GENETIC AND GENOMIC STUDIES OF HISTONE H3 METHYLATION AND ACETYLATION

By

YI JIN

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation/thesis of YI JIN find it satisfactory and recommend that it be accepted.

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GENETIC AND GENOMIC STUDIES OF HISTONE H3

METHYLATION AND ACETYLATION

ABSTRACT

By YI JIN, Ph.D.
Washington State University
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Chair: John Wyrick

Histone H3 methylation plays important roles in gene transcription regulation. A large body of literature has been devoted to investigating the functions of histone H3 methylation at individual residues. In contrast, very little is known about how combinations of methylated lysine residues act in concert to regulate genome-wide transcription. We have systematically mutated all three methylated histone lysine residues in *Saccharomyces cerevisiae*, and discovered that simultaneous mutation of H3K4, H3K36, and H3K79 to arginine (H3 K4,36,79R) is lethal. The histone H3 K4,36,79R mutant leads to a progressive transcription defect that initiates in telomere regions and later spreads further into the chromosome. In addition, this mutation results in a delay of the cell cycle progression. These defects are partially mediated by the increased binding of the SIR silencing complex to euchromatin regions adjacent to telomeres in the H3 K4,36,79R mutant. Intriguingly, the presence of intact histone lysine methyltransferase proteins, but not methylation activity, is required for the lethality of the H3 K4,36,79R mutant. These and other data suggest that the three histone H3 methylated lysine residues in concert are critical to transcription regulation in yeast.

Besides methylated lysine residues, acetylated lysine residues in histone H3 are also

integral to yeast gene expression and other biological processes. Multiple lines of evidence suggested that histone H3 acetylated and methylated lysine residues might functionally interact to regulate gene transcription. We tested this possible genetic interaction between histone mutants by simultaneously mutating acetylated or methylated lysine residues. The data indicate that various growth defects of the histone H3 methylated lysine mutant are rescued by the H3 acetylated lysine mutant. In contrast, histone H3 acetylated and methylated lysine residues generally make independent contributions to yeast gene transcription. Furthermore, the gene expression changes are preferentially associated with genomic regions in which histone H3 lysine residues are hypoacetylated and hypomethylated. Finally, our results reveal that acetylated and methylated lysine residues have different impacts on the binding of the SIR silencing complex to telomere heterochromatin regions, in turn regulating telomeric silencing.

Taken together, completion of the work described in this thesis provides novel and valuable information regarding to the combinatorial functions of histone H3 methylation and acetylation.

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INTRODUCTION: HISTONE MODIFICATIONS AND THEIR FUNCTIONS

Overview

Eukaryotic DNA is packaged with DNA-packaging proteins into chromatin, in order to allow the large eukaryotic genome to fit into a relatively small nuclear compartment. The main DNA-packaging proteins are the core histones: H2A, H2B, H3, and H4. The octameric histone proteins complex with 147 base pairs (bp) of DNA into nucleosome (Luger et al., 1997; Richmond and Davey, 2003), which is the principal repeating subunit of eukaryotic chromatin. The compaction of DNA with histone proteins into nucleosomes and into further higher order chromatin structures has profound effects on gene transcription and other diverse DNA-templated processes, including DNA replication, DNA repair, and chromosome segregation (Berger, 2002, 2007; Cosgrove and Wolberger, 2005; Kouzarides, 2007; Peterson and Laniel, 2004; Zhang and Reinberg, 2001).

One of the most important factors involved in organizing chromatin structure is post-translational modification of the histone proteins. Multiple modifications have been found on each core histone, including acetylation, methylation, ubiquitination, phosphorylation, ADP-ribosylation, and sumoylation. Different histone modifications or combinations of histone modifications can dramatically affect chromatin structure and lead to varying outcomes. For example, certain pattern of increased histone acetylation at a gene promoter region activates gene transcription. One attractive hypothesis, the histone code hypothesis, has been proposed as a model to describe how complex patterns of histone modifications determine biological outcomes, especially transcriptional outcomes (Jenuwein and Allis, 2001; Turner, 2002). The model suggested that multiple histone modifications might act in a combinatorial manner to regulate the association of

chromatin-binding proteins, leading to distinct biological readouts. Although a large body of scientific literature has supported the model, extensive studies are still required to further decipher that how all the possible combinatorial histone modifications affect gene regulation. In this introduction, I will briefly review the composition of eukaryotic chromatin, histone post-translational modifications and their roles in DNA-templated processes, especially in gene transcription regulation. Later chapters in this thesis describe work to characterize the roles of combinational histone H3 methylation and acetylation in regulating expression of the yeast genome and other important cellular processes.

Section I: Chromatin composition and histone post-translational modifications

Chromatin

In eukaryotic cells, DNA is compacted with histones and other DNA-packaging proteins to form chromatin. Chromatin fibers start with the simple "beads-on-a-string" structure of nucleosome arrays. This ~10 nm thick fiber is then folded into more condensed ~30 nm chromatin. With further condensation, chromatin fibers eventually form a highly compacted metaphase chromosome structure. Originally, chromatin was mainly thought to have a structural role, which permits the enormous length of DNA to fit into the relatively small nuclear volume. It was also believed that chromatin serves as a general repressor preventing DNA metabolic enzymes, such as RNA polymerase II (RNAPII), to access the DNA template (Grunstein, 1990). However, studies during the last several years suggested that chromatin structure is dynamic, fluctuating between condensed and loosed conformations. The dynamic changes of chromatin facilitate the

organization of eukaryotic chromosomes into distinct domains, such as transcriptionally active euchromatin and transcriptionally repressive heterochromatin. Euchromatin generally refers to regions of the genome that are less condensed outside of mitosis and mostly contains active genes (Kurdistani and Grunstein, 2003). In contrast, heterochromatin, which is constitutively condensed chromatin, is generally transcriptionally silent and replicates in late S phase (Kurdistani and Grunstein, 2003). These distinct chromatin domains profoundly influence the accessibility of all DNA metabolic processes. It has also been shown that eukaryotic chromatin is modified by chromatin-modifying enzymes. These covalent and non-covalent modifications alter local chromatin structure, enabling the dynamic changes of chromatin (More details will be discussed later in the introduction) (Aalfs and Kingston, 2000; Grunstein, 1997; Jenuwein and Allis, 2001; Li et al., 2007a; Strahl and Allis, 2000; Struhl, 1998).

The Nucleosome

Chromatin is composed of a polymer of nucleosomes, which are the fundamental repeating unit of DNA compaction (Kornberg and Lorch, 1999). Each nucleosome is composed of 147 bp of DNA wrapped 1.65 times around an octamer of core histone proteins H2A, H2B, H3, and H4 (Luger et al., 1997; Richmond and Davey, 2003). Crystal structure studies of the nucleosome revealed that the histone octamer contains four histone heterodimers, two each of H3/H4 and H2A/H2B (Figure 1-1) (Luger et al., 1997; White et al., 2001). The two H3 and H4 heterodimers form a central heterotetramer (Luger et al., 1997). Each side of the heterotetramer is flanked by a heterodimer of H2A and H2B (Luger et al., 1997). The DNA is tightly wrapped around

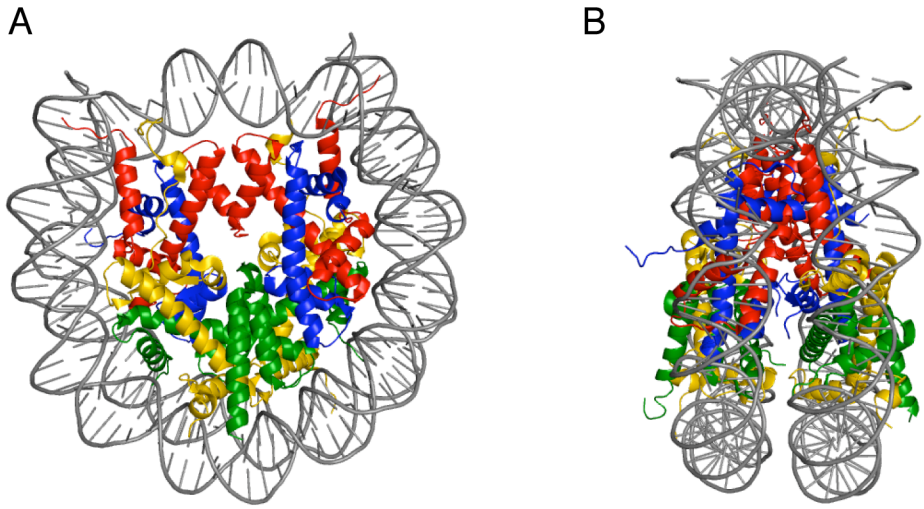

FIGURE 1-1: Crystal structure of the nucleosome core particle from *Saccharomyces cerevisiae*.

(**A**) The crystal structure of the yeast nucleosome core particle, viewed down the superhelical axis. Histone H2A is depicted in yellow color; histone H2B is depicted in green color; histone H3 is depicted in red color; and histone H4 is depicted in blue color. The DNA double strands are depicted in gray color. **(B)** Side view of the yeast nucleosome core particle. The protein structure data of yeast nucleosome core particle (Protein Data Bank accession number 1ID3) is from White *et al*. (White et al., 2001).

the histone octamer (Davey et al., 2002). It is important to note that the N-terminal tail domains of core histone proteins are largely unstructured and protrude from the nucleosome core particle. These unstructured histone tails provide a large number of residues for potential covalent post-translational modifications.

Adjacent nucleosomes are interconnected by a stretch of free DNA termed linker DNA, which varies in length depending on the species and tissue. Linker histones, such as histone H1 and its isoforms, are usually associated with linker DNA. Previous studies suggested that linker histones, as well as core histone N-terminal tails, play a key role in promoting the coiling of the long arrays of nucleosomes into higher order chromatin structure (~30 nm chromatin fibers) (Belikov and Karpov, 1998; Thomas, 1999; Vignali and Workman, 1998; Widom, 1998).

Histone proteins

Histones comprise the major protein component of chromatin. The four histone proteins H2A, H2B, H3, and H4 are core histones, which assemble to form histone octamers serving as scaffolding for nucleosomes. Histone H1 or linker histone associates with linker DNA between nucleosomes and functions in compacting nucleosomes into higher chromatin structures. The expression of most histone proteins is tightly and coordinately regulated during the cell cycle, and the deposition process of histones onto DNA is generally associated with DNA replication (Hereford et al., 1981; Sarma and Reinberg, 2005). Indeed, histone proteins are largely synthesized in S phase of the cell cycle, and subsequently incorporated with the newly synthesized DNA into chromatin (Hereford et al., 1981; Spellman et al., 1998). However, low levels of histone deposition

independent of DNA replication were also observed (Jackson, 1990; Kimura and Cook, 2001).

