylates H3 S10 in yeast (Lo et al. 2001). In addition to testing the H3 S10A mutation, *RTS1* suppression was examined in a *gcn5Δ snf1Δ* background in order to determine if suppression depends on the activity of Snf1. Deletion of *SNF1* had no effect on the ability of *RTS1* overexpression to rescue the temperature sensitivity of *gcn5Δ* (Figure 3-4), in agreement with the previous data generated with the H3 S10A mutation (Figure 3-3).

H2B T91 is necessary for suppression of *gcn5Δ* temperature sensitivity by *RTS1*. Since the known phosphorylated residues tested were not important for suppression by *RTS1*, the 70 remaining serine and threonine residues in each histone sequence were examined in *gcn5Δ* at elevated temperature. Each residue was analyzed for growth by dilution assay and it was discovered that the mutation of threonine 91 on H2B to alanine completely abrogates the rescue of *gcn5Δ* temperature sensitivity by *RTS1* overexpression (Figure 3-5A). This effect appears to be specific to the mutated residue and is not

Figure 3-3. Suppression of *gcn5Δ* temperature sensitivity by *RTS1* overexpression is independent of H3 S10, H3 S28, H3 T45, and H2A S121 phosphorylation. A) A *gcn5Δ* strain (LPY16434) containing the SHIMA plasmid bearing the H2A S121A mutation (pLP2501) was transformed with a 2μ *RTS1* plasmid (pLP2462) and compared to vector control (pLP136). Growth of *gcn5Δ* S121A showed no difference compared to the wild type control (pLP2492) at both the permissive temperature of 30°C and the restrictive temperature of 37°C. B) Growth of *gcn5Δ* (LPY16290) containing plasmids bearing the H3 T45A (pLP2515) and H3 S28A (pLP2439) mutations was rescued at high temperature with *RTS1* overexpression. H3 T45A was previously observed to be a mutation inducing temperature sensitivity in wild type cells which accounts for the poor growth. C) Growth of *gcn5Δ* (LPY16290) containing the plasmid bearing the H3 S10A mutation (pLP2436) was rescued at high temperature with *RTS1* overexpression.

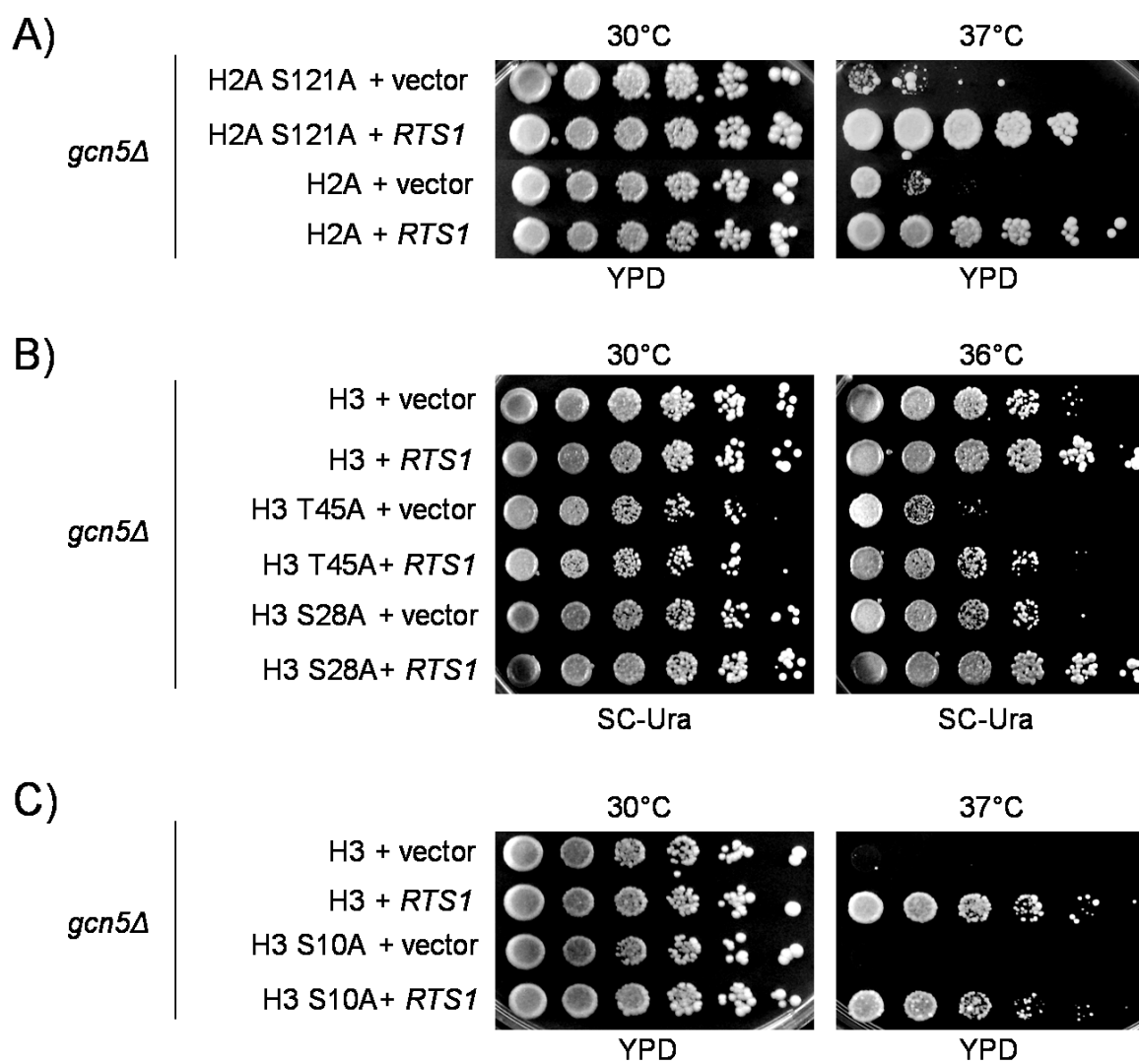


Figure 3-4. Suppression of *gcn5Δ* temperature sensitivity by *RTS1* overexpression does not require the kinase activity of Snf1. A *gcn5Δ snf1Δ* double mutant strain (LPY13486) was transformed with a 2μ plasmid containing *RTS1* (pLP2196) and compared to the strain containing vector (pLP135) and *GCN5* (pLP1524) control plasmids. A high dosage of *RTS1* was still able to suppress the temperature sensitivity of *gcn5Δ* even in the absence of *SNF1*.

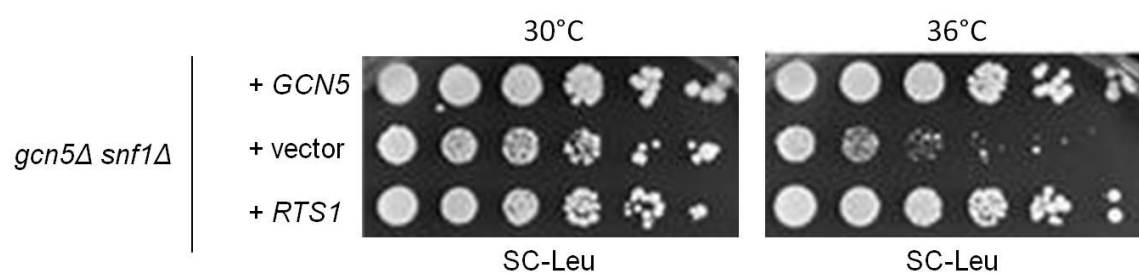
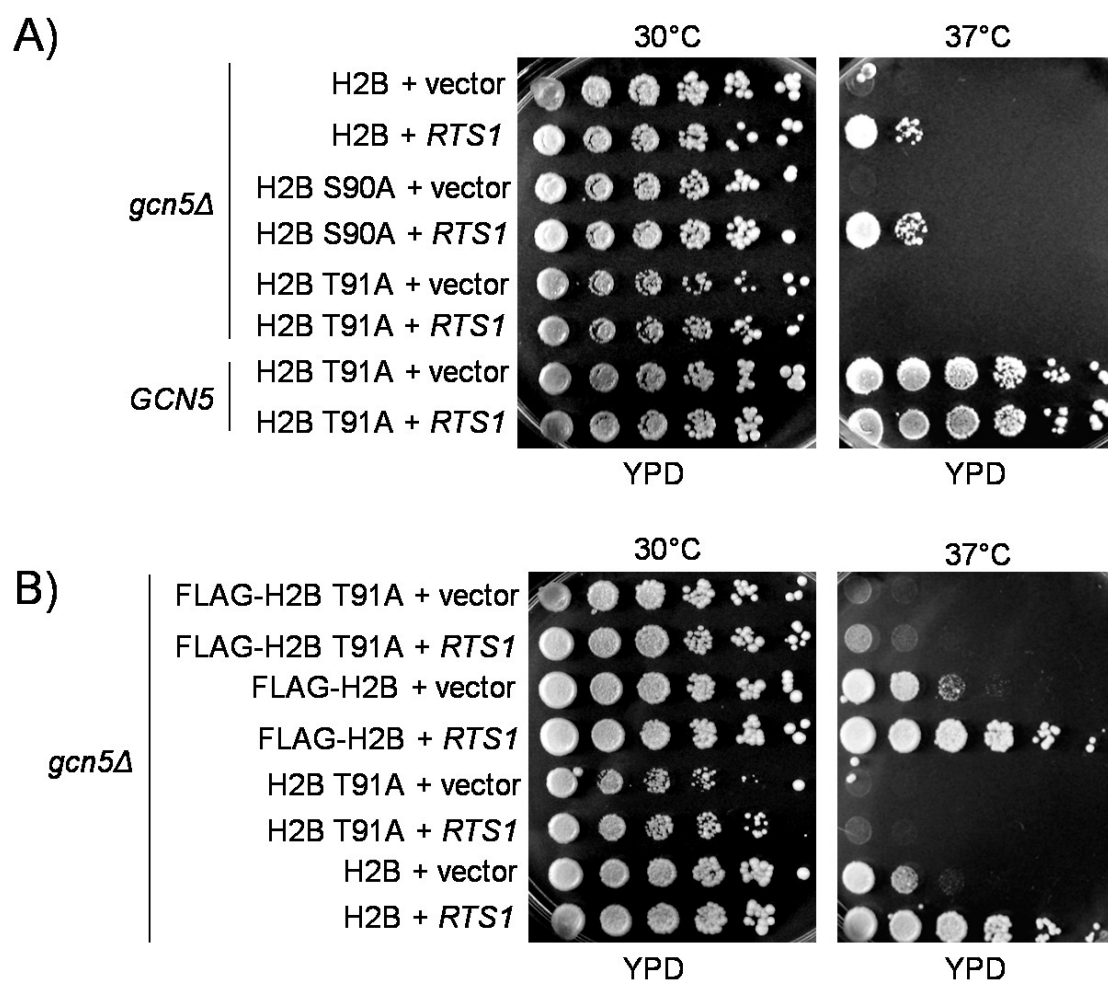


Figure 3-5. Suppression of *gcn5Δ* temperature sensitivity by *RTS1*

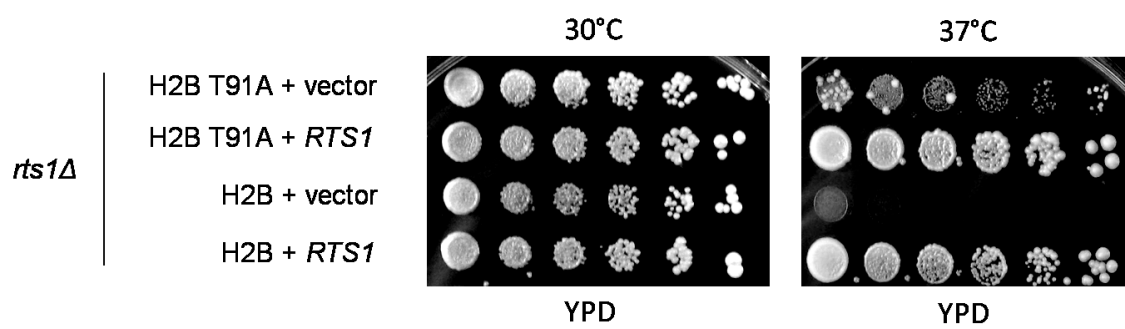
overexpression is abrogated by mutating H2B T91 to alanine. A) A *gcn5Δ* yeast strain with *HTA1-HTB1* and *HTA2-HTB2* deleted (LPY16434) was transformed with a *HIS3* plasmid bearing the H2B T91A mutation (pLP2482) and compared to the wild type H2B plasmid control (pLP2492) and H2B S90A (pLP2481). Each mutant was also tested with the 2μ *RTS1* plasmid (pLP2462) and vector control (pLP136) both at the permissive temperature of 30°C and the elevated temperature of 37°C. A wild type *GCN5* strain with *HTA1-HTB1* and *HTA2-HTB2* deleted (LPY14461) was also transformed with H2B T91A plasmid and the wild type control (pLP2492) and each of these with the 2μ *RTS1* plasmid (pLP2462) and vector control (pLP136) to test for temperature sensitivity of the H2B T91A mutation alone. B) To ask if the FLAG-tag on the SHIMA plasmid interfered with rescue by *RTS1*, a *HIS3* plasmid bearing wild type H2B (pLP2131) that is not part of the SHIMA library and does not contain a FLAG-tag was subjected to site-directed mutagenesis to T91A (pLP2714). Both the wild type plasmid (pLP2131) and the mutated plasmid as well as the 2μ *RTS1* plasmid (pLP2462) and vector control (pLP136) were transformed into a *gcn5Δ* yeast strain with *HTA1-HTB1* and *HTA2-HTB2* deleted and grown at the permissive temperature of 30°C and the elevated temperature of 37°C.



a regional effect because mutating the serine 90 residue immediately preceding threonine 91 to alanine does not interfere with the rescue by *RTS1*. The SHIMA plasmids have a FLAG-tag engineered into the N-terminus of the histone sequence so it was necessary to test the H2B T91A mutation without the FLAG-tag in order to be certain that it did not somehow interfere with *RTS1* suppression. A non-SHIMA and non-FLAG-tagged plasmid containing the gene for H2B was subjected to site-directed PCR mutagenesis to obtain the T91A mutation. Growth of *gcn5Δ* strains carrying either the non-FLAG plasmids or the SHIMA plasmids were compared and it was concluded that the FLAG-tag did not interfere with suppression as *RTS1* overexpression was still unable to rescue the growth of *gcn5Δ* at elevated temperature (Figure 3-5B). Thus, suppression of the growth defect of *gcn5Δ* at elevated temperature by *RTS1* overexpression is dependent on T91.

Given that the H2B T91A mutation results in abrogation of the rescue by *RTS1*, it appears that this residue is functionally linked to *RTS1*. In order to further understand the functional relationship, the plasmid containing the H2B T91A mutation was transformed into an *rts1Δ* strain and grown at high temperature. Deletion of *RTS1* results in temperature sensitivity at 37°C (Shu et al. 1997) and in Chapter 2 it was determined that overexpression of *GCN5* does rescue the temperature sensitivity of *rts1Δ* (Figure 2-3). However, mutating T91 to alanine allowed for moderate suppression of the temperature sensitivity

Figure 3-6. Mutation of H2B T91 to alanine improves the growth of *rts1Δ* at elevated temperature. An *rts1Δ* strain (LPY16346) containing a plasmid bearing the H2B T91A mutation (pLP2482) was grown at the permissive temperature of 30°C and the restrictive temperature of 37°C. The T91A mutation modestly improved the growth of *rts1Δ* at high temperature compared to the wild type histone control (pLP2492). This effect was only observed on YPD and not on SC. Subsequent repetitions also produced variable results.