Histones are between 100 to 200 amino acids in length and are basic proteins, which are highly enriched with lysine and arginine residues. These positively charged residues greatly promote histones binding to DNA. Core histone proteins mainly display three distinct structural domains, including the histone fold domains, the extensions of the fold domains, and the histone tail domains (Luger and Richmond, 1998). The histone fold domains are highly structured and conserved through eukaryotic species (Luger et al., 1997). The formation of the histone octamer is dependent on the interaction within the fold domains of different histones (Luger et al., 1997; Placek and Gloss, 2002). Unlike the histone fold domains, the histone tail domains found at the N- and/or C-termini of core histone proteins are largely unstructured, and the amino acid sequences are less conserved. The N-terminal tails of histones extend beyond the nucleosome core particle and are available to potential post-translational modifications, which will be discussed in the subsequent section of this introduction. It is worthy of note that although those tail domains are generally unstructured they are able to adopt partial secondary structures (Baneres et al., 1997; White et al., 2001). Previous studies have shown that the histone N-terminal tails are also involved in chromatin fiber condensation (Carruthers and Hansen, 2000) and internucleosomal histone-histone interactions (Luger et al., 1997). Studies of histone N-terminal tail deletions suggested that they have many different, yet partially redundant, functions (Grunstein, 1997; Grunstein et al., 1995; Hansen et al., 1998). It is believed that histone tails interact with a variety of different protein regulators and perform a large number of diverse regulatory functions.

Unlike higher eukaryotic species having several histone-encoding genes for each histone protein, there are only two copies of each histone gene, which code for nearly identical proteins in yeast, *Saccharomyces cerevisiae* (Hereford et al., 1979; Smith and Andresson, 1983; Smith and Murray, 1983). For example, histone H3 is encoded by two genes designated *HHT1* and *HHT2*, and H4 is encoded by *HHF1* and *HHF2*. *HHT1* and *HHF1* are located in a contiguous pair sharing a divergent promoter on chromosome II; *HHT2* and *HHF2* are in another contiguous pair on chromosome XIV. Since each gene pair shares a divergent promoter, the two histone genes in that particular pair are co-regulated and co-expressed (Spellman et al., 1998). Intriguingly, although the transcription level of the *HHT2-HHF2* gene pair is five to seven times higher than the *HHT1-HHF1* gene pair in yeast cells, yeast only needs one gene pair to survive and deletion of either gene pair causes very little, if any, growth phenotypes (Cross and Smith, 1988; Smith and Stirling, 1988). Similarly, two pairs of histone H2A and H2B genes are organized on Chromosome II and IV, respectively. Although only one gene pair of histone H2A and H2B is required for yeast viability, deletion of one of two loci causes distinct effects on cell growth and chromatin structure (Clark-Adams et al., 1988; Norris et al., 1988; Norris and Osley, 1987). Together, the exact biological difference between two almost identical histone gene copies is still unclear.

In addition to core histone proteins in eukaryotic cells, there are a small number of histone variants, which differ in their amino acid sequences from core histone proteins and have specialized functions (Brown, 2001; Kamakaka and Biggins, 2005; Malik and Henikoff, 2003; Sarma and Reinberg, 2005). Histone variants are expressed from orphan genes and their expression and deposition are not as strictly regulated during the cell

cycle like core histone proteins are (Kamakaka and Biggins, 2005; Sarma and Reinberg, 2005). Indeed, histone variants are expressed throughout the cell cycle and incorporated independent of DNA replication (Kamakaka and Biggins, 2005). Different histone variants can contribute to unique nucleosome architecture, regulating gene transcription, gene silencing, DNA replication, and DNA recombination (Brown, 2001; Kamakaka and Biggins, 2005; Sarma and Reinberg, 2005). For example, histone H3.3 is a histone H3 variant, which is generally deposited into chromatin during interphase of cell cycle (Ahmad and Henikoff, 2002b; Tagami et al., 2004). It shares a high sequence similarity with histone H3 and only differs by a few amino acids. Previous studies suggested that H3.3 is preferentially localized near and within transcribed active genes and upstream regulatory regions of certain genes (Chow et al., 2005; Jin and Felsenfeld, 2006; Mito et al., 2005; Schwartz and Ahmad, 2005; Wirbelauer et al., 2005). It has been proposed that H3.3 might play important roles in maintaining transcriptionally active chromatin state (Henikoff et al., 2004; Jin and Felsenfeld, 2007).

H2A.Z is a well-studied histone H2A variant, found through different eukaryotic species. The sequence of H2A.Z of different species is relatively conserved, which reflects its unique biological function. A crystal structure study of nucleosome core particles containing H2A.Z revealed significant differences from the normal nucleosome with histone H2A. In yeast, studies indicated that Htz1, a yeast orthologue of H2A.Z, preferentially localized within actively transcribed euchromatin regions, which flank heterochromatin regions that are mainly silenced by Silencing Information Regulator (SIR) complex. As a deletion of Htz1 causes the spreading of heterochromatin, it suggests that Htz1 aids in preventing the spreading of the SIR silencing complex and

establishing the boundary between heterochromatin and euchromatin regions (Meneghini et al., 2003). In mammals, depletion of H2A.Z results in early embryonic lethality, suggesting its functional role in early mammalian development (Faast et al., 2001).

Histone post-translational modifications

Histone proteins are targeted by a large number of post-translational modifications by distinct histone-modifying enzymes. These modifications include acetylation of lysines, methylation of lysines and arginines, ubiquitination of lysines, phosphorylation of serines and threonines, sumoylation of lysines, ribosylation of glutamates, and biotinylation of lysines, as well as less familiar deimination of arginines (Kouzarides, 2007). The majority of these post-translational modifications are on the histone N-terminal tail domains. However, more modifications have been discovered within the fold domains of histone proteins in recent years (Cosgrove et al., 2004; Mersfelder and Parthun, 2006). Although each histone protein has multiple potential modification residues, a single histone protein is rarely modified at every potential residue (Boyne et al., 2006; Smith et al., 2003; Thomas et al., 2006). Most of the histone post-translational modifications are dynamic and rapidly changing. More intriguingly, cross-talk between post-translational modifications have also been discovered. For example, methylation of histone H3 lysine 4 and 79 depends on ubiquitination of H2B lysine 123 (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002b; Sun and Allis, 2002). Histone post-translational modifications are able to alter and control nucleosome properties and chromatin structures, which in turn yields various biological outcomes (Table 1-1). Simply speaking, the functional roles of histone modifications can be divided

Table 1-1: Summary of current knowledge of histone post-translational modifications[a]

Histone proteins	Modifications	Potential residues	Major modifying enzymes	Functions regulated
H2A	Acetylation	Lys 4	Esa1	Transcription activation
		Lys 5	CBP/P300	Transcription activation
		Lys 7	Esa1	Transcription activation
	Phosphorylation	Ser 1	MSK1	Transcription repression; Mitosis regulation
		Thr 119	NHK-1	Mitosis regulation
		Ser 129	Mec1	DNA repair
	Ubiquitination	Lys 119	Bmi/Ring1/Ring2 HR6A/HR6B	DNA repair; Spermatogenesis
	Sumoylation	Lys 126	Ubc9	Transcription repression
H2B	Acetylation	Lys 5	ATF2	Transcription activation
		Lys 11	Gcn5	Transcription activation
		Lys 12	CBP/P300/ATF2	Transcription activation
		Lys 15	CBP/P300/ATF2	Transcription activation
		Lys 16	Gcn5/Esa1	Transcription activation
		Lys 20	P300	Transcription activation
	Phosphorylation	Ser 10	Ste20	Apoptosis regulation
		Ser 14	Mst1	Apoptosis regulation
		Ser 33	TAF1	Transcription activation
	Ubiquitination	Lys 120	RNF20/RNF40 HR6A/HR6B	Meiosis regulation
		Lys 123	Rad6	Euchromatin formation; Transcription activation
	Sumoylation	Lys 6	Ubc9	Transcription repression
		Lys 7	Ubc9	Transcription repression
		Lys 16	Ubc9	Transcription repression
		Lys 17	Ubc9	Transcription repression
	ADP ribosylation	Glu 2	MART	NA[b]
H3	Acetylation	Lys 9	Gcn5/SRC-1	Transcription activation
		Lys 14	Gcn5/PCAF/Esa1 Tip60/SRC-1 Hpa2/Elp3/TAF1 Sas2/Sas3/P300 hTFIIIC90	DNA repair; Transcription activation, elongation
		Lys 18	CBP/P300/Gcn5	DNA repair; DNA replication; Transcription activation

Table 1-1 (continued): Summary of current knowledge of histone post-translational modifications

Histone proteins	Modifications	Potential residues	Major modifying enzymes	Functions regulated
H3	Acetylation	Lys 23	CBP/P300/Gcn5 Sas3	DNA repair; Transcription activation
		Lys 27	Gcn5	Transcription activation
		Lys 36	Gcn5	Transcription regulation
		Lys 56	Rtt109/Spt10	Histone deposition; DNA replication; Genome stability; Heterochromatin formation
	Methylation	Arg 2	PRMT4/PRMT6	Transcription repression
		Lys 4	MLL/Trx/Ash1 Set7/Set9/Set1	Transcription activation, elongation; Euchromatin formation;
		Arg 8	PRMT5	Cell proliferation
		Lys 9	Suv39H/Cl4/G9a ESET/SETDB1 CLL8/Ezh2/Ash1 EuHMT/GLP	Transcription repression; Transcription silencing; Transcription activation; DNA methylation; Imprinting
		Arg 17	PRMT4	Transcription activation
		Arg 26	PRMT4	Transcription activation
		Lys 27	G9a/Ezh2	Transcription silencing; X inactivation
		Lys 36	NSD1/SYMD2 Set2	Transcription repression; Transcription elongation
		Lys 79	Dot1	Euchromatin formation; Transcription activation; DNA repair
	Phosphorylation	Thr 3	Haspin	Mitosis regulation
		Ser 10	Aurora-B/Snf1 MSK1/MSK2	Transcription activation; Mitosis regulation; Meiosis regulation; Chromosome condensation
		Thr 11	Dlk/ZIP	Mitosis regulation;
		Ser 28	MSK1/MSK2	Transcription activation; Mitosis regulation;

Table 1-1 (continued): Summary of current knowledge of histone post-translational modifications

Histone proteins	Modifications	Potential residues	Major modifying enzymes	Functions regulated
H4	Acetylation	Lys 5	Hat1/Tip60/Hpa2 ATF2/Esa1/P300 HBO1/Hpa2	Transcription activation; DNA repair; Histone deposition
		Lys 8	Gcn5/PCAF/Tip60 ATF2/P300/Esa1 HBO1/Elp3	Transcription activation; DNA repair; Transcription elongation
		Lys12	Hat1/Tip60/Esa1 HBO1/Hpa2	Transcription activation; Transcription silencing; DNA repair; Histone deposition
		Lys 16	Gcn5/Tip60/ATF2 Esa1/Sas2/MOF	Transcription activation; Euchromatin formation; DNA repair
	Methylation	Arg 3	PRMT1/PRMT4 PRMT5	Transcription activation;
		Lys 20	Pre-Set7/Suv4-20h Ash1/Set9	Transcription activation; Transcription silencing; Heterochromatin formation
	Phosphorylation	Ser 1	CKII	DNA repair; Sporulation regulation; Mitosis regulation
	Sumoylation	Lys 5	Ubc9	Transcription repression
		Lys 8	Ubc9	Transcription repression
		Lys 12	Ubc9	Transcription repression
		Lys 16	Ubc9	Transcription repression
		Lys 20	Ubc9	Transcription repression

[a] Table information is mainly gathered from recent reviews (Kouzarides, 2007; Peterson and Laniel, 2004; Shahbazian and Grunstein, 2007; Shilatifard, 2006).

[b] NA: Not Analyzed.

into two major categories: the establishment of global chromatin environments ("accessible" euchromatin and "inaccessible" heterochromatin), and orchestration of DNA-templated processes (gene transcription, DNA replication, DNA repair, and chromosome condensation) (reviewed in (Kouzarides, 2007)). These modifications are thought to accomplish their biological roles through two proposed distinct mechanisms. One of the potential mechanisms is that histone modifications alter the charge of a residue, which changes electrostatic interactions between histone proteins and DNA or disrupts internucleosomal interactions (Wolffe and Hayes, 1999). The electrostatic changes can further condense or lessen chromatin structure, thus controlling access of DNA-binding proteins, such as transcription factors. Among all the known modifications, acetylation and phosphorylation have the most potential to affect chromatin structure, since they change the basic charge of the residues (Kouzarides, 2007; Kurdistani and Grunstein, 2003; Shahbazian and Grunstein, 2007; Wolffe and Hayes, 1999). For instance, acetylation of one lysine residue in histone H3 will cause significant decrease in the total positive charge possessed by the protein (Peterson and Laniel, 2004). However, to date there is very limited experimental evidence to support this hypothesis *in vivo* (Kouzarides, 2007; Kurdistani and Grunstein, 2003), and one *in-vitro* study even showed that the histone-DNA interaction are not weakened by increased histone acetylation (Mutskov et al., 1998). A second model is that small chemical moieties that compose the histone post-translational modifications provide or prevent binding by chromatin regulatory proteins. Hence, these proteins dictate the biological consequences associated with those modifications. Evidence from the past decade strongly supports this model. For example, methylated histone H3 lysine 9 is bound specifically by Heterochromatin-

associated protein 1 (HP1) through its chromodomain, which facilitates chromatin condensation and transcription repression (Bannister et al., 2001; Lachner et al., 2001).

The histone code

These and other similar histone modification studies led to an appealing hypothesis, the histone code hypothesis (Jenuwein and Allis, 2001; Turner, 2002), thereby extending the information of the genome beyond the classic genetic code. This hypothesis suggests that multiple histone modifications on the same or different histones may act in a combinatorial manner to regulate the association of chromatin-binding proteins, leading to distinct biological readouts of those modifications, such as gene activation versus gene silencing (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2000). After the histone code hypothesis was proposed, a large number of studies have been carried on trying to identify different modifications and understand their functions. So far, significant progress has been made (Kouzarides, 2007). However, it also appears that comprehensively deciphering the histone code can be very challenging, because not only are there so many modifications on various histones and most of the modifications are unequally distributed, but also many of these modifications or combinations have several functional roles. Indeed, the biological functions of histone post-translational modifications can only be correctly interpreted by full consideration of their surrounding genomic and chromatin context. Apparently, more studies are required to further elucidate the complexity of the histone code.

Section II: Histone H3 acetylation and methylation in *Saccharomyces cerevisiae*

Histone acetylation and deacetylation

One of the first discovered and most well characterized histone post-translational modifications is histone acetylation. Histone acetylation refers to the covalent addition of the acetyl group from acetyl Coenzyme A to the ε–amino group of a lysine residue in histones. The added acetyl group can be subsequently removed by the deacetylation reaction. Histone proteins are subjected to both acetylation and deacetylation catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Most acetylation sites are found within the N-terminal tail domains of histones, which are thought to be more accessible for modifications. Recently, a lysine residue within the fold domain of histone H3 (lysine 56) has been discovered to be acetylated (Xu et al., 2005), which suggests that acetylation is not limited to the histone tail domains.

There are numerous HATs and HDACs present in eukaryotic cells. HATs are typically divided into three major families, CBP/p300, GNAT, and MYST (Sterner and Berger, 2000). Generally, each HAT modifies more than one lysine residue in histones but some HATs show limited specificity. For example, in *Saccharomyces cerevisiae*, the HAT ESA1 is responsible for acetylation of lysine 4, lysine 8 and lysine 12 in histone H4 (Allard et al., 1999; Suka et al., 2001), while HAT Sas2 mainly acetylates histone H4 lysine 16 (Kimura et al., 2002; Suka et al., 2002). In general, there are three distinct HDAC families, class I HDACs, class II HDACs, and class III HDACs. Class I and II HDACs are classical histone deacetylases and can be inhibited by trichostatin A (TSA). Class III HDACs, known as sirtuins (silent mating type information regulating homolog 2), are highly conserved nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases, therefore resistant to TSA inhibition. Although most HDACs do not show

much specificity for a particular histone lysine acetylation, a few of them do have specific targets. For example, the HDAC Sir2 mainly deacetylates the acetylated histone H4 lysine 16 (Imai et al., 2000). Many of HATs and HDACs have been identified as transcription activators or repressors (Kuo et al., 1998; Kurdistani and Grunstein, 2003; Shahbazian and Grunstein, 2007), as well as regulators of other DNA-based processes, such as DNA replication, DNA repair, heterochromatin establishment and maintenance, and chromosome condensation (Kurdistani and Grunstein, 2003; Shahbazian and Grunstein, 2007). It is important to note that HATs and HDACs generally function in multiprotein complexes and often the same enzyme exists within different complexes. The complex in which a HAT or a HDAC is present strongly affects its substrate preference and genomic occupancy, therefore regulating its functions (Shahbazian and Grunstein, 2007). For instance, the HDAC Rpd3 is known to present in two distinct complexes, Rpd3L and Rpd3S, in *Saccharomyces cerevisiae* (Kasten et al., 1997; Lechner et al., 2000; Rundlett et al., 1996). Evidence indicated that Rpd3L and Rpd3S vary in composition, genome-wide distribution, and function (Carrozza et al., 2005; Keogh et al., 2005; Li et al., 2007b; Li et al., 2007c).

The histone acetylation patterns across the genome are achieved from at least two distinct mechanisms. First, HATs and HDACs can modify the genome globally in an untargeted fashion (Krebs et al., 2000; Kuo et al., 2000; Vogelauer et al., 2000). This global background of histone acetylation throughout the genome by HATs and HDACs not only modulates basal genome transcription, but also allows gene activation or repression to be reversed by rapid changes in histone acetylation level (Kurdistani and Grunstein, 2003). Second, against a global background, HATs and HDACs can also be

recruited to specific promoter regions of genes through interaction with DNA-binding proteins, such as transcription factors (Bulger, 2005; Kurdistani and Grunstein, 2003; Shahbazian and Grunstein, 2007). This specific targeting mechanism contributes to the hyperacetylated and hypoacetylated regions in the genome (Bulger, 2005).

The function of histone acetylation and deacetylation has been extensively explored. As briefly mentioned previously, the effect of histone acetylation and deacetylation on transcription can be attributed to both direct structural changes and the recruitment of activator and repressors. From a structural viewpoint, histone hyperacetylation weakens DNA-histone interaction by neutralizing the positive charge of the histone tail domains. Histone hypoacetylation, in contrast, strengthens DNA-histone interactions by restoring the positive charge of histone proteins. Therefore, accessibility of transcription factors to gene promoters in chromatin is regulated by those direct chromatin structure changes. Other studies also suggested that histone acetylation and deacetylation can directly regulate higher order chromatin structures (Horn and Peterson, 2002; Tse et al., 1998).

Besides altering chromatin structure directly, histone acetylation and deacetylation might also regulate transcription by providing binding docks for transcriptional activators and repressors. For example, regulators involved in gene activation such as $TAF_{II}250$ and Bdf1 are recruited by histone acetylation through their bromodomain (Agalioti et al., 2002; Kurdistani et al., 2004), which interacts specifically with acetylated lysine residues in the histone tail domains (Tamkun et al., 1992; Winston and Allis, 1999). On the other hand, a number of co-repressors preferentially bind unacetylated histones through their SANT domain, which is thought to be attracted by

unacetylated histones (de la Cruz et al., 2005; Hartman et al., 2005; Yu et al., 2003). Generally, histone acetylation functions in promoting gene transcription, and histone deacetylation plays important roles in repressing transcription. However, recently studies showed that histone acetylation also functions at repressed genes (Gregory et al., 1999; Nourani et al., 2004), and that histone deacetylation contributes to transcription activation (De Nadal et al., 2004; Robyr et al., 2002; Wang et al., 2002). Besides their functions in transcription, histone acetylation and deacetylation are also involved in other DNA-templated processes, such as DNA replication, DNA repair, and chromosome condensation (Kouzarides, 2007). For example, depletion of HBO1, a HAT, causes a reduction of DNA synthesis and affects cell cycle progression (Doyon et al., 2006). This and other studies suggested that HBO1 functions in S phase initiation and firing replication origins through the acetylation of histone H4.