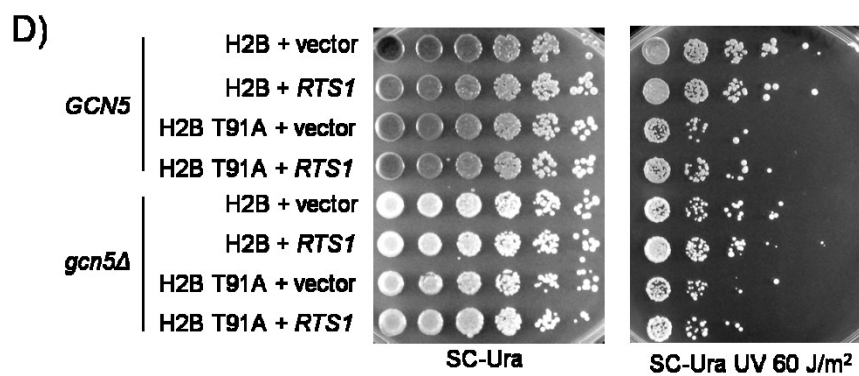
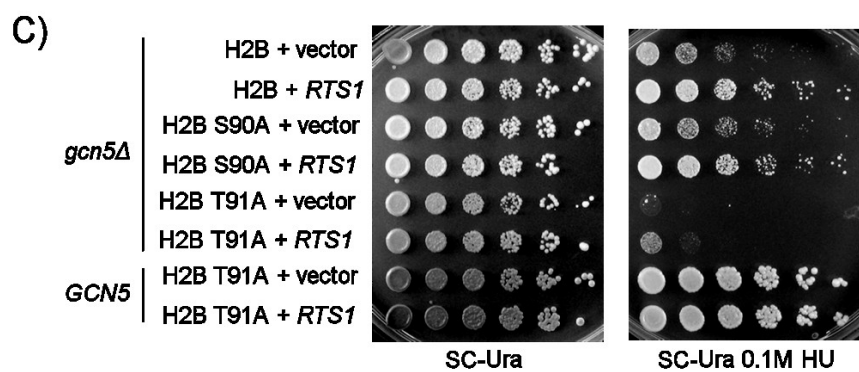
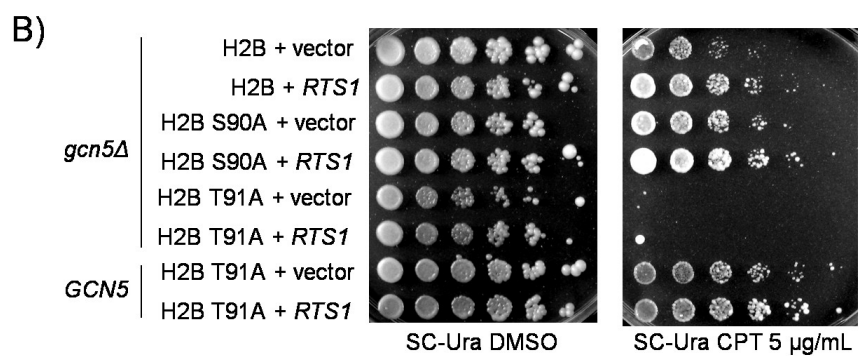
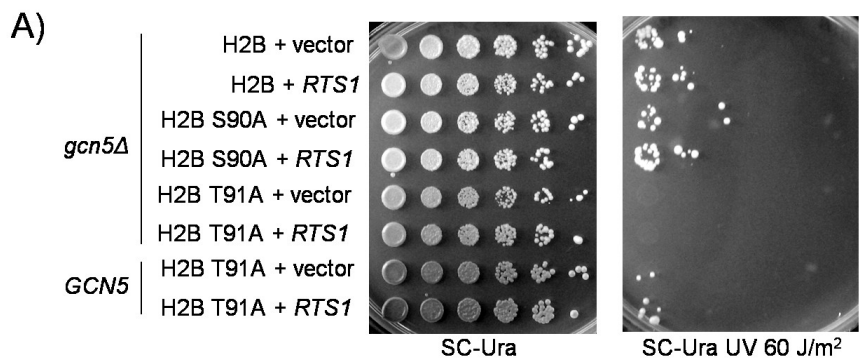


(Figure 3-6). Thus, it appears that H2B T91 links together the functional relationship of *GCN5* and *RTS1*.

H2B T91A exacerbates the DNA damage sensitivity of *gcn5Δ*. As previously mentioned, *gcn5Δ* cells are sensitive to DNA damaging agents [reviewed in (Burgess and Zhang 2010)]. In Chapter 2 it was shown that *RTS1* overexpression can also improve the growth of *gcn5Δ* strains when subjected to DNA damage-inducing agents. Since the T91 residue was established as necessary for the rescue of *gcn5Δ* by increased *RTS1* dosage in response to heat stress, it followed that this residue might be important for other forms of stress response like DNA damage repair. Growth of *gcn5Δ* cells with the H2B T91A mutation was evaluated with and without *RTS1* overexpression and subjected to DNA damage by CPT, HU, and UV exposure. The *gcn5Δ* strain bearing the H2B T91A mutation was more sensitive than *gcn5Δ* alone to all three of the DNA damaging agents tested and the sensitivity was not rescued by *RTS1* overexpression (Figure 3-7). In contrast to the broad spectrum of damage sensitivity in *gcn5Δ* cells, T91A specifically sensitized wild type cells to only UV-induced damage. The nature of this is not yet understood but is of interest (see Chapter 5).

Dynamic modification of H2B T91 is essential for viability. With H2B T91 established as important for the response of *gcn5Δ* cells to stress, and given that Gcn5-containing complexes and the PP2A complex are both involved in histone modification, it was hypothesized that T91 is post-translationally

Figure 3-7. Mutation of H2B T91 to alanine exacerbates the DNA damage sensitivity of *gcn5Δ* and inhibits the dosage-dependent suppression by *RTS1*. For all DNA damaging agents tested, a *gcn5Δ* strain (LPY16434) was transformed with a plasmid bearing the H2B T91A mutation (pLP2482) and growth was compared to the strain transformed with the S90A mutation (pLP2481) and wild type control (pLP2492), as well as a wild type strain (LPY14461) bearing the T91A mutation. Each strain transformed with the histone plasmids was also transformed with 2μ plasmids bearing *RTS1* (pLP2462) and vector control (pLP136). A) The T91A mutation causes wild type *GCN5* cells to become sensitive to UV damage and effectively eliminates the ability of *gcn5Δ* cells to survive UV damage. B) The T91A mutation severely impairs the ability of *gcn5Δ* cells to survive CPT exposure and growth cannot be rescued by *RTS1* overexpression. C) The T91A mutation is also important for DNA damage repair induced by hydroxyurea and *RTS1* overexpression does not significantly improve growth. D) A *gcn5Δ* strain (LPY16434) was transformed with a plasmid bearing the H2B T91A mutation (pLP2482) and growth was compared to the strain transformed with a wild type control (pLP2492). A wild type strain (LPY14461) was transformed with a plasmid bearing the H2B T91A mutation (pLP2482) and growth was compared to the strain transformed with a wild type control (pLP2492). The T91A mutation sensitized wild type cells to UV-induced DNA damage.

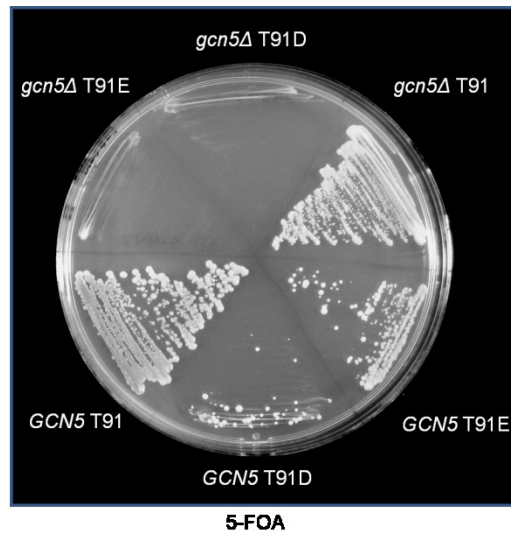
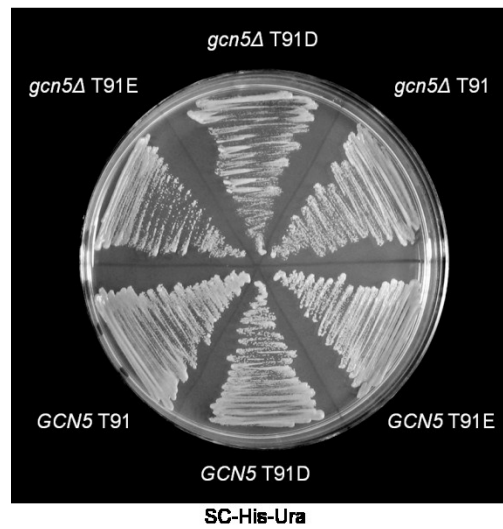


modified. Specifically, it was thought that H2B T91 might be phosphorylated, though this has not yet been established in yeast (Figure 3-1). Mutation of this threonine residue to an un-modifiable alanine residue blocked the rescue of *gcn5Δ* stress phenotypes by *RTS1* overexpression. Further mutational analysis was conducted on T91 by mutating this residue to both aspartic acid and glutamic acid, which are considered to mimic the phosphorylated state of a serine or threonine residue. The H2B T91D and H2B T91E phosphomimetics were examined in both wild type *GCN5* and in *gcn5Δ* and it was discovered that these mutations are viable in wild type *GCN5* but resulted in lethality in *gcn5Δ* (Figure 3-8). The H2B T91D and H2B T91E mutations were also found to be lethal in *rts1Δ*. These data support the idea that dynamic modification of T91 is essential for cells lacking *GCN5* or *RTS1* and that this residue is central to their functional relationship.

H4 T80 is essential for viability in *gcn5Δ* cells. In the process of screening the histone serine and threonine residues in *gcn5Δ*, a unique residue was found to be lethal in *gcn5Δ* cells during the shuffling of plasmids. Mutation of threonine 80 to alanine on histone H4 resulted in lethality (Figure 3-9) and *RTS1* overexpression could not suppress this phenotype (data not shown). As mentioned previously, Gcn5 and the NuA3 catalytic subunit Sas3 have overlapping acetylation targets on histone H3 (Rosaleny et al. 2007). To see if H4 T80 is functionally linked to both histone acetyltransferases, this mutant residue was examined in both *gcn5Δ* and *sas3Δ* in comparison to the wild type

Figure 3-8. Mutation of H2B T91 to aspartate and glutamate to mimic constitutive phosphorylation results in lethality in *gcn5Δ*. The SHIMA plasmid containing the T91A mutation (pLP2482) was subjected to site directed mutagenesis to obtain the T91D and T91E mutations. The plasmids were sequenced to verify the mutations. A) A *gcn5Δ* strain (LPY16434) with both sets of the *HTA-HTB* genes deleted was transformed with a plasmid containing the T91D (pLP2770) or T91E (pLP2689) mutation. The *HTA-HTB* gene deletions were already covered by a *URA3* plasmid containing wild type *HTA1-HTB1* (pLP2212) which was removed by streaking the cells on 5-FOA, thus making the strain dependent on the mutant histone plasmid. The *gcn5Δ* T91D and *gcn5Δ* T91E mutations were lethal in combination compared to the wild type histone control (pLP2492). Strains were plated on SC-His-Ura as a growth control. B) An *rts1Δ* strain (LPY16346) was examined in the same way with the T91D and T91E mutations. This also resulted in lethality. However, a strain wild type for both *GCN5* and *RTS1* (LPY14461) containing the T91E mutation grew almost as robustly as the wild type histone control and the T91D mutation allowed the cells to grow even though they appeared sick. Strains were plated on SC-His-Ura as a growth control.

A)



B)

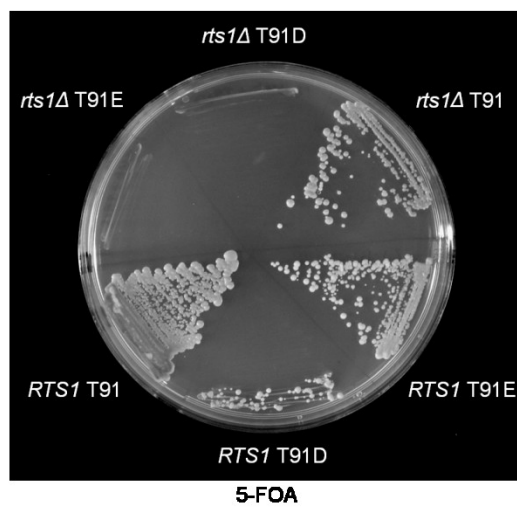
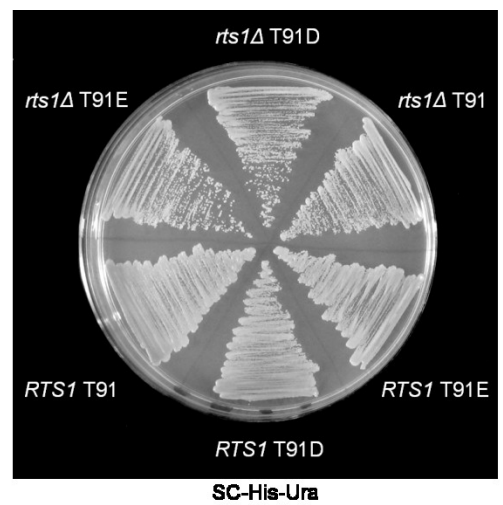
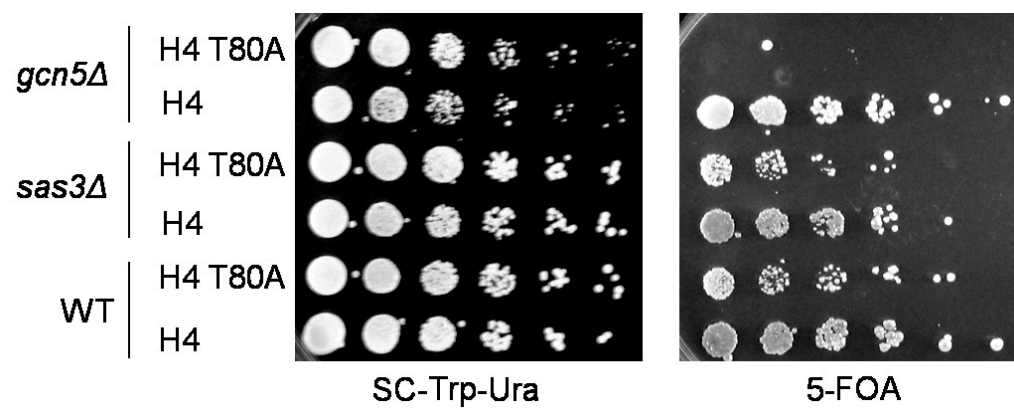


Figure 3-9. Mutation of H4 T80 to alanine is lethal in *gcn5Δ* but not in *sas3Δ*. A *gcn5Δ* strain (LPY16290) containing a plasmid bearing the H4 T80A mutation (pLP2647) resulted in lethality compared to a wild type histone control (pLP2646) when plated on 5-FOA during the plasmid shuffling process. The T80A mutation showed no effects in *sas3Δ* (LPY16432) or in a wild type strain (LPY12231). The SHIMA plasmids used in this experiment still contained almost the entire coding sequence of *SIW14*, a tyrosine phosphatase. To be sure that there were no genetic interactions between increased expression of *SIW14* and the T80A mutation the portion of the plasmid coding for *SIW14* was removed by PCR mutagenesis using primers OLP1616 and OLP1617.



strain. H4 T80A was found to be lethal in combination only with *gcn5Δ* and did not have any effect in *sas3Δ* (Figure 3-9). In addition, this lethality is specific to the W303 strain background. This was determined by mating a ResGen strain with *GCN5* deleted to a strain containing the H4 T80A histone mutant integrated into the genome (Dai et al. 2008). Full tetrads were recovered and genotyping confirmed both the *gcn5Δ* and T80A mutations indicating that the lethality observed is specific to W303 strains. In humans, proteomic studies have revealed that the residue homologous to T80 is phosphorylated (Hornbeck et al. 2012) (Figure 3-1). This is the first time that the importance of this residue has been characterized in yeast.

Acknowledgements. The experiment in Figure 3-4 was performed by Anne Lafon.