Histone H3 acetylation and deacetylation in *Saccharomyces cerevisiae*

There are seven acetylation sites found in histone H3 in *Saccharomyces cerevisiae,* including lysine 9, 14, 18, 23, 27, 36, and 56. The first six acetylated lysine residues are in the histone H3 N-terminal tail domain and lysine 56 resides in the fold domain (Figure 1-2). The HAT Gcn5, as part of the Spt-Ada-Gcn5-acetyltransferase (SAGA) multiprotein complex, and the HAT Sas3, as part of the Nucleosome Acetyltransferase of histone H3 (NuA3) complex, are primarily responsible for lysine acetylation within the histone H3 tail domain (Kurdistani and Grunstein, 2003; Millar and Grunstein, 2006; Morris et al., 2007). Conversely, HDACs Hda1, Hos2, and Rpd3 mainly deacetylate the histone H3 N-terminal tail (Kurdistani and Grunstein, 2003; Millar and

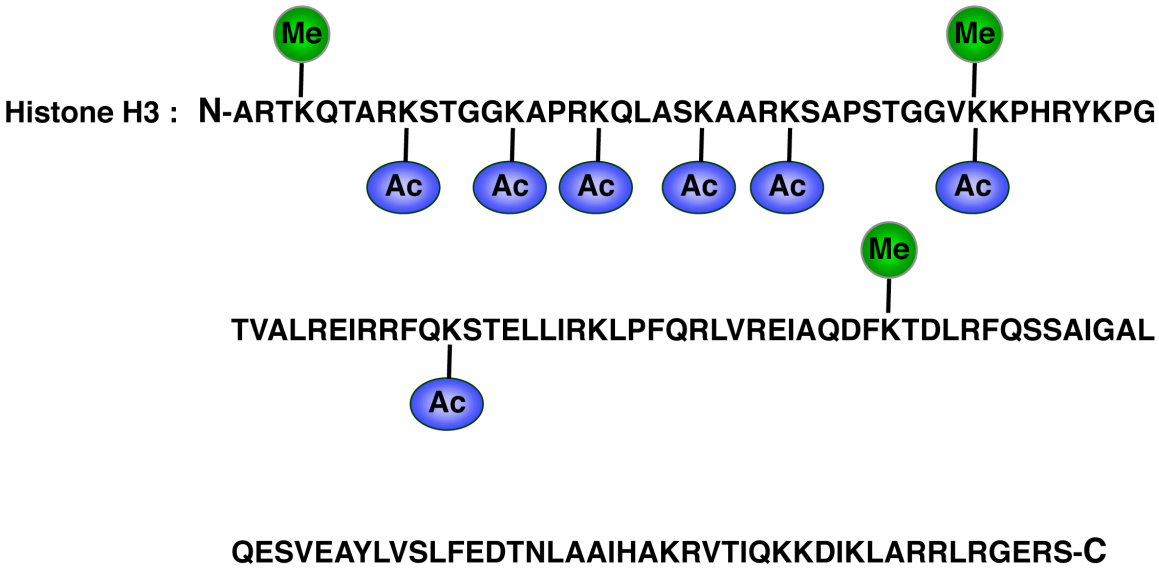

FIGURE 1-2: Histone H3 acetylation and methylation in *Saccharomyces cerevisiae*.

The histone H3 (HHT2) protein sequence from *Saccharomyces cerevisiae* is shown with the characterized histone acetylation (Ac) and methylation (Me) sites marked.

Grunstein, 2006). Previous studies have suggested that histone H3 acetylation and deacetylation function in various DNA processes (See introductions in Chapter Three). In contrast, histone H3 lysine 56 in the genome is acetylated by a novel HAT Rtt109 with no detectable sequence homology to other known HATs (Driscoll et al., 2007; Han et al., 2007; Schneider et al., 2006; Tang et al., 2008), whereas acetylation of histone H3 lysine 56 at the promoter of histone genes is mediated by the putative HAT Spt10 (Xu et al., 2005). Interestingly, Histone H3 lysine 56 is located at the lateral surface of the nucleosome facing toward the DNA major groove at the entry/exit point of the DNA in the nucleosome. It is possible that acetylation of lysine 56 could weaken histone-DNA interaction. The function of histone H3 lysine 56 acetylation has also been implicated in histone deposition, DNA replication, and genome stability (Driscoll et al., 2007; Han et al., 2007; Schneider et al., 2006; Xu et al., 2005). Intriguingly, although the histone H3 lysine acetylation is important in various biological processes, the histone H3 N-terminal tail domain is dispensable. Indeed, the histone H3 N-terminal tail is not required for nucleosome assembly and has redundant functions with the histone H4 N-terminal tail in histone deposition (Ling et al., 1996; Ma et al., 1998; Megee et al., 1990; Morgan et al., 1991).

Histone methylation

There are multiple residues in histone proteins subject to methylation. The methyl group is transferred from the common donor S-adenosyl-L methionine (SAM) to specific arginine or lysine residue in histones by histone methyltransferases. An arginine residue can accept one or two methyl groups. There are two configurations of dimethylated

arginines, a symmetric dimethylated arginine and an asymmetric dimethylated arginine (Figure 1-3A). In contrast, the ε–amino group of a lysine residue can take up to three methyl groups, forming mono-, di, or trimethylated lysine, respectively (Figure 1-3B). Although there are some variations among different species, the majority of methylation occurs in histone H3 and H4, at lysine 4, 9, 27, 36, and 79 of histone H3, lysine 20 of histone H4, arginine 2, 17, and 26 of histone H3, and arginine 3 of histone H4.

Histone methyltransferases

Over the past several years, a number of histone methyltransferases have been discovered. These histone methyltransferases have been divided into three major groups, including SET-domain containing histone lysine methyltransferases (HKMTs), non-SET-domain containing HKMTs, and protein arginine methyltransferases (PRMTs). The SET domain was first identified in three genes, Su(var)3-9, En(zeste), and Trithorax in Drosophila (Jenuwein et al., 1998). There are many SET-domain containing proteins (not limited to HKMTs) that have been found in both prokaryotes and eukaryotes. SET-domain containing HKMTs target the lysine methylation in the N-terminal domain of histone H3 and H4. The core histone H3 lysine 79 is exclusively methylated by the HKMT Dot1 (disruptor of telomeric silencing 1), which is the only HKMT identified without a SET domain. Initially, Dot1 was discovered as a high-copy disruptor for telomeric gene silencing in *Saccharomyces cerevisiae* (Singer et al., 1998). Unlike other HKMTs which methylate lysine residues in histone tail domains, Dot1 is responsible for methylation of lysine 79 of histone H3, which is in the core histone fold domain of histone H3 and at the surface of the nucleosome core particle (Luger et al., 1997). Indeed,

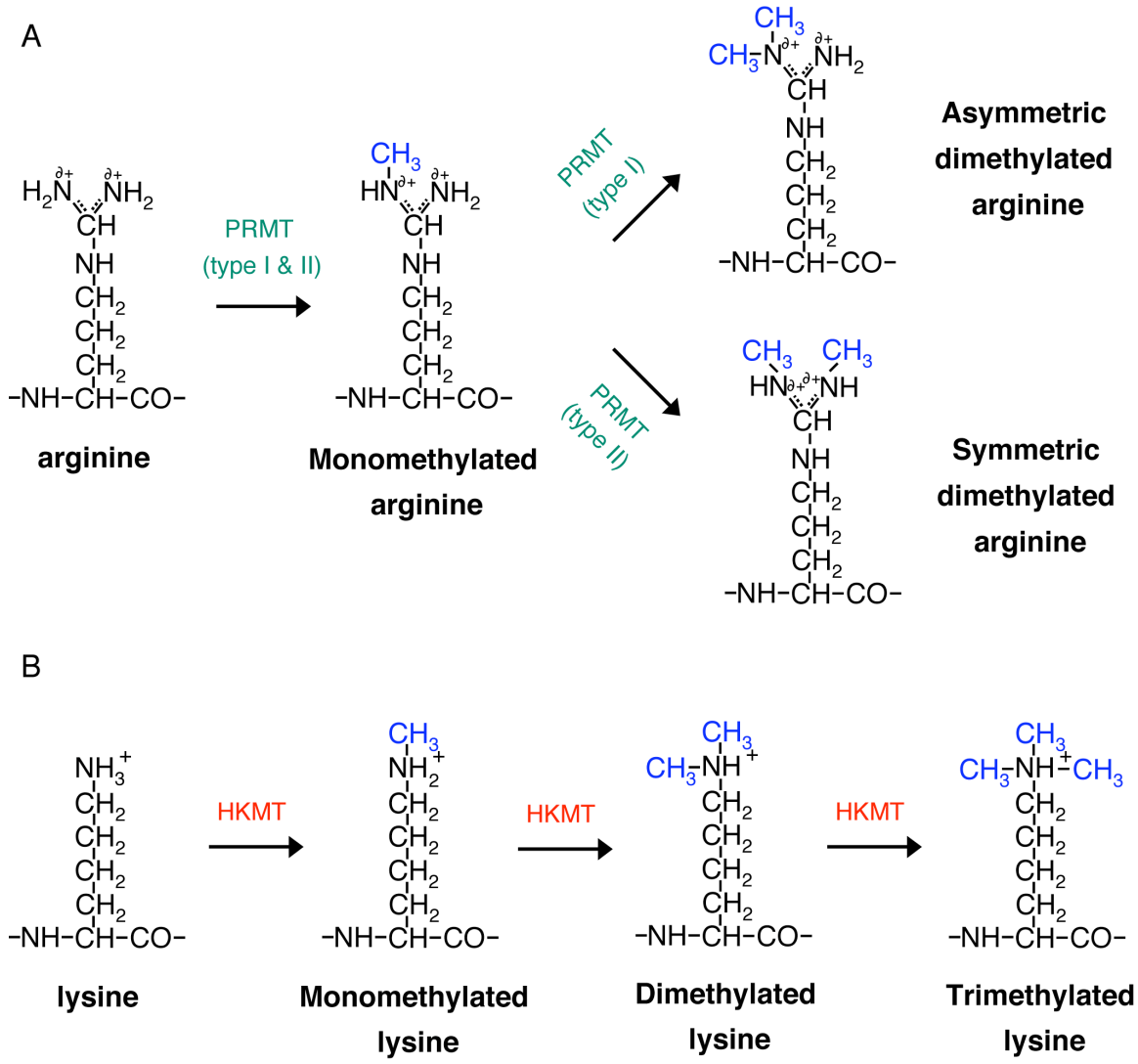

FIGURE 1-3: The chemical pathways for histone arginine and lysine methylation.

(A) Histone arginine residues can be methylated to mono- and dimethylated arginine. Type I protein arginine methyltransferases (PRMTs) catalyze asymmetric dimethylation; type II PRMTs catalyze symmetric dimethylation. **(B)** Histone lysine residues can be mono-, di-, and trimethylated by histone lysine methyltransferases (HKMTs).

almost 90% of lysine 79 of histone H3 in chromatin is methylated by Dot1 (van Leeuwen et al., 2002). The core domain of yeast Dot1, which is required for methylation activity, is conserved through Dot1 homologues in different species (Cheng et al., 2005).

PRMTs, another important type of histone methyltransferases, function in histone arginine methylation in eukaryotes. There are two types of PRMTs. Type I PRMTs catalyze monomethylated arginine and asymmetric dimethylated arginine (Figure 1-3A) (Branscombe et al., 2001). Type II PRMTs methylate arginine residues into monomethylated arginine and symmetric dimethylated arginine (Figure 1-3A) (Branscombe et al., 2001).