Chapter 4. Mutational analysis of histone lysine residues to dissect functional interactions between *GCN5* and *RTS1*

Introduction. The unstructured N-terminal tails of the histone proteins protrude from the histone core and are subjected to many different kinds of post-translational modifications [reviewed in (Kornberg and Lorch 1999)]. Lysine acetylation is a well studied histone modification that often occurs in combination with other modifications such as ubiquitination and methylation [reviewed in (Henikoff 2005)]. Crosstalk between phosphorylation and acetylation was first suggested as H3 S10 is phosphorylated by Snf1 (Lo et al. 2001) which leads to preferential binding of the SAGA complex and acetylation of H3 K14 [reviewed in (Baker and Grant 2007)]. The HAT Gcn5 is the catalytic subunit of the SAGA complex and it has lysine acetylation targets on the tails of histones H3 and H2B (Grant et al. 1997).

The N-terminal tails are not the only regions of the histone proteins to be modified. Increasingly more modifications of the core residues are coming to light through the application of mass spectrometry (Freitas et al. 2004). Core histone residues can be classified as belonging to three major groups: the solute accessible (or nucleosomal) face, the histone lateral surface, and the histone-histone interface [reviewed in (Mersfelder and Parthun 2006)]. The residues on the nucleosomal face are thought to be involved in chromatin structure. Residues in the lateral surface are thought to mediate histone-DNA interactions. Residues that lie in the histone-histone interface can affect the structure of the histone

octamer and, ultimately, chromatin structure. Mutation of some of the core histone residues results in defects in gene silencing, DNA damage repair, chromatin structure and assembly, and transcription [reviewed in (Mersfelder and Parthun 2006)]. In this chapter, lysine residues that lie along the lateral domain with H2B T91 were investigated for functional relationship to *GCN5* and *RTS1*. The Gcn5 acetylation targets of histone H2B were also examined. Since *GCN5* encodes a histone acetyltransferase and *RTS1* encodes a member of a phosphatase complex, it was hypothesized that the genetic interaction between the two is due to crosstalk between lysine residues that are potentially acetylated by Gcn5 and serine or threonine residues that are dephosphorylated by PP2A-Rts1.

Mutational analysis of lateral domain residues H2B K82, H2B K88, H4 K77, and H4 K79 reveals their importance for the heat stress response of *gcn5Δ* cells. In Chapter 3 it was demonstrated that the functional interaction between *GCN5* and *RTS1* involves T91 on histone H2B. The possible cross talk between acetylation of a specific lysine residue and H2B T91 in relationship to *GCN5* and *RTS1* was also investigated. H2B K82 is a conserved lysine residue that is found in the histone-DNA interface (Luger et al. 1997). H2B K88 also lies along the same surface and is close in proximity to T91 in the sequence of histone H2B. Similar to the approach carried out in Chapter 3, the plasmids from the SHIMA library bearing the H2B K82A and H2B K88A mutations were screened in a *gcn5Δ* background at high temperature with and without the *RTS1*

high-copy plasmid to see if mutation to alanine abrogated the rescue of *gcn5Δ* temperature sensitivity by *RTS1*. Neither of the single mutations inhibited rescue by *RTS1* but the combined mutations of H2B K82A T91A and H2B K88A T91A resulted in sickness at the permissive temperature of 30°C and lethality at higher temperatures (Figure 4-1). In wild type *GCN5* cells, the combined mutations had no impact on growth and showed no obvious phenotype (data not shown). The impaired growth of the double histone mutant could possibly be due to impeding chromatin remodeling activity, as residues in the histone-DNA interface are important for this mechanism [reviewed in (Cosgrove et al. 2004)]. H4 K77 and H4 K79 are residues that are located along the lateral surface of the nucleosome in line with H2B T91 and H4 T80. These two lysine residues have previously established roles in heterochromatic silencing at telomeric and rDNA loci as determined by mutational analysis (Hyland et al. 2005). However, a specific link to *GCN5* has not been established. Since H4 K77 and H4 K79 both lie along the lateral domain and are in close spatial proximity to H2B T91 and H4 T80, it was hypothesized that they might play a part in the functional relationship between *GCN5* and *RTS1*. The K77A and K79A mutations were analyzed in the same way as H2B K82A and H2B K88A. Mutation of K77 and K79 to alanine exacerbated the temperature sensitivity of cells lacking *GCN5* compared to the wild type histone control but did not impede the ability of *RTS1* to rescue the temperature sensitivity (Figure 4-2). The exacerbated temperature sensitivity of *gcn5Δ* cells could be due to a disturbance in chromatin remodeling and overall structure or perhaps is a result of an alteration in transcriptional activation, as

Figure 4-1. H2B K82 and H2B K88 are important for the response of *gcn5Δ* to heat stress in combination with H2B T91. A *gcn5Δ* strain (LPY16434) was transformed with a plasmid bearing the mutation of interest. The single K82A mutation (pLP2613) did not inhibit rescue by *RTS1* overexpression (pLP2462) at high temperature and is comparable to wild type H2B (pLP2492). In addition, the combined H2B K82A T91A (pLP2690) mutations made *gcn5Δ* cells sick at the permissive temperature of 30°C and near dead at an intermediate temperature of 34°C. Strains harboring the H2B K88A (pLP2601) single mutation and the H2B K88A T91A (pLP2688) combined mutations had similar phenotypes to those harboring the K82A and K82A T91A mutations, respectively.

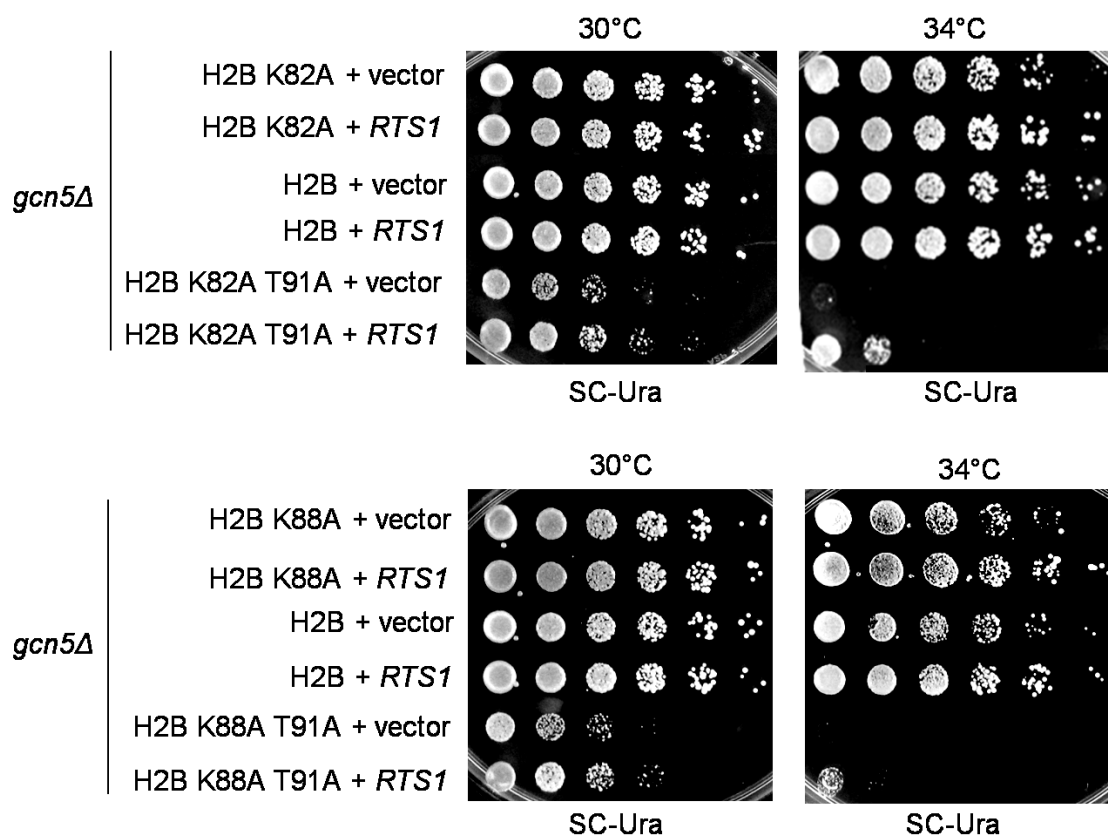
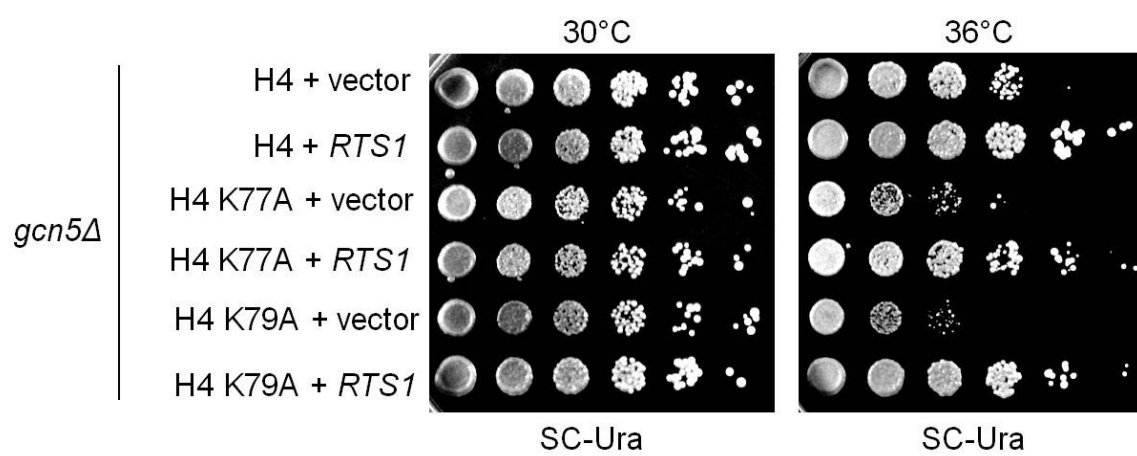


Figure 4-2. Lateral domain residues H4 K77 and H4 K79 exacerbate *gcn5Δ* temperature sensitivity but do not inhibit rescue by *RTS1* overexpression.

A *gcn5Δ* strain (LPY16290) was transformed with a plasmid bearing the mutation of interest. Mutation of both K77 (pLP2612) and K79 (pLP2603) to alanine exacerbated *gcn5Δ* temperature sensitivity compared to the wild type histone control (pLP2433) at the restrictive temperature of 36°C.



there is some evidence that both K77 and K79 are acetylated residues (Hyland et al. 2005).

The H2B acetylation targets of Gcn5 are not important for rescue of *gcn5Δ* temperature sensitivity by *RTS1* overexpression. The focus of this chapter so far has been on analyzing core residues of the nucleosome that lie in close proximity to H2B T91. However, the residues in the N-terminal tails of the histones receive the most modifications and have been the most well-characterized. Gcn5 has established acetylation targets on K11 and K16 of histone H2B (Grant et al. 1997). H2B K11 acetylation has been previously shown to be important for apoptotic signaling and chromatin condensation (Ahn et al. 2006) but not much is known about the role of H2B K16 acetylation. It was thought that post-translational modification of these residues might be important and that mutation of the residues to alanine might affect the suppression of *gcn5Δ* temperature sensitivity by *RTS1* overexpression. Plasmids containing the H2B K11A and H2B K16A mutations were transformed into *gcn5Δ* strains and grown at the permissive temperature of 30°C and the restrictive temperature of 37°C both with and without *RTS1* overexpression. It was determined that mutation of these two lysine residues to alanine does not inhibit the rescue by *RTS1* at high temperature (Figure 4-3). In order to investigate whether K11 and K16 have a functional connection to *RTS1*, these residues were also examined in the same way in an *rts1Δ* background. H2B K11A and H2B K16A did not improve the temperature sensitivity of *rts1Δ* mutants or have any observable phenotype

Figure 4-3. Suppression of *gcn5Δ* temperature sensitivity by *RTS1* overexpression does not require K11 or K16, the H2B acetylation targets of Gcn5. A *gcn5Δ* strain (LPY16434) was transformed with a plasmid bearing the mutation of interest. Growth of the *gcn5Δ* strain containing the H2B K11A (pLP2669) or H2B K16A mutations (pLP2670) was still rescued at high temperature by *RTS1* overexpression (pLP2462) at high temperature. The plasmids bearing the K11A and K16A mutations were also transformed into a wild type *GCN5* strain (LPY14461) and growth was examined at high temperature with and without *RTS1* overexpression. The K11A mutation in the wild type strain conferred a slight sensitivity to heat stress.

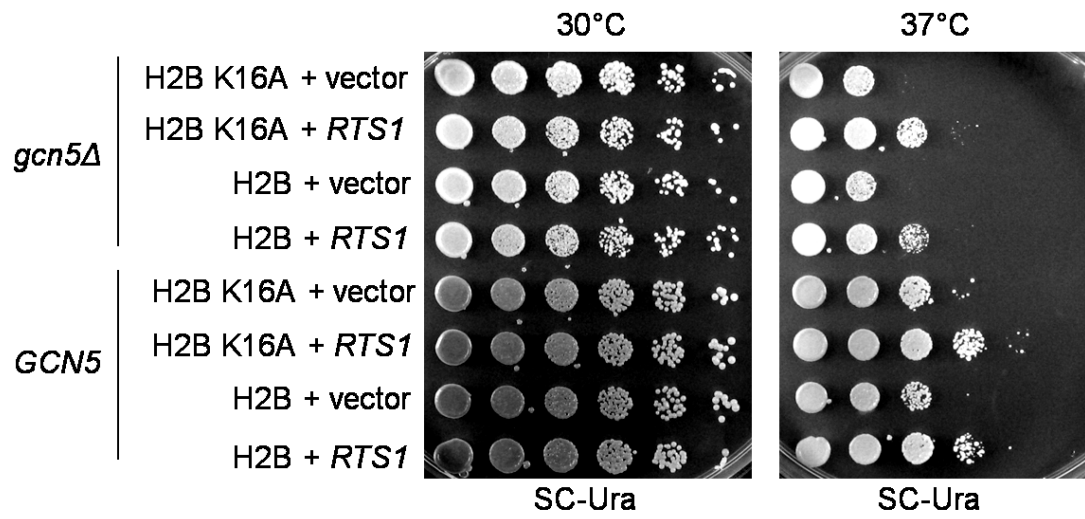
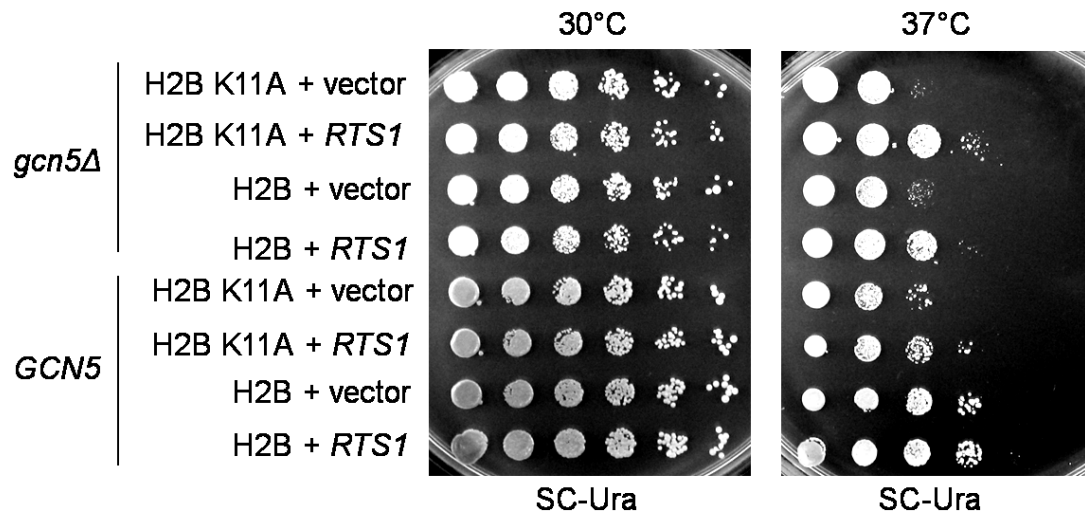
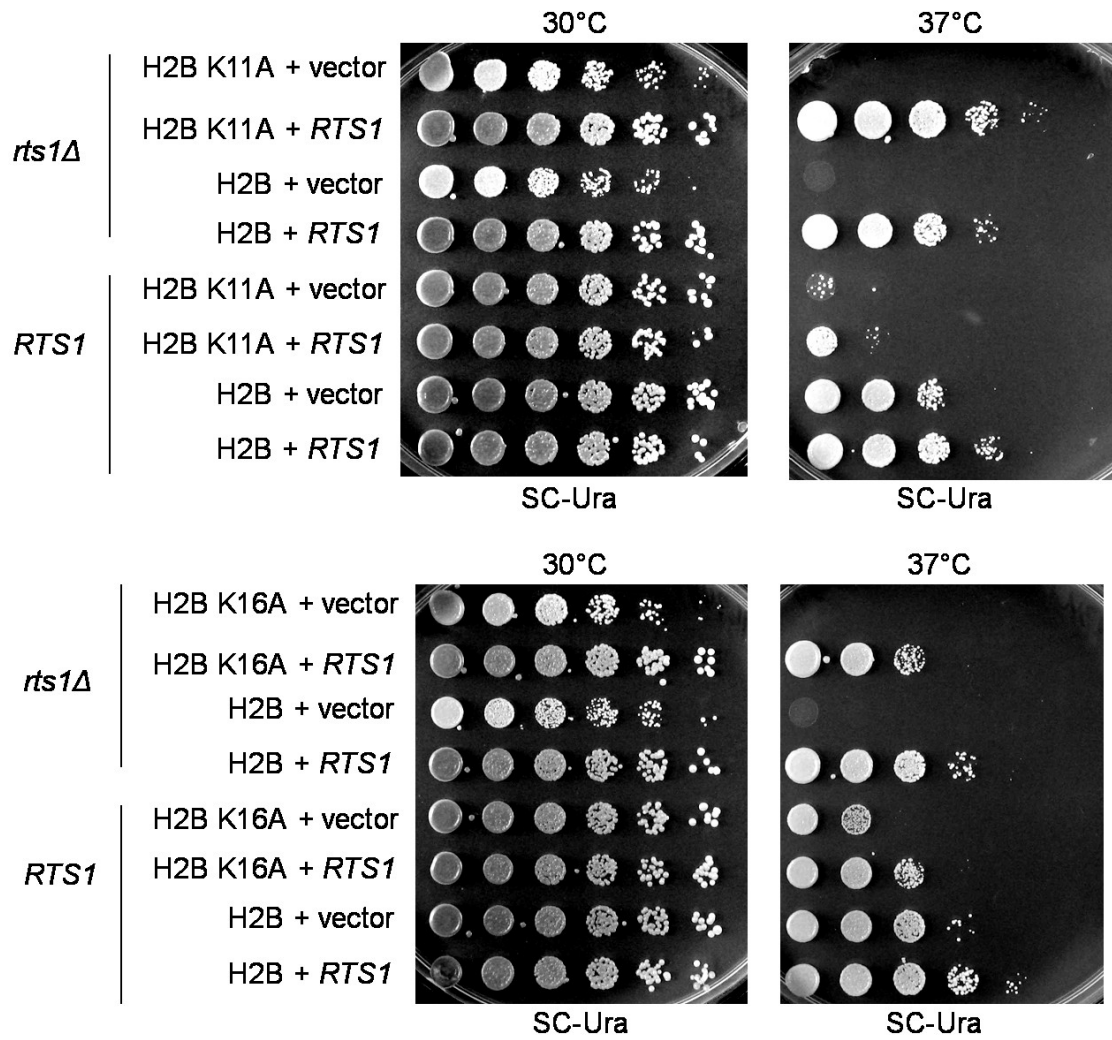