Histone demethylation

Unlike other readily reversible histone post-translational modifications (e.g., lysine acetylation), for a long time, histone methylation was considered to be relatively stable and irreversible. This idea was supported by the absence of known histone demethylases and the observations that histone proteins and histone methylation share similar half-life *in vivo* (Byvoet et al., 1972; Duerre and Lee, 1974). However, surprising discoveries in recent years have demonstrated that histone methylation is indeed reversible. One method of reversing histone methylation is that methylated histones can be substituted by unmodified histones or histone variants, in either a DNA replication dependent or independent manner. Multiple lines of evidence support this model (Ahmad and Henikoff, 2002a; Schwartz and Ahmad, 2005). More importantly, a number of histone demethylases have been discovered in the past four years. Indeed, three distinct classes of histone demethylases have been identified. Peptidylarginine deiminase 4

(PAD4) was the first identified histone demethylase. It converts the methylated arginine to citrulline and releases methylamine. PAD4 functions on arginine 17 of histone H3 and arginine 3 of histone H4 (Cuthbert et al., 2004; Wang et al., 2004). However, it should be acknowledged that PAD4 cannot produce unmodified arginine residues through this demethylation process. The second class of identified histone demethylases is lysine-specific demethylase 1 (LSD1). LSD1 is the first discovered histone lysine demethylase and is a nuclear amine oxidase homolog. LSD1 mainly demethylates lysine 4 and lysine 9 of histone H3 through a flavin adenine dinucleotide (FAD)-dependent manner and releasing formaldehyde as a byproduct (Metzger et al., 2005; Shi et al., 2004). Intriguingly, LSD1 is only capable of demethylating monomethylated and dimethylated lysine residues (Shi et al., 2004). The third class of histone demethylases is JmjC-domain-containing histone demethylases (JHDMs). This is also the largest class of histone demethylases identified so far. It has been shown that JHDMs demethylate all three histone lysine-methylation states through an oxidation reaction which requires α–ketoglutarate and iron Fe(II) as cofactors (Tsukada et al., 2006). Both lysine 36 and lysine 9 of histone H3 have been identified as demethylation targets of JHDMs (Cloos et al., 2006; Fodor et al., 2006; Klose et al., 2006b; Tsukada et al., 2006; Whetstine et al., 2006; Yamane et al., 2006). Phylogenetic and structural analyses reveal that members of JHDMs usually contain multiple protein domains besides the key JmjC domain, such as a plant homeobox domain (PHD) domain, a tetracopeptide repeat (TPR) domain, and a zinc-finger-like (ZF-like) domain (Klose et al., 2006a). It seems that the function of JHDMs depends on both the JmjC domain and other additional domains within each enzyme (Klose et al., 2006a). The discovery of histone demethylases represents an

important milestone of histone methylation studies. It suggests that histone methylation is readily reversible and can regulate dynamic biological functions.

During the past several years, benefiting from the extensive studies of histone methylation, our understanding of how histone methylation functions in regulating gene transcription, chromatin structure, DNA-repair, and epigenetic memory has grown explosively. Considering the scope of the work described in this thesis, I will mainly limit the introduction to the currently known functions of histone H3 methylation in chromatin structure and gene transcription regulation in *Saccharomyces cerevisiae.*

Histone H3 methylation and transcription elongation in *Saccharomyces cerevisiae*

Lysine 4 (H3K4), lysine 36 (H3K36), and lysine 79 (H3K79) of histone H3 are methylated in *Saccharomyces cerevisiae* (Figure 1-2). Histone H3K4 can be mono-, di-, and trimethylated by the Set1 HKMT (Briggs et al., 2001; Roguev et al., 2001; Santos-Rosa et al., 2002), a homologue of the human mixed lineage leukemia (MLL) oncogene (Milne et al., 2002). Histone H3K36 methylation is catalyzed by the Set2 HKMT (Strahl et al., 2002), and H3K79 is methylated by Dot1, a non-SET domain HKMT (Ng et al., 2002a; van Leeuwen et al., 2002).

In *Saccharomyces cerevisiae*, the three histone H3 HKMTs have been linked to transcription elongation machinery (Gerber and Shilatifard, 2003; Hampsey and Reinberg, 2003; Sims et al., 2004). The Set1 HKMT, responsible for H3K4 methylation, is associated with COMPASS, Complex of Proteins Associated with Set1 (Miller et al., 2001; Roguev et al., 2001). The COMPASS complex specifically binds to the phosphorylated serine 5 in the C-terminal domain (CTD) region of the early elongating

RNAPII (Krogan et al., 2003a; Ng et al., 2003c). This association is mediated by the polymerase II-associated factor 1 (Paf1) complex (Krogan et al., 2003a; Ng et al., 2003c). It has been shown that Set1 cannot properly methylate H3K4 without the presence of other components of the COMPASS complex (Shilatifard, 2006) and H3K4 methylation is dependent on monoubiquitination of histone H2B lysine 123 (Dover et al., 2002; Sun and Allis, 2002). Like Set1, the Set2 HKMT also associates with elongating RNAPII, but through the phosphorylated serine 2 in the CTD region, mediated by the Paf1 complex (Krogan et al., 2003b; Li et al., 2003; Li et al., 2002; Schaft et al., 2003; Xiao et al., 2003). Indeed, the Ctk1 kinase, which is responsible for the phosphorylation of serine 2 in the CTD region, is also required for H3K36 methylation by the Set2 HKMT (Krogan et al., 2003b; Xiao et al., 2003). Methylation of H3K36 can recruit Eaf3, a component of the Rpd3S complex, which leads to inhibition of RNAPII transcription initiation at cryptic start sites within open reading frames (Carrozza et al., 2005). Deletion of the *SET2* gene also causes defects in transcription elongation, supporting the argument that the Set2 HKMT is linked to transcription elongation (Krogan et al., 2003b; Li et al., 2003; Xiao et al., 2003). Interestingly, Set2-catalyzed methylation of H3K36 is facilitated by deubiquitination of histone H2B lysine 123 (Henry et al., 2003). Although there is no direct evidence to link the Dot1 HKMT to transcription elongation so far, evidence that Dot1-catalyzed methylation of histone H3K79 relies on the Paf1 complex (Krogan et al., 2003a) and methylation of H3K79 is largely associated with actively transcribed genes (Im et al., 2003), suggests a strong possibility that Dot1 is also linked to transcription elongation.

One appealing hypothesis termed "short-term memory" has been proposed to explain the function of histone methylation in transcription elongation. This model suggests that in addition to histone H3 methylation serving as a marker to recruit chromatin remodeling complexes, such as Isw1, facilitating transcription elongation and mRNA processing (Morillon et al., 2003; Santos-Rosa et al., 2003), histone H3 methylation may also serve as a short-term epigenetic marker of the recent transcription status of a given gene (Ng et al., 2003c; Shilatifard, 2006). Furthermore, the pattern of methylation may indicate how far the gene has been transcribed by RNAPII. However, this type of histone H3 methylation pattern can not be faithfully transmitted to all later generations of cells (Shilatifard, 2006).

Histone H3 methylation and euchromatin in *Saccharomyces cerevisiae*

Lysine methylation in histone H3 plays an important role in chromatin structure (Lachner and Jenuwein, 2002; Sims et al., 2003). In *Saccharomyces cerevisiae,* recent studies have shown that methylation of H3K4, H3K36, and H3K79 are generally associated with euchromatin formation. Previous studies of methylation of histone H3K4 showed that trimethylated H3K4 is selectively associated with the 5' coding region of actively transcribed genes (Millar and Grunstein, 2006; Ng et al., 2003c; Pokholok et al., 2005; Santos-Rosa et al., 2002), dimethylated H3K4 is generally enriched in the middle of transcriptionally active genes (Bernstein et al., 2002; Millar and Grunstein, 2006; Noma et al., 2001; Pokholok et al., 2005), and monomethylated H3K4 is found predominantly at the end of active genes (Pokholok et al., 2005) (Figure 1-4). More importantly, H3K4 methylation not only recruits activating chromatin remodeling

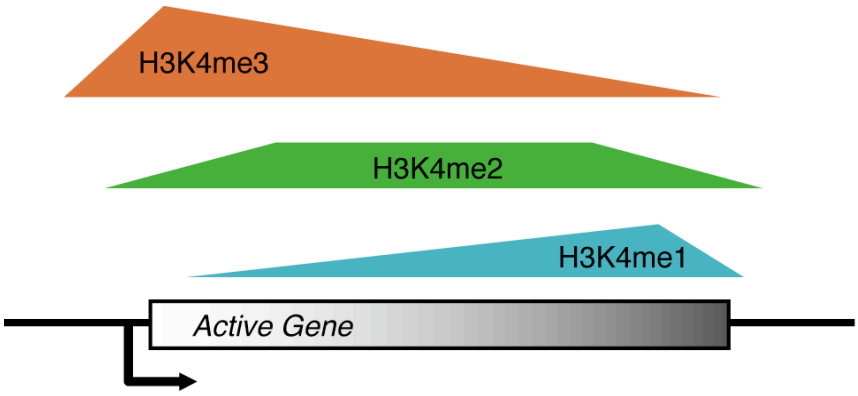

FIGURE 1-4: Histone H3K4 methylation profiles in active genes.

A schematic of an actively transcribed gene is shown. The profiles of histone H3K4 methylation are depicted by the colored shapes. Histone H3K4 trimethylation (orange) is most enriched at the 5' coding region of actively transcribed genes; H3K4 dimethylation (green) is generally present in the coding region of active genes; H3K4 monomethylation (blue) is found predominantly at the 3' end of active genes.

complexes, such as the Isw1 chromatin remodeling ATPase (Morillon et al., 2003; Santos-Rosa et al., 2003), but also blocks the binding of repressive chromatin remodeling complexes, like the repressive NuRD HDAC (Nishioka et al., 2002; Zegerman et al., 2002) and the SIR silencing complex (Santos-Rosa et al., 2004). Recruitment of Isw1 by H3K4 methylation better coordinates transcription elongation and termination (Morillon et al., 2003; Santos-Rosa et al., 2003). Similarly to H3K4 methylation, there is also a good correlation between methylation of H3K36 and H3K79 and actively transcribed euchromatin regions. Trimethylation of H3K36 is preferentially associated with the coding regions of actively transcribed genes, peaking near the 3' ends of open reading frames (Krogan et al., 2003b; Li et al., 2003; Pokholok et al., 2005; Xiao et al., 2003), and trimethylation of H3K79 is largely enriched within the transcribed regions of genes (Im et al., 2003; Pokholok et al., 2005). H3K79 methylation blocks the association of the SIR silencing complex (the SIR complex will be further introduced later), facilitating euchromatin establishment and maintenance (Ng et al., 2003a; van Leeuwen et al., 2002).