Figure 4-4. Mutation of the Gcn5 acetylation targets H2BK11 and H2BK16 to alanine does not rescue the temperature sensitivity of *rts1Δ*. An *rts1Δ* strain (LPY16346) was transformed with a plasmid bearing the mutation of interest. There was still no growth of the *rts1Δ* strain containing the H2B K11A (pLP2669) or H2B K16A mutations (pLP2670) at high temperature.



(Figure 4-4). The Gcn5 acetylation targets K11 and K16 therefore do not play an apparent role in the functional relationship of *GCN5* and *RTS1*.

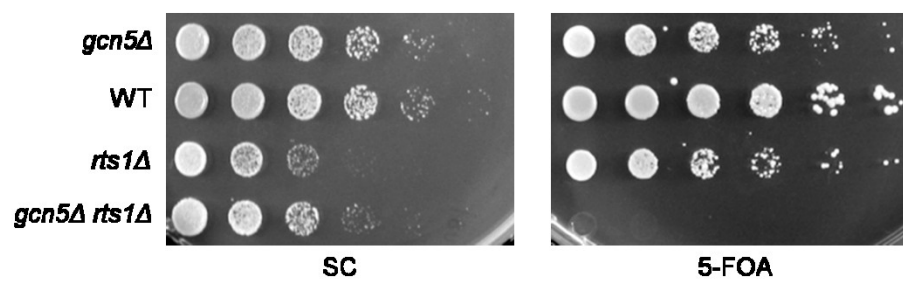
Chapter 5. Discussion and future directions

Genetic analysis reveals a synthetic lethal interaction between *GCN5* and *RTS1*. In Chapter 2 it was shown that an increased dosage of the gene encoding the PP2A regulatory subunit Rts1 suppresses the temperature sensitivity of cells lacking the Gcn5 histone acetyltransferase. Subsequent genetic analysis of *GCN5* and *RTS1* revealed that deletion of both of these genes results in synthetic lethality which has not been previously reported (Figure 5-1A). Synthetic lethality could be due to loss of catalytic activity or some other property of the enzyme. This was examined by transforming a *gcn5Δ rts1Δ* double mutant with a plasmid encoding a catalytically dead mutant of Gcn5 in which amino acid residues 126, 127, and 128 (K, Q, and L, respectively) were substituted by alanines (Wang et al. 1998). Thus, the synthetic lethality is dependent on the catalytic HAT activity of Gcn5 (Figure 5-1B).

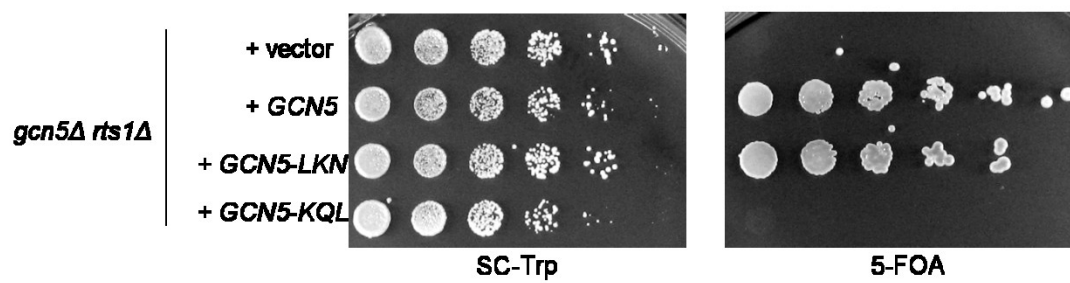
In order to further characterize the functional relationship between *GCN5* and *RTS1* it was important to dissect the genetic interactions between *GCN5* and the different subunits of the PP2A complex, and to determine the genetic interactions between *RTS1* and the subunits of the different Gcn5-containing complexes. Gcn5 is incorporated into at least three different complexes in yeast so determining which of the Gcn5 complexes mediates the functional relationship in yeast is vital for understanding the nature of the relationship and the pathways involved.

Figure 5-1. The *gcn5Δ rts1Δ* double mutant is synthetically lethal and lethality is dependent on the catalytic activity of Gcn5. A) A *gcn5Δ* strain (LPY13435) covered by a *URA3* plasmid containing wild type *GCN5* was crossed to an *rts1Δ* strain (LPY14653). The diploid was sporulated and then dissected. A tetrad bearing all four meiotic recombination possibilities was analyzed by dilution assay on 5-FOA to select against the *URA3* plasmid and demonstrate the lethality of the *gcn5Δ rts1Δ* double mutant (LPY15178). B) A *gcn5Δ rts1Δ* double mutant strain (LPY15178) covered by a wild type *URA3 GCN5* plasmid (pLP1640) was also transformed with a plasmid bearing the previously characterized catalytically dead *GCN5-KQL* mutant (pLP1521) and a plasmid bearing a catalytically active *GCN5-LKN* mutant. These were compared to vector control (pLP61) and *GCN5* wild type (pLP1518) on 5-FOA to select against the *URA3* plasmid. Only the strains containing the *GCN5* and catalytically active *GCN5-LKN* plasmids were viable. Thus, the lethality of the double mutant is dependent on the catalytic activity of Gcn5.

A)



B)

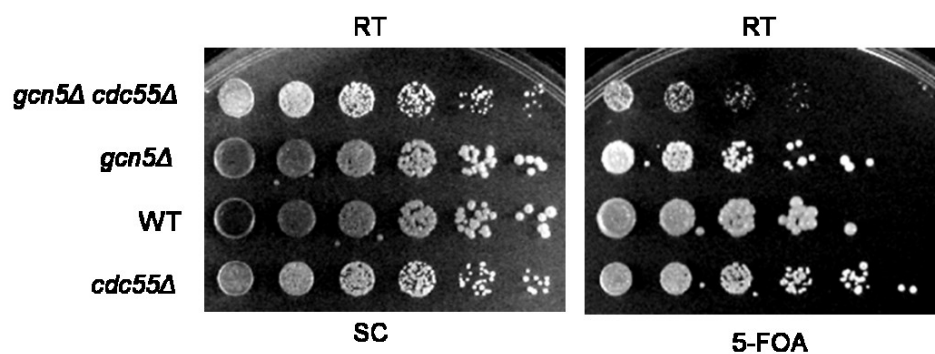


Dissecting the genetic interactions between *GCN5* and the members of the PP2A complex. After discovery of the synthetic lethal interaction between *GCN5* and *RTS1*, it was important to further dissect the genetic interactions between *GCN5* and the other subunits of the PP2A complex in order to determine if the interaction is between *GCN5* and *RTS1* specifically or with the functional activity of PP2A as a whole. First, the other regulatory subunit of the PP2A complex, Cdc55, was examined. In Chapter 2 it was determined that overexpression of *CDC55* does not rescue the temperature sensitivity of *gcn5Δ* as *RTS1* does (Figure 2-2). *GCN5* and *CDC55* are also not lethal when deleted in combination, although the double mutant is sick and grows poorly compared to either single mutant (Figure 5-2A). Deletion of *TPD3*, the gene encoding the structural subunit of PP2A, results in lethality when *GCN5* is also deleted (Figure 5-2B). The *gcn5Δ pph21Δ* and *gcn5Δ pph22Δ* double mutants displayed even greater temperature sensitivity than the *gcn5Δ* single mutant. Overexpression of *RTS1* in the *gcn5Δ pph21Δ* and *gcn5Δ pph22Δ* double mutants only modestly improved growth at high temperature compared to the *GCN5* control (Figure 5-2C). It appears that the catalytic activity of the PP2A complex and especially that of the Pph22 subunit contributes to the rescue observed by *RTS1* overexpression. Thus, it was concluded that Rts1 in the context of the PP2A complex is important for the genetic interaction between *RTS1* and *GCN5*.

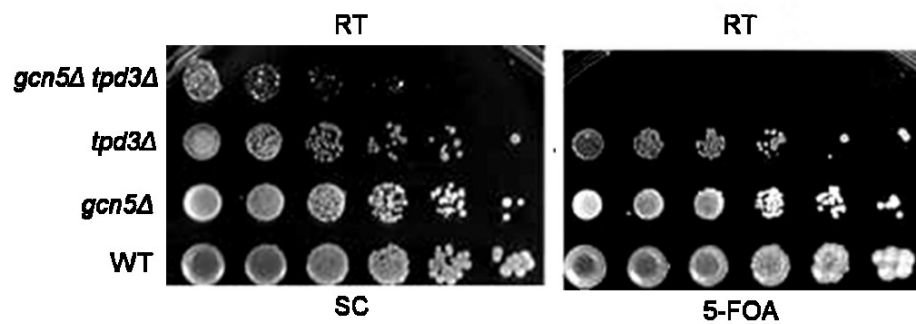
Dissecting the genetic interactions between *RTS1* and the structural subunits of the Gcn5-containing complexes. As discussed earlier, the HAT

Figure 5-2. GCN5 has genetic interactions with members of the PP2A complex. A) A *gcn5Δ* strain (LPY13435) covered by a *URA3* plasmid containing wild type *GCN5* (pLP1640) was crossed to a *cdc55Δ* strain (LPY15213). The diploid (LPY15265) was sporulated and dissected and the resulting genotypes were compared by dilution assay on 5-FOA to select against the *URA3* plasmid. The *gcn5Δ cdc55Δ* double mutant (LPY15296) was very sick compared to wild type and the *cdc55Δ* (LPY15297) and *gcn5Δ* single mutants. B) A *gcn5Δ* strain (LPY13435) was crossed to a wild type strain (LPY5) to obtain a diploid. A *tpd3Δ::kanMX* deletion cassette was transformed into the diploid strain. The resulting diploid (LPY15328) was sporulated and dissected and the resulting genotypes were compared by dilution assay on 5-FOA to select against the *URA3* plasmid. The *gcn5Δ tpd3Δ* double mutant (LPY15416) was very sick compared to wild type and the *tpd3Δ* (LPY15417) and *gcn5Δ* single mutants. C) The *gcn5Δ pph21Δ* double mutant (LPY14644) was transformed with a high-copy plasmid containing *RTS1* (pLP2462) and compared to vector (pLP136) and *GCN5* (pLP1641) controls. The *gcn5Δ* (LPY13435) and *pph21Δ* (LPY14642) single mutants containing vector control plasmids (pLP136) were also included for comparison. *RTS1* overexpression improved growth of the double mutant but suppression was not as complete as with the addition of *GCN5*. In the same way, the *gcn5Δ pph22Δ* double mutant (LPY14692) and *pph22Δ* single mutants (LPY14690) were compared. *RTS1* overexpression only modestly improved the growth of the double mutant in the absence of *PPH22*.

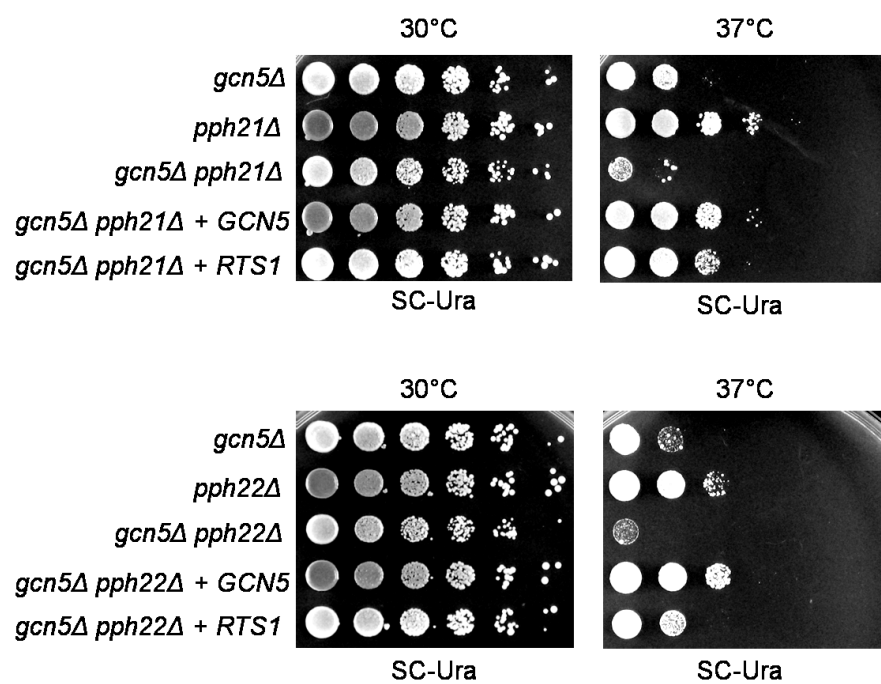
A)



B)



C)



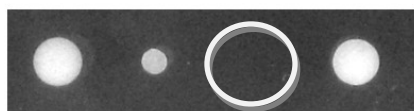
Gcn5 is part of at least three different complexes in yeast: SAGA, ADA, and SLIK/SALSA. The SAGA complex functions primarily in the transcriptional activation of genes involved in stress response [reviewed in (Baker and Grant 2007)]. Not much is yet known about the function of the ADA complex but it has been speculated that it is involved in kinetochore assembly. The absence of *GCN5* results in slow growth and temperature sensitivity and since these are not dependent on SAGA complex integrity, they might be dependent on the ADA complex (Vernarecci et al. 2008). The SLIK/SALSA complex functions in the yeast retrograde response pathway in sensing mitochondrial dysfunction (Pray-Grant et al. 2002). Each of the three complexes has a unique structural subunit that is required for complex integrity. The Spt20 subunit is required for SAGA complex integrity, as SAGA is undetectable by Western analysis in *spt20Δ* mutants (Grant et al. 1997). The ADA complex is believed to be completely disrupted in the absence of the Ahc1 structural subunit (Eberharter et al. 1999). The SLIK/SALSA complex contains many of the same Spt and Ada proteins as the SAGA complex but it contains a truncated version of Spt7 and uniquely contains the Rtg2 subunit. Cells lacking Rtg2 show a complete disruption of the SLIK/SALSA complex (Pray-Grant et al. 2002). Therefore, crossing an *rts1Δ* strain to a strain bearing *spt20Δ*, *ahc1Δ*, or *rtg2Δ* mutations should allow determination the complex or complexes through which *RTS1* and *GCN5* interact.