Histone H3 methylation and heterochromatin in *Saccharomyces cerevisiae*

In higher eukaryotes, heterochromatin is well defined by the presence of methylation at H3K9, H3K27, and H4K20, as well as the absence of methylation at H3K4, H3K36, and H3K79 (Grewal and Moazed, 2003; Lachner and Jenuwein, 2002; Shilatifard, 2006; Sims et al., 2003). Heterochromatin-associated protein 1 (HP1) binds specifically to methylated H3K9 through its chromodomain (Bannister et al., 2001; Lachner et al., 2001), and directs chromatin condensation and transcriptional repression. H3K27 methylation, a characteristic of the inactive X chromosome, recruits the

chromodomain-containing Polycomb repressor complex, which is required for gene silencing (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002).

Interestingly, H3K9, H3K27, and H4K20 are not methylated in *Saccharomyces cerevisiae*. So far, no homologues of HP1 or the Polycomb proteins have been discovered in *Saccharomyces cerevisiae.* However, there are well-established heterochromatin regions, in which the genes mainly remain transcriptionally silenced due to the condensed chromatin structure regardless of their promoter sequences, present in *Saccharomyces cerevisiae*. These heterochromatin regions include the silent mating loci (*HML* and *HMR* cassettes) (Klar et al., 1981; Nasmyth et al., 1981), ribosomal DNA (rDNA) repeats (Fritze et al., 1997; Smith and Boeke, 1997), telomere proximal regions (Gottschling et al., 1990; Lustig, 1998), and subtelomeric regions (Robyr et al., 2002). Over the past several years, evidence has emerged indicating that the absence of H3K4, H3K36, and H3K79 methylation facilities heterochromatin formation in *Saccharomyces cerevisiae*. The Set1 HKMT is required for telomeric silencing (Krogan et al., 2002), and hypomethylated H3K4 is enriched in telomeric and subtelomeric heterochromatin regions (Bernstein et al., 2002). Like Set1 and hypomethylated H3K4, the Dot1 HKMT is also essential for telomeric silencing and H3K79 is hypomethylated in telomeric heterochromatin (Ng et al., 2003a; Ng et al., 2002a; van Leeuwen et al., 2002). Further studies demonstrated that hypomethylation of H3K4 and H3K79 are involved in the recruitment of the heterochromatin-associated SIR silencing complex at yeast telomeres (Ng et al., 2003a; Santos-Rosa et al., 2004). It is not clear whether hypomethylation of H3K36 is associated with heterochromatin. However, one very recent study showed that the Set2 HKMT regulates SIR silencing complex binding (Tompa and Madhani, 2007).

Taken together, heterochromatin is characterized by hypomethylation of H3K4, H3K79, and perhaps H3K36 in *Saccharomyces cerevisiae.*

SIR silencing complex in *Saccharomyces cerevisiae*

Previous studies revealed that heterochromatin in silent mating loci and telomere regions is characterized by the major structural component, the SIR silencing complex, in *Saccharomyces cerevisiae*. The SIR silencing complex consists of the Sir2 HDAC and the structural components Sir3 and Sir4. Indeed, the SIR silencing complex is composed of a stable heterodimer of Sir2/Sir4, dimers of Sir3, and some higher-order oligomers of Sir3 (Liou et al., 2005; Rudner et al., 2005; Tanny et al., 2004). Sir2 is a NAD-dependent HDAC that deacetylates H4K16, among other histone residues (Cubizolles et al., 2006; Imai et al., 2000). It appears that deacetylation of H4K16 is crucial to the assembly of the SIR silencing complex in chromatin (Hoppe et al., 2002; Luo et al., 2002). Further study indicated that the byproduct O-acetyl-ADP-ribose (AAR or OAADPR) released from Sir2 deacetylation triggers a conformational change in the SIR silencing complex and is required for SIR mediated heterochromatin formation (Liou et al., 2005).

Sir3 and Sir4 preferentially bind the hypoacetylated N-terminal tails of histone H3 and H4 (Carmen et al., 2002; Hecht et al., 1995). H4K16 deacetylation is particularly important for Sir3 binding (Johnson et al., 1990; Liou et al., 2005). Sir3 also interacts with Sir4, and the interaction is essential for silencing and SIR silencing complex spreading, although the interaction between Sir3 and Sir4 is not as strong as between Sir2 and Sir4 (Hecht et al., 1996; Moazed et al., 1997; Moretti et al., 1994; Rudner et al., 2005).

A general model of heterochromatin assembly mediated by the SIR silencing complex has been proposed based on current knowledge. Briefly, this assembly process can be divided into two steps, nucleation (initiation) at the silencer site and spreading along the chromatin (Hoppe et al., 2002; Luo et al., 2002; Moazed, 2001; Rusche et al., 2002). At the nucleation step, the SIR silencing complex is first recruited by certain DNA-binding proteins to the silencing initiation sites. Then Sir2 deacetylates histone H4K16, facilitating further SIR silencing complex binding. The cycle of Sir2 deacetylation and more SIR silencing complex binding sets the stage for spreading of the SIR silencing complex along the adjacent chromatin (Hoppe et al., 2002; Luo et al., 2002; Rusche et al., 2002). Aside from H4K16 acetylation, there are several other distinct mechanisms to regulate spreading of the SIR silencing complex, including Sir3 oligomerization (Liou et al., 2005), and incorporation of histone Htz1 (H2A.Z variant) as a heterochromatin boundary element (Meneghini et al., 2003). Methylation of histone H3 lysine residues also prevents spreading of Sir proteins (Bernstein et al., 2002; Fingerman et al., 2005; Katan-Khaykovich and Struhl, 2005; Ng et al., 2002a; Nislow et al., 1997; Santos-Rosa et al., 2004; van Leeuwen et al., 2002).

In *Saccharomyces cerevisiae*, the best studied heterochromatin region mediated by the SIR silencing complex is telomeric heterochromatin. In accordance with the proposed model above, the DNA-binding protein Rap1 with the Ku70/Ku80 protein complex first localizes to the end of a chromosome, subsequently recruiting Sir4 to the telomere end through Sir4 interaction with Rap1 (Luo et al., 2002; Moretti and Shore, 2001). After Sir4 brings Sir2 to form the Sir2/Sir4 heterodimer, the Sir2 HDAC deacetylates histone H4K16. This deacetylation will further promote Sir3 and Sir4 binding to the chromatin

(Moazed, 2001). The subsequent spreading of the SIR silencing complex relies on Sir2 deacetylation, the interaction among Sir proteins, the interaction of the SIR silencing complex with the N-terminal tails of histone H3 and H4, and Sir3 oligomerization (Kurdistani and Grunstein, 2003; Liou et al., 2005; Shahbazian and Grunstein, 2007), as well as Htz1 distribution (Meneghini et al., 2003). Interestingly, recent studies showed that hypomethylation of histone H3K4 and H3K79 is associated with telomeric heterochromatin regions and hypermethylation of histone H3 in euchromatin regions blocks binding of the SIR silencing complex, suggesting that the SIR silencing complex potentially prefers binding to the hypomethylated H3 N-terminal domain (Bernstein et al., 2002; Fingerman et al., 2005; Katan-Khaykovich and Struhl, 2005; Ng et al., 2002a; Nislow et al., 1997; Santos-Rosa et al., 2004; Shilatifard, 2006; van Leeuwen et al., 2002). A similar mechanism of heterochromatin assembly has been revealed at the mating type loci (Hoppe et al., 2002; Rusche et al., 2002).

However, it is important to note that rDNA and subtelomeric heterochromatin regions do not require the entire SIR silencing complex. Instead, rDNA silencing requires a different silencing protein complex containing Sir2, Net1, and Cdc14 (Moazed, 2001). The silencing in subtelomeric heterochromatin regions requires the Hda1 HDAC (Robyr et al., 2002), but the molecular mechanism has yet to be uncovered.

Histone H3 methylation and transcription regulation in *Saccharomyces cerevisiae*

Many studies have been performed to elucidate the function of methylation of H3K4 by the Set1 HKMT in transcription regulation in *Saccharomyces cerevisiae*. Genome-wide expression profiling experiments revealed that a *SET1* deletion results in

small magnitudes down-regulation of 480 genes (Santos-Rosa et al., 2002). Set1 also regulates rDNA silencing via H3K4 methylation (Bryk et al., 2002). Another study showed that Set1 is required for the induction of meiotic genes during sporulation (Sollier et al., 2004). Furthermore, hypermethylation of H3K4 has been strongly implicated in gene activation across the genome (Bernstein et al., 2002; Pokholok et al., 2005). Taken together, all the evidence suggests that methylation of H3K4 regulates transcription at a subset of yeast genes.

Set2-catalyzed methylation of H3K36 also regulates gene transcription in *Saccharomyces cerevisiae*. Previous studies showed that H3K36 methylation is essential for the maintenance of the low basal expression of the *GAL4* gene. Deletion of *SET2* causes an increase in the basal expression level of the *GAL4* gene (Landry et al., 2003; Strahl et al., 2002). A similar result was also obtained by mutating the histone H3K36 residue (Landry et al., 2003; Strahl et al., 2002). On the other hand, a mutant with a *SET2* deletion is sensitive to 6-azauracil, indicating that the mutant suffers transcription elongation defects (Krogan et al., 2003b; Li et al., 2003; Xiao et al., 2003). These studies shed light on the roles of H3K36 methylation in both activation and repression of gene transcription.

The function of methylated H3K79 in transcription regulation is less well understood. In *Saccharomyces cerevisiae*, approximately 90% of histone H3K79 in chromatin was methylated (van Leeuwen et al., 2002). Although trimethylated H3K79 is enriched within the transcribed regions of genes, there was no obvious correlation between the level of H3K79 trimethylation and transcriptional activity (Pokholok et al., 2005). So far, the only known function of H3K79 methylation in transcription regulation

is that both the Dot1 HKMT and H3K79 are required for telomeric gene silencing. Lack of the Dot1 HKMT or a mutation of H3K79 causes telomeric silencing defects by affecting the binding of the SIR silencing complex (Ng et al., 2003a; Ng et al., 2002a; van Leeuwen et al., 2002).

Cross-talk between histone modifications in *Saccharomyces cerevisiae*

As more and more histone post-translational modifications have been identified, it is important to study the potential cross-talk or interplay of these modifications with each other. Indeed, several examples of cross-talk between histone modifications in the same histone protein and different histone proteins have been discovered (Latham and Dent, 2007).

The simplest case of histone modification cross-talk is a residue in histone proteins targeted by multiple histone post-translational modifications, such as lysine residues can be alternatively acetylated, methylated, sumoylated, and ubiquitinated. Thus one type of modification can block the addition of a different modification. For example, H3K36 has been recently discovered to be either methylated or acetylated in *Saccharomyces cerevisiae* (Morris et al., 2007). Methylation of H3K36 blocks subsequent H3K36 acetylation, and vice versa. It has been shown that methylation of H3K36 occurs mainly within the coding regions of active genes, whereas acetylation of H3K36 is enriched in the promoter regions of RNAPII transcribed genes, indicating their distinct roles in transcription regulation (Morris et al., 2007). Similar types of cross-talk have also been seen on other lysine residues of histone H2B and H4 (Nathan et al., 2006).