The *rts1Δ spt20Δ* double mutant was not viable as it failed to be recovered by tetrad dissection and (Figure 5-3A). The dissection was performed a second time and covered by a *URA3* plasmid containing *RTS1* so the double mutant could be recovered from the cross. Four independent *rts1Δ spt20Δ* double mutants were recovered and analyzed by dilution assay on 5-FOA to verify that lethality resulted from loss of both genes (Figure 5-3B). Thus, it appears that Gcn5 needs to be present in the context of the SAGA complex in cells lacking Rts1. This agrees with the results presented in Chapter 2 as *RTS1* overexpression rescued both temperature sensitivity and DNA damage phenotypes in *gcn5Δ* and would seem to implicate SAGA function as it controls transcriptional activity in stress response pathways.

RTS1 has functional overlap with the SAGA complex but could also have functional interactions with ADA and SLIK/SALSA as well. Over 80 tetrads were dissected in an attempt to isolate the *ahc1Δ rts1Δ* double mutant (LPY17370 crossed to LPY16980) for evaluation of a possible interaction between *RTS1* and the ADA complex. However, both genes are linked on chromosome XV thus not allowing the double mutant to be recovered by genetic dissection. To recover the double mutant, it will be necessary to take approaches involving direct knockouts or stimulation of recombination in the tetrads to date. Due to time constraints, there was no attempt yet made to isolate the *rts1Δ rtg2Δ* double mutant but this should be performed to determine if it is the SAGA complex alone that functionally interacts with *RTS1*, or if it interacts with additional complexes.

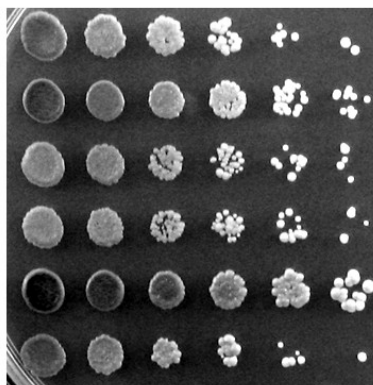
Figure 5-3. The *rts1Δ spt20Δ* double mutant is synthetically lethal, suggesting that *RTS1* functionally interacts with the SAGA complex. An *rts1Δ* strain (LPY16980) was crossed to an *spt20Δ* strain (LPY16914) transformed with the *LEU2* plasmid containing the *SPT20* open reading frame from the Yeast Genomic Tiling Collection (Jones et al. 2008) in order to cover the mating defect of *spt20Δ* mutants. A diploid was selected and grown overnight in YPD in order to lose the *LEU2* plasmid. Cultures were normalized to 1 OD₆₀₀ /mL and 100 μL of a 1:15625 dilution was plated on YPD to get single colonies which were plated on SC-Leu to determine if loss of the plasmid occurred. A) A diploid that no longer contained the *LEU2* plasmid was sporulated and dissected but full tetrads were not obtained and inferred genotypes of the missing spores (white circle) was consistent with the double mutant. B) The diploid was then transformed with a 2μ *URA3*-marked *RTS1* plasmid (pLP2462) and sporulated and dissected. Four independent *rts1Δ spt20Δ* double mutants were recovered (from top to bottom: LPY17484-17487) and all were covered by the *RTS1* plasmid. The double mutants were analyzed by dilution assay on 5-FOA to select against the *URA3* plasmid and demonstrate their lethality compared to the parent strains with a vector control plasmid (pLP136).

A)

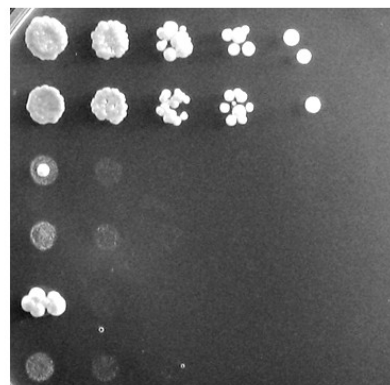


B)

spt20Δ
rts1Δ
rts1Δ spt20Δ
rts1Δ spt20Δ
rts1Δ spt20Δ
rts1Δ spt20Δ



SC-Ura



5-FOA

***GCN5* and *RTS1* might function together to regulate the TOR**

pathway. The SAGA complex and the PP2A complex are both involved in many different cellular pathways so narrowing down the most likely location of interaction proved to be difficult. One potential model investigated involves the TOR pathway. The TOR pathway is a highly conserved pathway in eukaryotes that controls cell growth by sensing nutrients in the environment and relaying that information to downstream signaling pathways that affect growth, autophagy, ribosome synthesis, translation initiation, and stress response [reviewed in (Wei and Zheng 2011)]. The TOR proteins phosphorylate S6 kinase 1 (S6K1), a prominent player in the downstream TOR signal cascade [reviewed in (Sarbasov et al. 2005)]. It was recently discovered in *Drosophila* that the PP2A complex containing the B' regulatory subunit (yeast Rts1 homolog) dephosphorylates S6K1, negatively regulating its activity (Hahn et al. 2010). In mammals, S6K1 is acetylated by p300, the mammalian homolog of the yeast Gcn5 histone acetyltransferase (Fenton et al. 2010). In yeast, Sch9 is the protein believed to be the S6 kinase homolog (Urban et al. 2007). It was hypothesized that this same sort of regulation might also occur in yeast, given that the TOR pathway is highly conserved in eukaryotes (Figure 5-4). If *RTS1* overexpression suppresses *gcn5Δ* temperature sensitivity by negatively regulating Sch9 then deletion of *SCH9* in *gcn5Δ* mutants might also produce the same effect. A strain lacking *SCH9* was constructed and then crossed to a *gcn5Δ* mutant strain with the deletion covered by a *URA3* plasmid containing *GCN5*. When plated on 5-FOA to remove the wild type *GCN5* plasmid, this resulted in

Figure 5-4. Model of potential Sch9 regulation in yeast. In mammals, Gcn5 was shown to acetylate S6K1, the homolog of yeast Sch9 (Fenton et al. 2010). In *Drosophila*, PP2A negatively regulates the activity of S6K1 by dephosphorylation (Hahn et al. 2010). Gcn5 and PP2A-Rts1 may work together to regulate the activity of Sch9 in yeast as well.

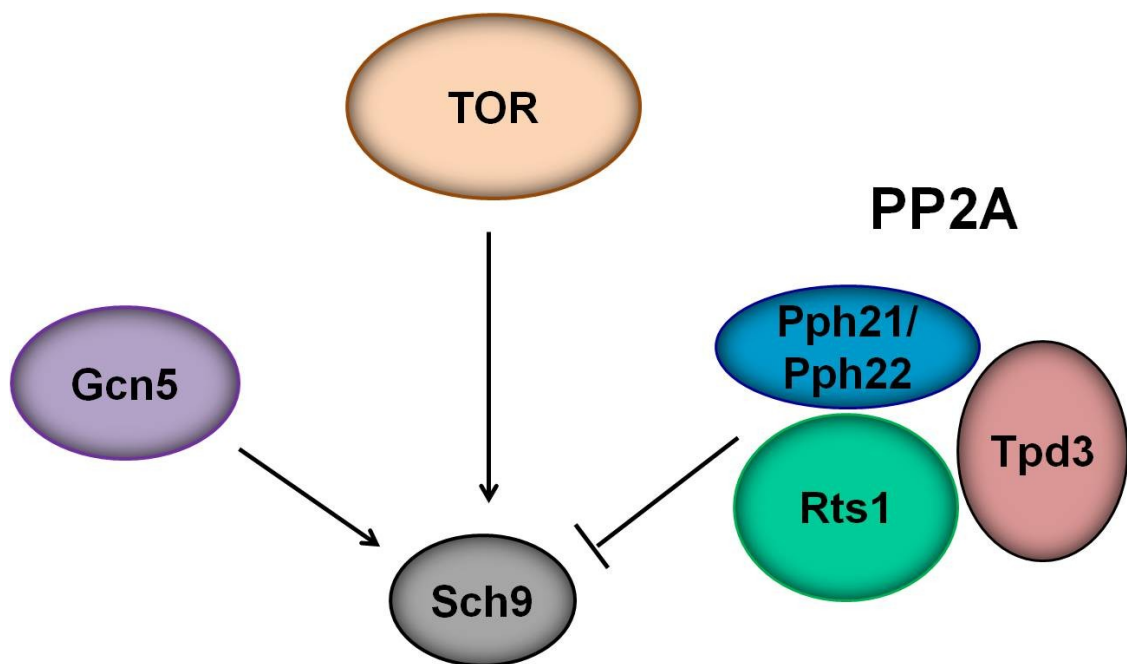
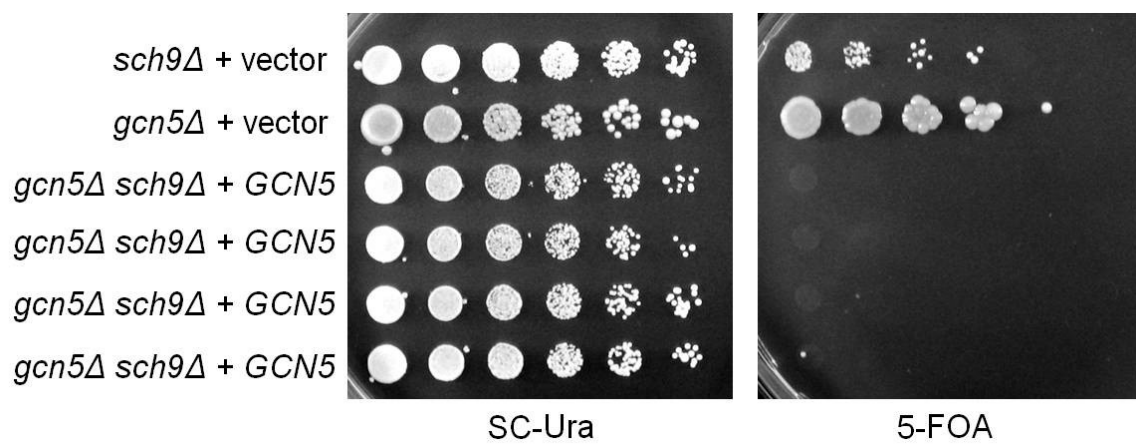


Figure 5-5. The *gcn5Δ sch9Δ* double mutant is synthetically lethal suggesting intersection with the TOR pathway. A *gcn5Δ* strain (LPY13435) was transformed with a *URA3* plasmid containing wild type *GCN5* (pLP1640) and then crossed to an *sch9Δ* mutant strain (LPY16456). The diploid was sporulated and dissected and four independent *gcn5Δ sch9Δ* double mutants covered by the *GCN5* wild type plasmid were recovered (from top to bottom: LPY17138-17141). The double mutants were analyzed by dilution assay on 5-FOA to select against the *URA3* plasmid and demonstrate their lethality compared to the parent strains with a vector control plasmid (pLP126).



lethality in the *gcn5Δ sch9Δ* double mutant, a genetic interaction that has not been previously reported (Figure 5-5). As *GCN5* has lethal interactions with both *SCH9* and *RTS1*, it was hypothesized that deletion of all three genes would alleviate the stress caused by misregulation of Sch9. Initial attempts to construct the *gcn5Δ rts1Δ sch9Δ* triple mutant have not yet been successful, but with additional time, I expect this to be possible and will help address the interactions modeled in Figure 5-4.

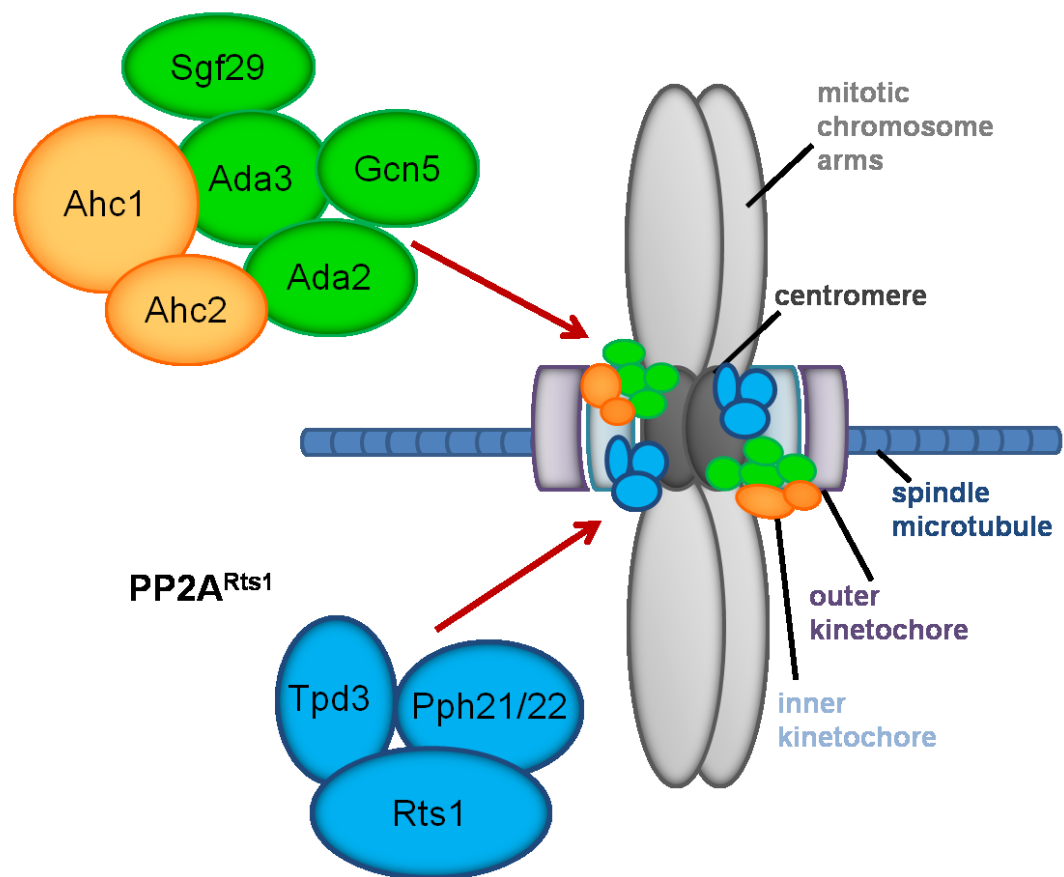
***GCN5* and *RTS1* could have vital roles in parallel pathways at the kinetochore.** It was also speculated that Gcn5 and PP2A-Rts1 could be functioning in parallel pathways at the kinetochore during cell division. The lethality observed with the loss of both proteins could be due to mitotic defects. In a study done by Vernarecci and colleagues it was found that *gcn5Δ* mutants progress slowly through G₂/M, have short spindles and defective nuclear migration, and have altered centromeric chromatin. They also found through ChIP analysis that Gcn5 physically interacts with centromeric DNA (Vernarecci et al. 2008). It was hypothesized that Gcn5 functions at the kinetochore as part of the ADA complex and not the SAGA complex, as deletion of *SPT20* did not result in slow growth or temperature sensitivity phenotypes. In a study done by Gentry and Hallberg, Rts1 was also found to coprecipitate centromeric DNA in a manner dependent on the structural integrity of the kinetochore (Gentry and Hallberg 2002). We propose a model in which Gcn5 and PP2A-Rts1 have parallel functions at the kinetochore and the synthetic lethal interaction between *GCN5*

and *RTS1* is a result of mitotic defects (Figure 5-6). Vernarecci and colleagues implicate the ADA complex at the kinetochore (Vernarecci et al. 2008) but my findings (Figure 5-3) implicate the SAGA complex, with the ADA and SLIK/SALSA questions unresolved. Thus it remains possible that *RTS1* has genetic interactions with more than one Gcn5-containing complex and that there might be more than one pathway where Gcn5 and PP2A-Rts1 interact.