Another common histone modification cross-talk is between modifications located in the same histone protein. In this case, generally the addition of one histone modification on a residue can either promote or inhibit another histone modification to different residues in the same histone protein. One example is histone H2B monoubiquitination and sumoylation in *Saccharomyces cerevisiae*. Previous studies suggested that H2B monoubiquitination and sumoylation have opposing effects in regulating gene transcription. H2B monoubiquitination functions in gene activation, and H2B sumoylation contributes to gene repression (Nathan et al., 2006). Indeed, sumoylation of H2BK6, H2BK7, and possibly H2BK16 and H2BK17, represses H2BK123 monoubiquitination, and vice versa (Nathan et al., 2006). Histone H2BK11 acetylation and H2BS10 phosphorylation also share a similar cross-regulation (Ahn et al., 2006).

Cross-talk between histone modifications in different histone proteins has also been identified in *Saccharomyces cerevisiae*. One of the most well studied examples is that the methylation of H3K4 and H3K79, which depends on the monoubiquitination of H2BK123. The ubiquitin-conjugating enzyme Rad6, complexed with Bre1 and other components, is responsible for H2BK123 monoubiquitination (Robzyk et al., 2000; Wood et al., 2003a). Deubiquitination of H2BK123 is catalyzed by the enzyme Ubp8, a component of the SAGA HAT complex (Daniel et al., 2004; Henry et al., 2003). Surprisingly, further studies indicated that H2BK123 monoubiquitination is only required for di- and trimethylation of H3K4 and H3K79, but not for monomethylation (Briggs et al., 2002; Dehe et al., 2005; Dover et al., 2002; Ng et al., 2002b; Shahbazian et al., 2005; Sun and Allis, 2002; Wood et al., 2003a). Furthermore, H2BK123 monoubiquitination

does not depend on the methylation level of H3K4 or H3K79 (Briggs et al., 2002; Ng et al., 2002b). As mentioned previously, the methylation levels of H3K4 and H3K79 are critical to direct euchromatin and heterochromatin formation; therefore it has been proposed that regulation of H3K4 and H3K79 methylation by H2BK123 monoubiquitination is one of the major regulators of chromatin organization (Briggs et al., 2002; Fingerman et al., 2008; Ng et al., 2002b). Although many other factors involved in this cross-talk have already been identified, such as the Paf1 complex, Swd2, and the Bur1/Bur2 complex (Lee et al., 2007; Ng et al., 2003b; Wood et al., 2003b, 2005), the molecular mechanism of this cross-talk is still currently unknown. It is also intriguing to note that H2BK123 monoubiquitination does not regulate methylation of H3K36.

Recently, multiple lines of emerging evidence have suggested appealing potential cross-talk between histone H3 methylation and H3 acetylation in *Saccharomyces cerevisiae*. First, gene specific promoter studies revealed that the trimethylation of H3K4 is strongly correlated with hyperacetylation of histone H3 at the 5' promoter regions of active genes in euchromatin (Ng et al., 2003c; Santos-Rosa et al., 2002; Schneider et al., 2004). Later, genome-wide analysis of histone H3K4 methylation and H3 acetylation confirmed that this correlation is observed throughout the whole genome (Pokholok et al., 2005). This strong co-existence suggests potential cross-talk between H3K4 methylation and H3 acetylation. In fact, previous studies established the fact that histone H3K4 methylation can recruit HAT complexes, SAGA and SLIK (SAGA-like), through one of their shared components, the chromodomain-containing protein Chd1 (Pray-Grant et al., 2005). Another HAT complex, NuA3, can also be recruited by histone H3K4

methylation, due to one of the components, Yng1, binds to trimethylated H3K4 via its PHD domain (Lee and Workman, 2007; Martin et al., 2006a; Martin et al., 2006b; Taverna et al., 2006). Intriguingly, histone H3 acetylation can also promote H3 methylation, as the Gcn5 HAT is required for trimethylation of H3K4 (Govind et al., 2007; Jiang et al., 2007). Like H3K4, methylation of H3K36 promotes the recruitment of the NuA3 HAT complex (Martin et al., 2006b). Set2-catalyzed H3K36 methylation also recruits the Rpd3S HDAC complex through the chromodomain-containing protein Eaf3, a subunit of this complex, which subsequently deacetylates histones H3 and H4 in the coding regions of actively transcribed genes (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005). Further studies are still required to characterize this fascinating cross-talk in great detail.

Summary

Histone proteins are subject to various post-translational modifications. These modifications are regulated by different factors and dramatically affect the functions of histone proteins involved in most of the DNA-templated processes. Furthermore, cross-talk between histone modifications adds another layer of complexity to histone modifications. The histone code hypothesis has been proposed as a model to describe that complex patterns of histone modifications determine distinct biological outcomes. So far, thanks to a large body of studies on histone modifications, our understanding of the histone code has progressed greatly.

Many previous studies have been devoted to deciphering the functions of specific histone modifications on a certain histone residue. However, little is known about how

histone modifications function in combinatorial fashions. The work described in this thesis focuses on addressing the functions of histone H3 methylation and acetylation in a combinatorial manner and elucidating the potential genetic interaction between histone H3 acetylated and methylated lysine residues in *Saccharomyces cerevisiae*.

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CHAPTER TWO

SIMULTANEOUS MUTATION OF METHYLATED LYSINE RESIDUES IN HISTONE H3 CAUSES ENHANCED GENE SILENCING, CELL CYCLE DEFECTS, AND CELL LETHALITY IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

The methylation of specific lysine residues in histone H3 is integral to transcription regulation; however, little is known about how combinations of methylated lysine residues act in concert to regulate genome-wide transcription. We have systematically mutated methylated histone lysine residues in yeast, and found that the triple mutation of H3K4, H3K36, and H3K79 to arginine (H3 K4,36,79R) is lethal. The histone H3 K4,36,79R mutant causes a mitotic cell cycle delay and a progressive transcription defect that initiates in telomere regions and then spreads into the chromosome. This effect is mediated by the SIR silencing complex, as we observe increased binding of the SIR complex to genomic regions adjacent to yeast telomeres in the H3 K4,36,79R mutant, and deletion of *SIR2*, *SIR3*, or *SIR4* rescues the lethal phenotype. Curiously, a yeast strain in which the histone methyltransferase genes are simultaneously deleted is viable. Indeed, deletion of the histone methyltransferase genes can suppress the H3 K4,36,79R lethal phenotype. These and other data suggest that the cause of lethality may in part be due to the association of histone methyltransferase enzymes with a histone substrate that cannot be methylated.

My contribution to this project:

When I joined Dr. Wyrick's lab in 2004, this work was just initiated on systematically mutating all the methylated lysine residues in histone H3. Amy Rodriguez discovered that the triple mutation of H3K4, H3K36, and H3K79 to arginine (H3 K4,36,79R) is lethal in *S. cerevisiae*. I chose to further characterize the growth phenotype of this lethal mutant and confirmed that the mutant protein is stable. In collaboration with Amy Rodriguez, I aided in profiling the genome-wide transcription change of the H3 K4,36,79R mutant. In addition, I was responsible for the genetic screen to identify extragenic mutations that suppressed the lethal phenotype, and I conducted chromatin immunoprecipitation (ChIP) assays to measure the association of the SIR silencing complex with a series of DNA regions near telomere V-L. Finally, I characterized the effects of histone methyltransferases deletion on the H3 K4,36,79R lethal mutant. The data from these experiments, combined with the FACS analysis and microscopic analysis of the H3 K4,36,79R mutant done by Ana Kitazono, are described in this chapter.

INTRODUCTION

Eukaryotic chromosomes are organized into distinct domains of transcriptionally active euchromatin and repressive heterochromatin. These distinct chromatin domains profoundly influence the transcription, replication, repair, and segregation of their concomitant chromosomal sequences. Histone modifications, particularly histone lysine methylation, have important roles in initiating and maintaining these distinct chromatin domains. For example, methylation of histone H3 lysine-4 (H3K4), lysine-36 (H3K36), and lysine-79 (H3K79) directs the formation of euchromatin (reviewed in (Martin and Zhang, 2005)).

In the yeast *Saccharomyces cerevisiae*, the euchromatin-specific lysine residues are methylated by three histone lysine methyltransferases: Set1 (H3K4), Set2 (H3K36), and Dot1 (H3K79). These methyltransferases are recruited by the transcription elongation machinery, and their subsequent methylation of histone H3 is associated with actively transcribed genes (reviewed in (Shilatifard, 2006; Sims et al., 2004)). However, while H3K4, H3K36, and H3K79 methylation is intricately coupled with transcription elongation, mutations in these residues, or their cognate histone methyltransferases, have relatively little effect on cell viability and gene expression. Deletion of *SET1* results in decreased mRNA levels for a large number of genes (Boa et al., 2003; Santos-Rosa et al., 2002), in accordance with its proposed role in transcription elongation and euchromatin formation, but the magnitude of these transcriptional defects is relatively minor. In contrast, Set2-catalyzed methylation appears to primarily repress transcription by recruiting the Rpd3 histone deacetylase complex (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005).

These studies suggest that histone H3 methylation plays only a modest role in transcription elongation and euchromatin formation in *S. cerevisiae*. We have investigated this possibility by systematically mutating the methylated lysine residues in histone H3. Surprisingly, we find that the triple mutation of H3K4, H3K36, and H3K79 to arginine (H3 K4,36,79R) is lethal in *S. cerevisiae*. Our data indicate that this lethal phenotype is not due to a defect in histone H3 stability or its incorporation in the nucleosome, but instead due to a progressive gene expression defect that initiates in telomeric heterochromatin and then spreads into the chromosome. We show that the H3 K4,36,79R mutation elicits the binding of the heterochromatin-associated SIR (Silent Information Regulator) silencing complex (Guarente, 1999) into regions of euchromatin adjacent to yeast telomeres. In addition, mutations in SIR complex rescue this lethal phenotype. Curiously, mutations in the histone methyltransferase genes (e.g., *SET1*) also rescue the lethal H3 K4,36,79R mutant. These and other data suggest the cause of lethality may in part be due to the association of histone methyltransferase enzymes with a histone substrate that cannot be methylated.