***RTS1* overexpression suppresses *gcn5Δ* temperature sensitivity and improves DNA damage sensitivity but does not rescue the sporulation defect.** In Chapter 2, *RTS1* was found to be a high copy suppressor of *gcn5Δ* *sas3^{ts}* conditional lethality (Figure 2-1). Subsequent analysis revealed that *RTS1* overexpression specifically suppresses the temperature sensitivity of *gcn5Δ* mutants (Figure 2-2). Furthermore, the other PP2A regulatory subunit, *CDC55*, could not suppress the temperature sensitivity of *gcn5Δ* mutants (Figure 2-2). Cdc55 and Rts1 are not functionally redundant in their activity and each regulatory subunit targets the PP2A complex to specific locations in the cell, therefore, it was not expected that an increased dosage of *CDC55* would rescue the growth of *gcn5Δ* mutants at high temperature.

Deletion of *RTS1* also results in temperature sensitivity at 37°C. However, overexpression of *GCN5* in *rts1Δ* mutants does not rescue the temperature sensitivity and thus, the suppressor relationship does not work in both directions (Figure 2-3). The respective temperature sensitive phenotypes of *gcn5Δ* and *rts1Δ* mutants most likely have unrelated causes. For the *gcn5Δ* temperature

Figure 5-6. Proposed model of Gcn5 and PP2A-Rts1 interaction in regulating kinetochore function. It was discovered that Gcn5 is physically present at the centromere and might be important for kinetochore assembly as *GCN5* has a genetic interaction with several kinetochore subunits. This function is most likely mediated by the ADA complex as *spt20Δ* mutants showed no slow growth or temperature sensitivity phenotypes (Vernarecci et al. 2008). Rts1 was found to coprecipitate centromeric DNA in a manner dependent on the structural integrity of the kinetochore (Gentry and Hallberg 2002). The PP2A-Rts1 complex interacts with shugoshin proteins to protect centromeric cohesion (Haase et al. 2012). It is possible that Gcn5 and Rts1 interact to regulate kinetochore dynamics, an outstanding question that will be the focus of future studies.

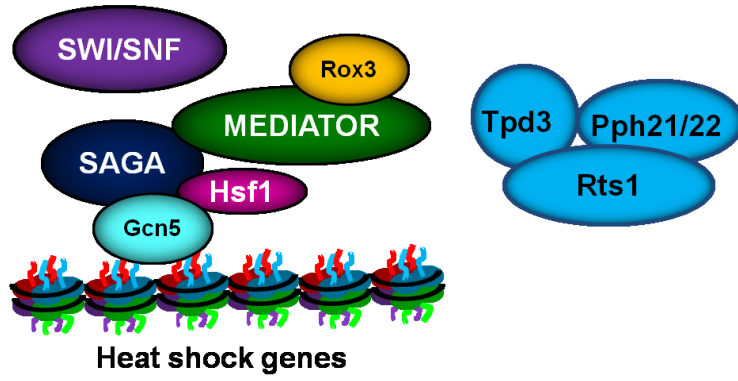
ADA complex

sensitivity, it can be speculated that it is the result of transcriptional defects at the heat shock genes. The SAGA complex was found to be present at heat shock genes mediated by heat shock transcription factor 1 (Hsf1) and an increase in SAGA occupancy was observed upon actual heat shock (Kremer and Gross 2009). Perhaps the absence of Gcn5 alters the transcriptional activation of heat shock genes. The suppression of the temperature sensitivity observed when *RTS1* is overexpressed could be contributed to it being able to somehow overcome the absence of Gcn5 in SAGA. It was discussed earlier that *RTS1* is a high-copy suppressor of a temperature sensitive allele of *ROX3* which encodes a subunit of the mediator complex (Evangelista et al. 1996). Mediator is a transcriptional coactivator complex that has been shown to be important for regulation of heat shock genes (Singh et al. 2006). *RTS1* was also found to be a high-copy suppressor of a temperature sensitive mutation of *HSF1* (Imazu and Sakurai 2005). If mediator, SAGA, and Hsf1 work together to regulate heat shock gene activity and *RTS1* overexpression rescues temperature sensitivity phenotypes involving each component, then this is a plausible model for the suppression relationship between *GCN5* and *RTS1* (Figure 5-7). More work would need to be done in order to validate this model. For example the temperature sensitive mutation of *HSF1* could be constructed in a *gcn5Δ* mutant and examined at high temperature with *RTS1* overexpression to see if the temperature sensitivity is still rescued when Hsf1 is non-functional.

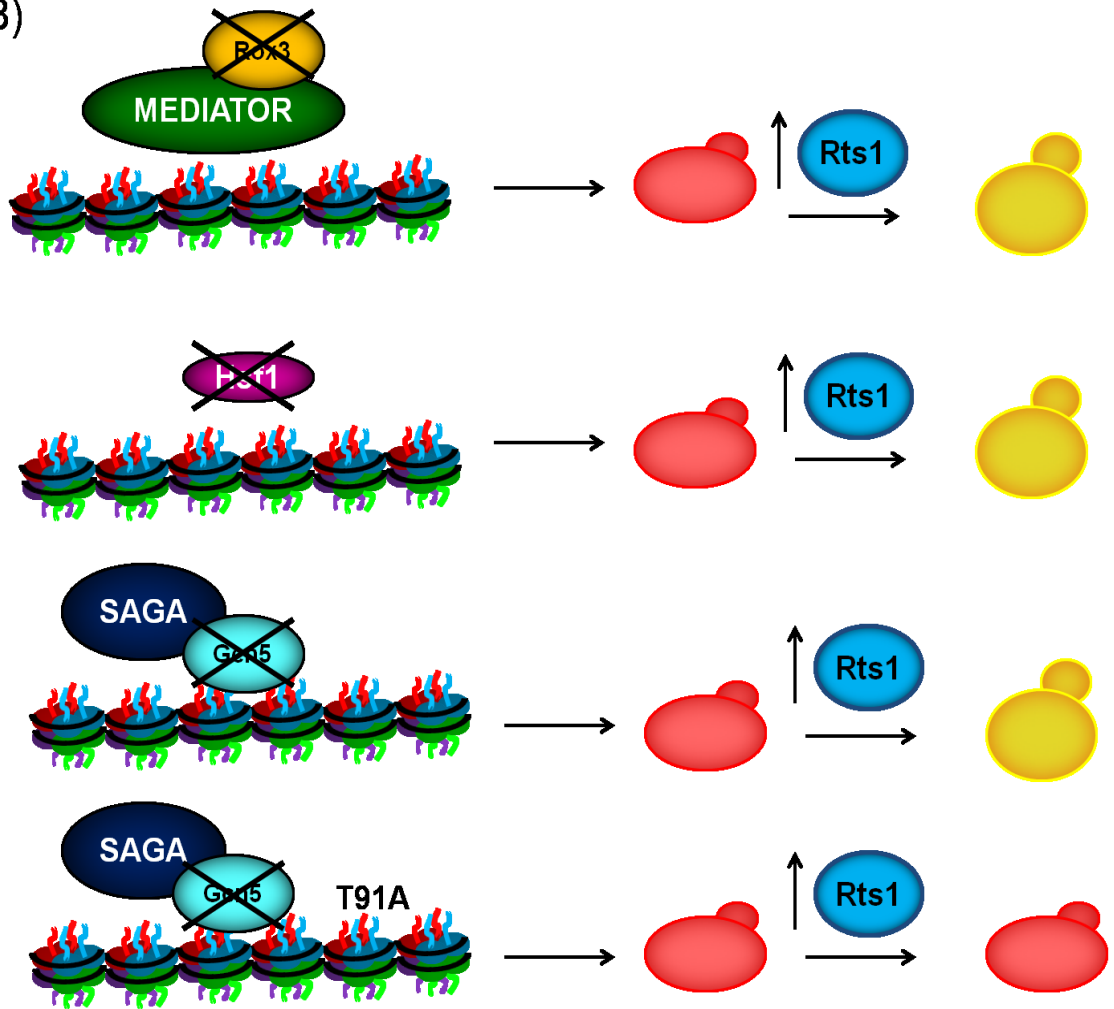
Figure 5-7. Proposed model of *gcn5Δ* suppression by *RTS1*

overexpression. A) The SAGA and mediator complexes both participate in regulation of genes involved in heat shock response. Hsf1 is a transcription factor that also contributes to regulation. The SWI/SNF chromatin remodeling complex is also localized to heat shock genes to aid nucleosome eviction but its exact role has not been determined. B) *RTS1* overexpression rescues temperature sensitive phenotypes of a *ROX3* mutant (mediator subunit), an *HSF1* mutant, and *gcn5Δ* mutants. The red cartoon of the budding yeast represents a cell that is sensitive to high temperature. The yellow cartoon of the budding yeast represents a cell where increased levels of Rts1 has suppressed the temperature sensitivity phenotype and returned the cell to wild type function. It is proposed that a high dosage of *RTS1* overcomes defects in transcription at the heat shock genes to restore viability at high temperature. In addition, the H2B T91A mutation inhibits rescue of *gcn5Δ* mutants at the restrictive temperature by *RTS1*. Perhaps this residue is important for nucleosome eviction by SWI/SNF.

A)



B)



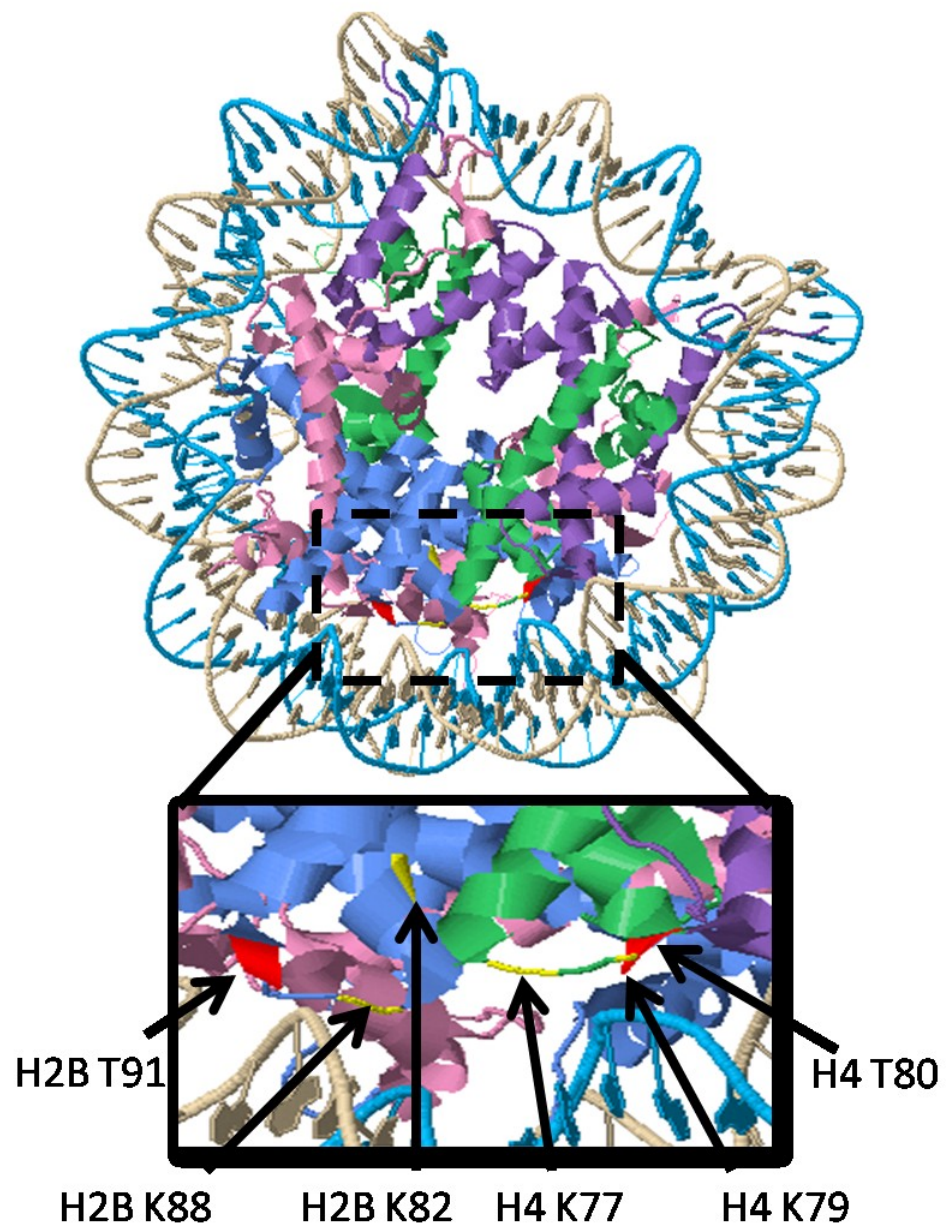
In addition to rescuing the temperature sensitivity of *gcn5Δ* mutants, a high dosage of *RTS1* also improved the growth when exposed to CPT, HU, and UV DNA damaging agents (Figure 2-5). The SAGA complex has roles at other stress response genes besides those involved in heat shock. Perhaps *RTS1* functions to restore transcriptional activation to SAGA regulated DNA damage response genes in a way similar to that proposed for heat shock genes. *RTS1* overexpression did not restore the ability of *gcn5Δ* cells to undergo sporulation (Figure 2-4). In a recent study, it was found that Gcn5 (as part of SAGA) acetylates Ume6, a repressor of early meiotic genes, in order to facilitate its degradation and relieve the repression of the meiotic program (Mallory et al. 2012). Since the acetyltransferase activity of Gcn5 appears to be vital to this process, failure of *RTS1* overexpression to restore sporulation is consistent with these new observations.

Specific histone residues are important for *gcn5Δ* mutants. In Chapters 3 and 4, serine, threonine, and lysine residues were screened in order to determine if post-translational modifications and specific histone residues were important for high-copy suppression of *gcn5Δ* temperature sensitivity by *RTS1*. A summary of the results from the screen are listed in Table 5-1. It was found that the suppression did not depend on serine or threonine residues that had been previously characterized and shown to be phosphorylated in yeast such as H3 S10, H3 T45, H3 S28, and H2A S121 (Figure 3-3). The suppression by *RTS1* also does not depend on the activity of Snf1, in agreement with the previous data

(Figure 3-4). Instead, it was discovered that suppression of *gcn5Δ* temperature sensitivity by *RTS1* overexpression is dependent on H2B T91, a previously uncharacterized residue that lies along the lateral domain of the nucleosome (Figure 3-5). Mutation of T91 to alanine made it impossible for *gcn5Δ* mutants to cope with any of the DNA damaging agents and resulted in lethality, even with an increased dosage of *RTS1* (Figure 3-7). The T91A mutation also sensitized the wild type strain specifically to UV-induced damage. Perhaps mutation of this residue interferes with nucleotide excision repair. Mutation of T91 to aspartate or glutamate resulted in lethality in both *gcn5Δ* and *rts1Δ* mutants (Figure 3-8). Therefore, dynamic modification of this residue appears required for viability in *gcn5Δ* and *rts1Δ* mutants. Even though there is evidence that the T91 homologue in humans is phosphorylated, this residue should be analyzed by mass spectrometry to be certain that it is phosphorylated in yeast (histone isolation protocol is proposed in Chapter 6). It was also hypothesized that there could be some crosstalk amongst modified residues as the combined mutations of H2B K82A T91A and H2B K88A T91A resulted in sickness in *gcn5Δ* mutants at the permissive temperature of 30°C and lethality at the restrictive temperature of 37°C (Figure 4-1). Another threonine residue along the lateral domain, H4 T80, was also found to be required for viability in *gcn5Δ* mutants and this genetic interaction appeared to be specific to *GCN5* as *sas3Δ* mutants bearing the T80A mutation were viable (Figure 3-9).

Figure 5-8. Important histone residues for the *GCN5-RTS1*

interaction. H2B T91 and H4 T80 (highlighted in red) have not been previously characterized in yeast but mass spectrometry data reveal that the homologous residues in humans are phosphorylated (Hornbeck et al. 2012). H2B K82, H2B K88, H4 K77, and H4 K79 (highlighted in yellow) are lysine residues close by. Modification of H4 K77 and H4 K79 has previously been demonstrated to be important for silencing at telomeric and rDNA loci in yeast but H2B K82 and H2B K88 are previously uncharacterized in yeast. In this work, the combined mutations of H2B K82A T91A and H2B K88A T91A conferred sickness to *gcn5Δ* mutants but not in wild type strains. Since all of these residues lie along the lateral domain of the nucleosome at the histone-DNA interface, they could be important for chromatin remodeling and overall structure. This image of the nucleosome (PDB 1id3) was generated using the Jmol program.



The H2B acetylation targets H2B K11 and K16 were not necessary for suppression by *RTS1* (Figure 4-3). However, mutation of lysine residues H4 K77 and H4 K79 which lie along the lateral domain in line with H2B T91, H4 T80, H2B K82, and H2B K88 (Figure 5-8) exacerbated the temperature sensitivity of *gcn5Δ* mutants (Figure 4-2). Since all of these residues are in the lateral domain, it could be hypothesized that chromatin remodeling plays an important role when *GCN5* is deleted, a result consistent with other data in the lab (Lafon, et al. in review). In Figure 5-7 it was proposed that mediator, SAGA, and Hsf1 all work together to regulate heat shock genes. The SWI/SNF chromatin remodeling complex localizes to heat shock factor mediated gene promoters and aids in nucleosome eviction. The histone residues investigated here could be important for proper nucleosome eviction by SWI/SNF and efficient transcription of stress response genes (Erkina et al. 2008). This potential interaction could be investigated by genetic dissection of *gcn5Δ* mutants with components of the SWI/SNF complex to see if *RTS1* overexpression can still suppress *gcn5Δ* temperature sensitivity in the absence of a fully functioning chromatin remodeling complex.

Future directions. Gcn5 and Rts1 are each members of a protein complex that has numerous vital functions in the cell. Narrowing down the point of interaction and further characterizing the functional relationship between *GCN5* and *RTS1* is going to be vital for our understanding cellular processes. In human cells, the PP2A complex functions as a tumor suppressor and there are

often mutations in the subunits that compose the holoenzyme (Eichhorn et al. 2009). Gcn5 and the SAGA complex also contribute to cancer progression by regulating c-Myc, an oncogene [reviewed in (Koutelou et al. 2010)]. Both SAGA and the PP2A complex contribute to cell-cycle progression and tumorigenesis so it is critical to further define their functional interaction. The experimental results presented in this work provide the foundation for future discoveries to ultimately contribute to our understanding of the inner workings of the cell.

Acknowledgements. The experiments presented in Figure 5-1 and 5-2A,B were performed by Bryce Mendelsohn.

Table 5-1. Summary of histone mutant phenotypes in *gcn5Δ*

Histone Mutant	<i>gcn5Δ</i>	
	Growth at 30°C	Growth at high temp with <i>RTS1</i>
H2A S121	+++	+++
H2B K11A	+++	+++
H2B K16A	+++	+++
H2B K82A	+++	+++
H2B K88A	+++	+++
H2B T91A [*]	+++	Lethal
H2B T91D	Lethal	NA
H2B T91E	Lethal	NA
H2BK89A	+++	+++
H2B K82A T91A ^{**}	++	Lethal
H2B K88A T91A ^{***}	++	Lethal
H3 S10A	+++	+++
H3 T45A	++	++
H3 S28A	+++	+++
H4 K77A	+++	++
H4 K79A	+++	+++
H4 T80A ^{****}	Lethal	NA

^{*} This mutation results in increased DNA damage sensitivity in *gcn5Δ*. This mutation modestly suppresses the temperature sensitivity of *rts1Δ* but results varied.

^{**} These mutations did not have any observable phenotype in the wild type *GCN5* strain

^{***} These mutations did not have any observable phenotype in the wild type *GCN5* strain

^{****} This mutation was examined in *sas3Δ* but was not observed to be lethal as it is with *gcn5Δ*

Chapter 6. Materials and methods

General yeast protocol and growth assays. Protocol for the handling of yeast and growth conditions can be found as described in (Sherman 1991).

Yeast cells were grown at 30°C unless otherwise stated. All dilution assays were normalized to 1.0 OD₆₀₀/mL and diluted 5 fold. Pictures of growth assays were taken after 3-7 days; most commonly at 4 days. Ultraviolet (UV) damage was assessed by subjecting YPD, or SC-dropout plates without the lid to 60-80 Joules /m² for 2 seconds. The Stratlinker instrument was disinfected with ethanol and pre-warmed by running twice at 100 J/m² before use. Strains used in the research can be found in Table 6-1.

Media preparation. Yeast media preparation can be found in (Sherman 1991). Camptothecin (CPT) plates for DNA damage analysis were prepared by adding between 1-30 µg/mL of CPT dissolved in DMSO to normal media concentrations prepared in pH 7.5 phosphate buffer. DMSO plates were prepared as a solvent control in mirror concentration of the CPT plates as described in (Nitiss and Wang 1988). Hydroxyurea (HU) plates for DNA damage analysis were prepared by adding filter sterilized HU (aqueous) to normal media for a final concentration of 0.1M.

Plasmid construction and recovery. This research extensively utilized the SHIMA plasmid library of histone mutants which can be found in Table 6-2 (Nakanishi et al. 2008). Initially, an attempt at isolating plasmids from yeast was performed by growing cells in 2 mL of dropout media, pelleting, resuspending in

lysis buffer (2% Triton X-100, 1% SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA), vortexing with glass beads, extracting with PCI, and precipitating with 3M NaOAc and ethanol. However, bacterial transformation failed to yield any colonies so all plasmids extracted from yeast were performed with the Zymoprep Yeast Plasmid Miniprep II kit from Zymo Research. Site-directed mutagenesis by PCR was performed using a protocol derived from (Wang and Malcolm 1999). Individual oligo amplification reactions (25 μ L) were prepared in PCR tubes (1 reaction for forward oligo, 1 reaction for reverse oligo): 2.5 μ L 10x Pfu Buffer, 1 μ L 1/50 dilution of miniprep plasmid DNA, 0.6 μ L 10 pm/ μ L primer, 2.5 μ L, 2.5 mM dNTPs, 1 μ L homemade Pfu (or 0.6 μ L commercial Pfu), 17.4 μ L deionized (MilliQ or MQ) water. Initial amplification of forward and reverse reactions separately: 94°C 1' then 3 cycles: 94°C 30'', 52°C 1', 65°C 18'. Combined 25 μ L forward/reverse reactions in 1 PCR tube, added 1 μ L Pfu then returned to PCR block for amplification: 94°C 1' then 18 cycles: 94°C 30'', 52°C 1', 65°C 2 x kb length of plasmid + 1-2'. 10 units of Dpn1 restriction enzyme were added (to destroy unmutated template DNA) and incubated at 37°C for 3 hours. 1-2 μ L of PCR product were transformed into DH5 α . DNA prepared from candidate colonies was sequenced commercially to verify the mutagenesis. Plasmids and oligo primers used in this research can be found in Table 6-2 and Table 6-3, respectively.

Simple transformation of yeast. Yeast were scraped from a plate with a flat toothpick into 20 μ L of mQ water. 5 μ L of miniprep plasmid DNA, 2.5 μ L

10mg/mL ssDNA, and 0.5 mL PLATE (81mL 50% PEG-4000, 1 mL 1M Tris-HCl pH 7.5, 10 mL 1M lithium acetate, 0.2 mL 0.5M EDTA, 9 mL mQ) solution were added and the solution vortexed. Transformation mixtures were incubated overnight at room temperature then 50 μ L was plated on selective media. The transformants used in this research can be found in Table 6-4. For the shuffling of histone genes to assay individual mutations, transformants were struck on 5-FOA to select for loss of the *URA3* marked plasmid bearing the wild type histone genes after being transformed with the plasmid bearing the mutant histone gene of interest. A single colony was taken from the 5FOA plate and the same colony was struck on both ura- medium and the appropriate selectable dropout plate to ensure that the plasmid bearing the histone mutation was retained and that the wild type *URA3* plasmid was cured.

Bacterial transformation. 100 μ L of calcium-competent *E. coli* DH5 α cells were added to 1-10 μ L DNA and incubated 30 minutes on ice. Cells were heat shocked at 42°C for 90 seconds then recovered on ice for 1-2 minutes. 1 mL of LB medium was added and the cells were incubated for 1-2 hours at 37°C before being plated on selective medium.

Yeast gene deletions. This research utilized the *Saccharomyces* Deletion Consortium (Winzeler et al. 1999) strain collection of *S. cerevisiae* gene deletions. For deletions made in the W303 background, the *kanMX* knockout construct was amplified by PCR from the stock strain and then transformed into LPY5 or LPY79. Gene deletions were confirmed by PCR molecular genotyping.

Genomic DNA preparation. Adapted from (Hoffman and Winston 1987).

Cells were grown overnight in 3 mL of YPD. 1.5 mL were spun down in a microfuge tube and the pellet resuspended in 0.5 mL of mQ water vortexed. Cells were spun again and the pellet was resuspended in 0.2 mL of breaking buffer (10 mM Tris (pH 8.0), 1 mM EDTA, 100 mM NaCl, 2% Triton-X 100, 1% SDS). Glass beads were added to the meniscus and then 0.2 mL of PCI (24 phenol: 23 chloroform: 1 isoamyl alcohol) were added. Cells were vortexed at maximum speed for 3 minutes. 0.3 mL of TE were added and the tubes were vortexed for 5 seconds. The tubes were spun at top speed in a microcentrifuge for 5 minutes. The top layer was then transferred to a new tube and 1 mL of ethanol was added and the tube inverted to mix. The tube was spun for 2 minutes at maximum speed, the supernatant removed and the pellet resuspended in 0.2 mL of TE. 5 μ L of 10 mg/mL RNase A were added and the tube was incubated at 37°C for 15 minutes. 0.2 mL of PCI were added and the tube was spun at maximum for 2 minutes. The top, aqueous layer was again transferred to a new tube and the DNA was precipitated by adding 20 μ L 3M NaOAc and 0.5 mL EtOH and incubating at -20°C for 1-2 hours. The DNA was pelleted at maximum speed in the cold for 10 minutes. The pellet was washed with 0.3 mL of 70% EtOH then spun for 2 minutes at maximum speed. The tube was inverted and the pellet air dried for 20-30 minutes before resuspending in 50 μ L TE. Genomic DNA was diluted 1:20 in water before use in PCR machine.

Bulk preparation of histones. Cells were prepared by growing 3 mL YPD starter cultures over night. 25 mL YPD cultures were inoculated early the next morning using the starter cultures and grown all day, The cells were then diluted back to OD₆₀₀ 0.005 in 500 mL YPD cultures and grown overnight. Cells were harvested at an OD₆₀₀ 1 and spun at 5000 rpm for 5 min in a Sorvall F12-6X500 LEX rotor. The cells were washed with 20 mL of mQ water and then transferred to pre-weighed 50 mL Oakridge tubes and spun at 13000 rpm in a Sorvall SS-34 rotor for 10 min. The pellet was weighed and then resuspended in 1/20 the starting volume (25 mL) of Buffer A (50mM Tris pH 7.5, 30mM DTT) and incubated for 15 min at 30°C while shaking. Cells were spun for 10 min at 13000 rpm (SS-34) and the pellet washed with 1/10 the starting volume (50 mL) of Buffer S (1.2M sorbitol, 20mM HEPES pH 7.4). The cells were spun for 10 min at 13000 rpm (SS-34) then resuspended in 1/10 the starting volume (50 mL) Buffer S with 2 mg of zymolyase per gram of the pellet. The cells were spheroplasted by shaking at 30°C for approximately 1 hour with the cells being examined under the microscope every 20 minutes to observe a change in the appearance of the refractile properties of the cell. The tube containing the cells was placed on ice and 1/10 the starting volume (50 mL) of ice-cold Buffer B (1.2M sorbitol, 20mM PIPES pH 6.8, 1mM MgCl₂) were added to the sample. Cells were spun for 10 min at 5000 rpm at 4°C in Oakridge tubes (SS-34). The samples were moved to the 4°C cold room where the supernatant was removed and the pellet was resuspended in 5 mL ice-cold NIB buffer (250mM sucrose, 60mM KCl, 14mM NaCl, 5mM MgCl, 1mM CaCl₂, 15mM MES pH 6.6, 0.8% TritonX-100, 1mM

PMSF, 1mM NaF, 1mM sodium orthovanadate, 2nm okadaic acid). The cells were placed on ice for 20 min and then spun at 6000 rpm for 5 min (SS-34). NIB was added, the cells incubated on ice and then spun at 6000 rpm for a total of 3 times. The pellet was then resuspended in 5 mL ice cold Wash Buffer A (10mM Tris pH 8.0, 30 mM sodium butyrate, 75 mM NaCl, 0.5% NP-40, 1 mM NaF, 1mM PMSF, 1mM sodium orthovanadate, 2nm okadaic acid), incubated on ice for 15 min, then spun at 6000 rpm (SS-34) for 5 min. Wash Buffer A was added, the cells incubated on ice and then spun at 6000 rpm for a total of 3 times. The pellet was then resuspended in 5 mL ice cold Wash Buffer B (10mM Tris pH 8.0, 30 mM sodium butyrate, 400mM NaCl, 1mM NaF, 1mM PMSF, 1mM sodium orthovanadate, 2nm okadaic acid), incubated on ice for 20 min, then spun at 6000 rpm (SS-34) for 5 min. Wash Buffer B was added, the cells incubated on ice and then spun at 6000 rpm for a total of 3 times. Acid-soluble proteins including the histones were then extracted using 1 mL of ice cold 0.25N HCl and transferred to a 1.5 mL Eppendorf tube. The sample was incubated on ice for 1 hour and vortexed occasionally. The sample was spun at 10,000 rpm in a microcentrifuge for 10 min in the cold. The supernatant was placed in a new 1.5 mL Eppendorf tube. 100% TCA was added for a final concentration of 20% and the sample was incubated overnight at 4°C. The sample was spun for 30 min at 12,000 rpm in a microcentrifuge in the cold and the pellet containing histones was washed with 500 µL cold acidified acetone (acetone + 0.1% HCl) and then spun in a microcentrifuge in the cold for 10 min at 12,000 rpm. The pellet was washed with 500 µL of cold 100% acetone and spun again in the cold for 10 min

at 12,000 rpm. The acetone was removed without disturbing the pellet and the pellet was air dried while on ice. The histone pellet was resuspended in 250 μ L 10 mM Tris pH 8.0 and stored at -20°C. All buffers were made the day of use for buffer NIB, Wash Buffer A, and Wash Buffer B, the NaF, PMSF, sodium orthovanadate, and okadaic acid were added immediately before use.

Table 6-1. Strains used in this research

Strain	Genotype	Source
LPY5	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL</i>	Pillus Lab
LPY1552	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11/his3-11 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 GAL/GAL</i>	Pillus Lab
LPY10182	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::kanMX</i>	Pillus Lab
LPY11437	<i>MATα ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::kanMX sasΔ::HIS3 ura3::C357Y, P375A – URA3</i>	Pillus Lab
LPY12231	<i>MATα ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL hht1-hhf1Δ::kanMX hht2-hhf2Δ::kanMX hta2-htb2Δ::HPH + pLP2212</i>	Pillus Lab
LPY13435	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::kanMX</i>	Pillus Lab
LPY13846	<i>MATα ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::kanMX snf1Δ::TRP1</i>	Pillus Lab
LPY14461	<i>MATα ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL hht1-hhf1Δ::kanMX hta1-htb1Δ::natMX hta2-htb2Δ::HPH + pLP2212</i>	Pillus Lab
LPY14642	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL pph21Δ::kanMX</i>	Pillus Lab
LPY14644	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::kanMX pph21Δ::kanMX</i>	Pillus Lab
LPY14653	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL rts1Δ::kanMX</i>	This study
LPY14690	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL pph22Δ::kanMX</i>	Pillus Lab
LPY14692	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::kanMX pph22Δ::kanMX</i>	Pillus Lab
LPY15178	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::natMX rts1Δ::kanMX + pLP1640</i>	Pillus Lab
LPY15180	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11/his3-11 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 GAL/GAL gcn5Δ::natMX/gcn5Δ::natMX</i>	Pillus Lab

Table 6-1 – Strains used in this research (continued)

Strain	Genotype	Source
LPY15213	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL psi+ Kin4-3HA:TRP1, cdc55Δ::kanMX6</i>	A. Amon
LPY15265	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11/his3-11 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 GAL/GAL cdc55Δ::kanMX6/CDC55 gcn5Δ::natMX/GCN5 Kin4-3HA:TRP1/KIN4</i>	Pillus Lab
LPY15296	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 ura3-1 GAL cdc55Δ::kanMX6 gcn5Δ::natMX +pLP1640</i>	Pillus Lab
LPY15297	<i>MATα ade2-1 can1-100 his3-11 leu2-3,112 ura3-1 GAL cdc55Δ::kanMX6 +pLP1640</i>	Pillus Lab
LPY15328	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11/his3-11 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 GAL/GAL gcn5Δ::natMX/GCN5 tpd3Δ::kanMX/TPD3</i>	Pillus Lab
LPY15416	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::natMX tpd3Δ::kanMX +pLP1640</i>	Pillus Lab
LPY15417	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL tpd3Δ::kanMX +pLP1640</i>	Pillus Lab
LPY15460	<i>MATa/MATα ade2-1/ade2-1 can1-100/can1-100 his3-11/his3-11 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 GAL/GAL gcn5Δ::natMX/GCN5</i>	Pillus Lab
LPY16290	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL hht1-hhf1Δ::kanMX hht2-hhf2Δ::kanMX hta2-htb2Δ::HPH gcn5Δ::kanMX + pLP2212</i>	This study
LPY16346	<i>MATα ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL hht1-hhf1Δ::kanMX hta1-htb1Δ::NAT hta2-htb2Δ::HPH rts1Δ::kanMX pLP2212</i>	This study
LPY16432	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL hht1-hhf1Δ::kanMX hht2-hhf2Δ::kanMX hta2-htb2Δ::HPH sas3Δ::kanMX + pLP2212</i>	This study
LPY16434	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL hht1-hhf1Δ::kanMX hta1-htb1Δ::natMX hta2-htb2Δ::HPH gcn5Δ::kanMX + pLP2212</i>	This study

Table 6-1. Strains used in this research (continued)

Strain	Genotype	Source
LPY16456	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL sch9Δ::kanMX</i>	This study
LPY16914	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 spt20Δ::HIS3</i>	David Stillman
LPY16980	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL rts1Δ::kanMX</i>	This study
LPY17138	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::natMX sch9Δ::kanMX + pLP1640</i>	This study
LPY17139	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::natMX sch9Δ::kanMX</i>	This study
LPY17140	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::natMX sch9Δ::kanMX</i>	This study
LPY17141	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL gcn5Δ::natMX sch9Δ::kanMX</i>	This study
LPY17370	<i>MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL ahc1Δ::kanMX</i>	This study
LPY17484	<i>MAT ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL rts1Δ::kanMX spt20Δ::HIS3 + pLP2462</i>	This study
LPY17485	<i>MAT ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL rts1Δ::kanMX spt20Δ::HIS3 + pLP2462</i>	This study
LPY17486	<i>MAT ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL rts1Δ::kanMX spt20Δ::HIS3 + pLP2462</i>	This study
LPY17487	<i>MAT ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 GAL rts1Δ::kanMX spt20Δ::HIS3 + pLP2462</i>	This study

Table 6-2. Plasmids used in this research

pLP Number	Gene	Marker/Copy Number	Source
61	Vector	<i>TRP1/CEN</i>	Pillus Lab
126	Vector	<i>URA3/CEN</i>	Pillus Lab
135	Vector	<i>LEU2/2μ</i>	Pillus Lab
136	Vector	<i>URA3/2μ</i>	Pillus Lab
645	<i>SAS3</i>	<i>LEU2/2μ</i>	Pillus Lab
1518	<i>GCN5</i>	<i>TRP1/CEN</i>	L. Howe
1521	<i>GCN5-KQL</i>	<i>TRP1/CEN</i>	Wang et al. 1998
1524	<i>GCN5</i>	<i>LEU2/2μ</i>	Pillus Lab
1640	<i>GCN5</i>	<i>URA3/CEN</i>	Pillus Lab
1641	<i>GCN5</i>	<i>URA3/2μ</i>	Pillus Lab
2131	<i>HTA1 HTB1</i>	<i>HIS3/CEN</i>	K. Ingvarsdottir
2196	<i>RTS1</i>	<i>LEU2/2μ</i>	M. Smith
2197	<i>RTS1</i>	<i>LEU2/2μ</i>	Pillus Lab
2212	<i>HTA1 HTB1 HHT2 HHF2</i>	<i>URA3/CEN</i>	Mitch Smith
2330	<i>CDC55</i>	<i>LEU2/2μ</i>	Pillus Lab
2433	<i>HHT2 HHF2</i>	<i>TRP1/CEN</i>	Shima Library
2436	<i>hht2-S10A HHF2</i>	<i>TRP1/CEN</i>	Shima Library
2439	<i>hht2-S28A HHF2</i>	<i>TRP1/CEN</i>	Shima Library
2462	<i>RTS1</i>	<i>URA3/2μ</i>	Pillus Lab
2481	<i>HTA1 htb1-S90A-FLAG</i>	<i>HIS3/CEN</i>	Shima Library
2482	<i>HTA1 htb1-T91A-FLAG</i>	<i>HIS3/CEN</i>	Shima Library
2492	<i>HTA1 HTB1-FLAG</i>	<i>HIS3/CEN</i>	Shima Library
2501	<i>hta-S121A HTB-FLAG</i>	<i>HIS3/CEN</i>	Shima Library

Table 6-2. Plasmids used in this research (continued)

pLP Number	Gene	Marker/Copy Number	Source
2515	<i>hht2-T45A HHF2</i>	<i>TRP1/CEN</i>	Shima Library
2601	<i>HTA1 htb1-K88A-FLAG</i>	<i>HIS3/CEN</i>	Shima Library
2602	<i>HTA1 htb1-K89A-FLAG</i>	<i>HIS3/CEN</i>	Shima Library
2603	<i>HHT2 hhf2-K79A</i>	<i>TRP1/CEN</i>	Shima Library
2612	<i>HHT2 hhf2-K77A</i>	<i>TRP1/CEN</i>	Shima Library
2613	<i>HTA1 htb1-K82A-FLAG</i>	<i>HIS3/CEN</i>	Shima Library
2646	<i>HHT2 HHF2</i> ; <i>SIW14</i> removed	<i>TRP1/CEN</i>	Shima Library; this study
2647	<i>HHT2 hhf2-T80A</i> ; <i>SIW14</i> removed	<i>TRP1/CEN</i>	Shima Library; this study
2669	<i>HTA1 htb1-K11A-FLAG</i>	<i>HIS3/CEN</i>	Shima Library
2670	<i>HTA1 htb1-K16A-FLAG</i>	<i>HIS3/CEN</i>	Shima Library
2688	<i>HTA1 htb1-K88A T91A-FLAG</i>	<i>HIS3/CEN</i>	Shima Library; this study
2689	<i>HTA1 htb1-T91E-FLAG</i>	<i>HIS3/CEN</i>	Shima Library; this study
2690	<i>HTA1 htb1- K82A T91A-FLAG</i>	<i>HIS3/CEN</i>	Shima Library; this study
2714	<i>HTA1 htb1-T91A</i> in pRS313	<i>HIS3/CEN</i>	K. Ingvarsdottir; this study
2770	<i>HTA1 htb1-T91D-FLAG</i>	<i>HIS3/CEN</i>	Shima Library; this study
2802	<i>SPT20</i>	<i>LEU2/2μ</i>	G. Prelich. Yeast Genomic Tiling Library

Table 6-3. Primers used in this research

OLP Number	Gene	Use	Sequence
1298	<i>HTB1</i>	Sequencing	GAT CAT CTC AGA TGG TCA G
1586	<i>SCH9</i>	Knockout	GCG CCA GTT CCC GCC TGC
1587	<i>SCH9</i>	Knockout	CGC GCA TCG ATG AGC CCT GCC
1588	<i>HHT2</i>	Sequencing	CGA ATA ACA ACA GCC CAG GCG CG
1589	<i>HHF2</i>	Sequencing	CGC GAG AGA GCA CAA CAC GC
1616	<i>SIW14</i>	Mutagenesis	GCA GAC AAT TCC CGC CAG ATG GCA GGG CGC TTG ATC AGC AGT TC
1617	<i>SIW14</i>	Mutagenesis	GAA CTG CTG ATC AAG CGC CCT GCC ATC TGG CGG GAA TTG TCT GC
1643	<i>HTB1</i>	Mutagenesis	CGT ATA ACA AGA AGT CTG AGA TCT CTG CTA GAG
1644	<i>HTB1</i>	Mutagenesis	CTC TAG CAG AGA TCT CAG ACT TCT TGT TAT ACG
1645	<i>HTB1</i>	Mutagenesis	GCT ACT GAA GCT TCT GCA TTG GCT GCG TAT AAC
1646	<i>HTB1</i>	Mutagenesis	GTT ATA CGC AGC CAA TGC AGA AGC TTC AGT AG
1647	<i>HTB1</i>	Mutagenesis	GGC TGC GTA TAA CGC GAA GTC TGC TAT C
1648	<i>HTB1</i>	Mutagenesis	GAT AGC AGA CTT CGC GTT ATA CGC AGC C
1685	<i>HTB1</i>	Mutagenesis	CGT ATA ACA AGA AGT CTG CTA TCT CTG CTA GAG
1686	<i>HTB1</i>	Mutagenesis	CTC TAG CAG AGA TAG CAG ACT TCT TGT TAT ACG
1754	<i>HTB1</i>	Mutagenesis	GCT GCG TAT AAC AAG AAG TCT GAC ATC TCT GCT AGA G
1755	<i>HTB1</i>	Mutagenesis	CTC TAG CAG AGA TGT CAG ACT TCT TGT TAT ACG CAG C

Table 6-3. Primers used in this research (continued)

OLP Number	Gene	Use	Sequence
1789	<i>AHC1</i>	Knockout	GCC ACT GTG CAT AGC CG
1790	<i>AHC1</i>	Knockout	GGG TAC GTC TAT GGC

Table 6-4 - Transformants used in this research

Strain	Strain + plasmid	Source
13603	LPY13486 + pLP1524	This study
13604	LPY13486 + pLP135	This study
13605	LPY13486 + pLP2196	This study
16635	LPY14461 + pLP2670 + pLP136	This study
16636	LPY14461 + pLP2670 + pLP2462	This study
16637	LPY14461 + pLP2669 + pLP136	This study
16638	LPY14461 + pLP2669 + pLP2462	This study
16639	LPY14461 + pLP2492 + pLP136	This study
16640	LPY14461 + pLP2492 + pLP2462	This study
16641	LPY14461 + pLP2482 + pLP136	This study
16642	LPY14461 + pLP2482 + pLP2462	This study
16645	LPY16434 + pLP2688 + pLP2462	This study
16646	LPY16434 + pLP2492 + pLP136	This study
16647	LPY16434 + pLP2492 + pLP2462	This study
16648	LPY16434 + pLP2482 + pLP136	This study
16649	LPY16434 + pLP2482 + pLP2462	This study
16650	LPY16434 + pLP2690 + pLP136	This study
16651	LPY16434 + pLP2690 + pLP2462	This study
16658	LPY16346 + pLP2482 + pLP136	This study
16659	LPY16346 + pLP2482 + pLP2462	This study
16660	LPY16346 + pLP2670 + pLP136	This study
16661	LPY16346 + pLP2670 + pLP2462	This study
16663	LPY16346 + pLP2492 + pLP136	This study
16664	LPY16346 + pLP2492 + pLP2462	This study
16665	LPY16346 + pLP2669 + pLP136	This study
16666	LPY16346 + pLP2669 + pLP2462	This study
16667	LPY16434 + pLP2669 + pLP136	This study
16668	LPY16434 + pLP2669 + pLP2462	This study
16669	LPY16434 + pLP2670 + pLP136	This study
16670	LPY16434 + pLP2670 + pLP2462	This study
16671	LPY16434 + pLP2601 + pLP136	This study
16672	LPY16434 + pLP2601 + pLP2462	This study
16673	LPY16434 + pLP2613 + pLP136	This study
16674	LPY16434 + pLP2613 + pLP2462	This study
16675	LPY16432 + pLP2647 + pJH33	This study
16676	LPY16432 + pLP2646 + pJH33	This study
16677	LPY12231 + pLP2646 + pJH33	This study
16678	LPY12231 + pLP2647 + pJH33	This study
16679	LPY16290 + pLP2647 + pJH33	This study
16680	LPY16290 + pLP2646 + pJH33	This study
16951	LPY16434 + pLP2689	This study
16952	LPY16434 + pLP2482	This study
16953	LPY16434 + pLP2492	This study
16954	LPY14461 + pLP2689	This study
16955	LPY14461 + pLP2482	This study

Table 6-4. Transformants used in this research (continued)

Strain	Strain + plasmid	Source
16956	LPY14461 + pLP2492	This study
16958	LPY16434 + pLP2481 + pLP136	This study
16959	LPY16434 + pLP2481 + pLP2462	This study
16961	LPY16434 + pLP2688 + pLP136	This study
17021	LPY16434 + pLP2131 + pLP136	This study
17022	LPY16434 + pLP2131 + pLP2462	This study
17023	LPY16434 + pLP2714 + pLP136	This study
17024	LPY16434 + pLP2714 + pLP2462	This study
17135	LPY14653 + pLP136	This study
17136	LPY14653 + pLP1641	This study
17137	LPY14653 + pLP2462	This study
17480	LPY16290 + pLP2515 + pLP136	This study
17481	LPY16290 + pLP2515 + pLP2462	This study
17482	LPY16290 + pLP2439 + pLP136	This study
17483	LPY16290 + pLP2439 + pLP2462	This study
17572	LPY16434 + pLP2501 + pLP136	This study
17573	LPY16434 + pLP2501 + pLP2462	This study
17574	LPY16290 + pLP2433 + pLP136	This study
17575	LPY16290 + pLP2433 + pLP2462	This study
17576	LPY16290 + pLP2436 + pLP136	This study
17577	LPY16290 + pLP2436 + pLP2462	This study
17578	LPY16290 + pLP2612 + pLP136	This study
17579	LPY16290 + pLP2612 + pLP2462	This study
17580	LPY16290 + pLP2603 + pLP136	This study
17581	LPY16290 + pLP2603 + pLP2462	This study
17582	LPY14642 + pLP136	This study
17583	LPY14644 + pLP136	This study
17584	LPY14644 + pLP2462	This study
17585	LPY14644 + pLP1641	This study
17586	LPY14690 + pLP136	This study
17587	LPY14692 + pLP136	This study
17588	LPY14692 + pLP2462	This study
17589	LPY14692 + pLP1641	This study
17590	LPY13435 + pLP136	This study
17649	LPY5 + pLP136	This study
17650	LPY5 + pLP2462	This study
17651	LPY10182 + pLP136	This study
17652	LPY10182 + pLP2462	This study

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