MATERIALS AND METHODS

Yeast strain and growth conditions. A complete list of yeast strains used in this study is listed in Table 2-1. In general, yeast strains were propagated according to standard procedures in either rich media (YPD) or in appropriate selective media (SC). For the growth and viability assays, yeast cells were grown to mid-log phase in selective media containing galactose and then shifted to selective media containing glucose (see Yeast strain and growth conditions). Cell density was monitored by OD_{600} at consecutive time points, and later normalized to the highest starting OD_{600}. Each growth was performed in triplicate. For the viability assay, after the shift, approximately 500 yeast cells from glucose media at consecutive time points were plated on galactose plates. Colonies on the plates were counted after 72 hours incubation. Each viability assay was repeated three times. For the spotting assay, yeast cells were grown to mid-log phase, normalized by OD_{600}, 5-fold serially diluted, and spotted on appropriate SC plates or SC plates containing 5-fluoroorotic acid (5-FOA) (Zymo Research). All spotted plates were incubated at 30°C. SC plates were photographed after 48 hours incubation and 5-FOA plates were photographed after 72-96 hours. All spotting assays were repeated to ensure reproducibility.

Plasmid construction and site-directed mutagenesis. Details of plasmid construction are available upon request. Each histone H3 mutant was generated from plasmid pJW028 by site-directed mutagenesis (QuikChange Kit, Stratagene). The *set1-N1016Q* and *set1-C1068A* mutants were generated from plasmid pJW048 (Fingerman et al., 2005) by site-directed mutagenesis. All mutants were confirmed by DNA sequencing. The complete list of mutagenic primer sequences is listed in Table 2-2.

Yeast growth conditions for genome-wide expression profiling. For genome-wide expression analysis of the H3 K4,36,79R mutant time course, duplicate or triplicate cultures of the H3 K4,36,79R mutant strain (AMY102) were grown in selective media containing galactose until mid-log phase, and then shifted to selective media containing glucose, and harvested at the indicated time point (0, 6, 9 hours) following the shift to glucose media. The initial cell density following the shift to glucose media was carefully chosen so that the final OD_{600} for all time points was 0.5 - 0.7. Wild-type yeast cultures (AMY101) were grown in parallel with the mutants and harvested at 0, 6, and 9 hours post-shift. For the microarray analysis of the H3 K4,79R mutant, triplicate mutant and wild-type yeast cultures were grown in parallel to a final OD_{600} of 0.5-0.7 in YPD media. All the cell cultures were harvested as described previously (Holstege et al., 1998).

Genome-wide expression profiling. Total RNA was isolated from each yeast culture. Equal amounts of five exogenous poly-A control RNAs were added to each total RNA sample (Holstege et al., 1998). The total RNA samples were used to prepare complementary DNA and biotin-cRNA, as previously described (Martin et al., 2004). The cRNA was then hybridized to a single S98 genome oligonucleotide array and scanned following standard protocols (Affymetrix). Intensities were captured using GeneChip software (Affymetrix) and a single raw expression level for each gene was determined. Complete data sets are available at http://wyrick.sbs.wsu.edu/HistoneMethylation/. The data discussed in this publication have also been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/), and are accessible through GEO Series accession numbers GSE6319, GSE6326, GSE6327, GSE6328, and GSE6331.

Data analysis. The data from each chip were normalized using a normalization factor calculated from the signal intensities of five exogenous poly-A controls, as previously described (Holstege et al., 1998). A change in mRNA levels was deemed significant based on the following criteria: (A) the average fold change up or down was greater than 2 fold, (B) the fold change (up or down) in each replicate experiment was greater than 1.5 fold; and (C) the absolute intensity change was above background levels. See Martin *et al*. (Martin et al., 2004) for more details.

Telomere-proximal gene analysis and statistics. For the chromosome plots, all yeast genes were ordered according to their distance from a telomere, and divided into consecutive 50-gene bins. The fraction of genes down-regulated in the mutant strain and the average distance of the genes from the telomere were plotted for each bin. Only those genes within ~210 kb of a telomere were plotted. Statistical analysis of enrichment for telomere-proximal genes (0 to 10 kb from a telomere) was calculated using a hypergeometric probability distribution (Martin et al., 2004).

Micrococcal nuclease (MNase) digestion assay. MNase digestion assays were performed as described previously (Kent and Mellor, 1995). Briefly, yeast cells were grown and harvested at similar conditions as genome-wide expression experiments. Cells were spheroplasted with zymolyase (Zymo Research) and treated with doubling concentrations of MNase as indicated. After digestion, DNA was purified and resolved on a 1.5% agarose TBE gel. The gels were stained with ethidium bromide and photographed.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were performed as described previously (Kuo and Allis, 1999), with slight modifications. Briefly, yeast cells

were grown to similar conditions as genome-wide expression experiments and cross-linked with 1% formaldehyde. Cells were lysed by glass beads and sonicated to shear the chromatin to fragments sizes of 150 to 400 bp. Cross-linked chromatin fragments were immunoprecipitated with anti-Myc antibody (Biosource, AHO0052) or anti-Sir2 antibody (Santa Cruz, sc-6666) bound to magnetic beads (Dynal Biotech). After the cross-links were reversed, the DNA fragments were extracted for PCR. Taq DNA polymerase (New England Biolabs) and appropriate primer pairs were used in the PCR amplification reactions. PCR products were resolved on 2% agarose TBE gels, stained with ethidium bromide and quantified using GelDoc EQ imager with Quantity One software (Bio-Rad). The complete list of ChIP primer sequences is listed in Table 2-2.

Cell cycle analysis. Cells were grown in selective media containing 2% galactose and 2% raffinose at 32°C, washed once, and resuspended into media containing glucose to a final OD_{600} of 0.35. Further incubations were done at 32°C and aliquots taken every hour were either fixed with 3.7% formaldehyde or 70% ethanol. The latter samples were used for the flow cytometry analysis as follows. Approximately $5x10^6$ cells were washed once with 50 mM Tris [pH 8.0], incubated for 3.5 hours at 50°C in 50 mM Tris [pH 8.0] containing 0.25 mg/ml of RNase A (Sigma). The cells were washed once with water, pelleted, resuspended in 0.5 ml of 5 mg/ml pepsin in 0.05 M HCl and incubated at 37 °C for 1 hour. The cells were pelleted, resuspended in 1 x PBS, lightly sonicated, pelleted, resuspended in 1 ml of 2.5 μM Sytox Green (Molecular Probes) or 0.05 mg/ml propidium iodide (Sigma) in 1 x PBS, incubated for 1 hour at 23°C or overnight at 4°C and then analyzed in a BD Facscalibur flow cytometer (Beckton Dickinson). For each sample, data from at least 30,000 events were collected for analysis. Cells from these

preparations, and formaldehyde fixed cells stained with DAPI were used for microscopic analysis of cell morphology and cell-cycle profile.

Table 2-1: List of yeast strains and genotypes used in this study

Strain	Experiment	Genotype
WY121	H3	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pJL001 *(CEN6 URA3 HHT2 HHF2)*
AMY201	H3	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW028 *(CEN6 ADE2 HHT2 HHF2)*
AMY202	H3 K36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM003 *(CEN6 ADE2 HHF2 hht2 K36,79R)*
AMY203	H3 K4,36R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM005 *(CEN6 ADE2 HHF2 hht2 K4,36R)*
AMY204	H3 K4,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM006 *(CEN6 ADE2 HHF2 hht2 K4,79R)*
AMY205	H3 K4,36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM007 *(CEN6 ADE2 HHF2 hht2 K4,36,79R)*
AMY206	H3 K4,36,79G	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM018 *(CEN6 ADE2 HHF2 hht2 K4,36,79G)*
AMY216	H3 K9,36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAR027 *(CEN6 ADE2 HHF2 hht2 K9,36,79R)*
WY139	H3	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3*, plus pJW028 *(CEN6 ADE2 HHT2 HHF2)*

Table 2-1 (continued): List of yeast strains and genotypes used in this study

Strain	Experiment	Genotype
AMY001	H3 K36R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3*, plus pAM001 *(CEN6 ADE2 HHF2 hht2 K36R)*
AMY002	H3 K79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3*, plus pAM002 *(CEN6 ADE2 HHF2 hht2 K79R)*
AMY004	H3 K4R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3*, plus pAM004 *(CEN6 ADE2 HHF2 hht2 K4R)*
AMY006	H3 K4,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3*, plus pAM006 *(CEN6 ADE2 HHF2 hht2 K4,79R)*
WY151	Glucose shutdown	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pRM102 *(CEN4 URA3 pGAL10-HHT2 pGAL1-HHF2)*
AMY101	H3	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pRM102 *(CEN4 URA3 pGAL10-HHT2 pGAL1-HHF2)* pJW028 *(CEN6 ADE2 HHT2 HHF2)*
AMY102	H3 K4,36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pRM102 *(CEN4 URA3 pGAL10-HHT2 pGAL1-HHF2)* pAM007 *(CEN6 ADE2 HHF2 hht2 K4,36,79R)*
AMY103	H3 Δ4-45	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pRM102 *(CEN4 URA3 pGAL10-HHT2 pGAL1-HHF2)* pRM445 *(CEN4 TRP1 HHF2 hht2 Δ4-45)*
WY160	set1Δset2Δdot1Δ	*MAT**a** ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 SET1::KanMX4 SET2::LEU2 DOT1::HIS3*

Table 2-1 (continued): List of yeast strains and genotypes used in this study

Strain	Experiment	Genotype
YJ113	set1Δ H3	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::TRP1,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW028 *(CEN6 ADE2 HHT2 HHF2)*
YJ413	set1Δ H3 K36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::TRP1,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM003 *(CEN6 ADE2 HHF2 hht2 K36,79R)*
YJ1213	set1Δ H3 K4,36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::TRP1,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM007 *(CEN6 ADE2 HHF2 hht2 K4,36,79R)*
YJ613	set1Δ Set1 H3	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW048 *(TRP1 ADH1 FLAG-SET1)* pJW028 *(CEN6 ADE2 HHT2 HHF2)*
YJ713	set1Δ Set1 H3 K36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW048 *(TRP1 ADH1 FLAG-SET1)* pAM003 *(CEN6 ADE2 HHF2 hht2 K36,79R)*
YJ1313	set1Δ Set1 H3 K4,36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW048 *(TRP1 ADH1 FLAG-SET1)* pAM007 *(CEN6 ADE2 HHF2 hht2 K4,36,79R)*
YJ167	set1Δ Set1-N1016Q H3	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW050 *(TRP1 ADH1 FLAG-set1-N1016Q)* pJW028 *(CEN6 ADE2 HHT2 HHF2)*

Table 2-1 (continued): List of yeast strains and genotypes used in this study

Strain	Experiment	Genotype
YJ267	set1Δ Set1-N1016Q H3 K36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW050 *(TRP1 ADH1 FLAG-set1-N1016Q)* pAM003 *(CEN6 ADE2 HHF2 hht2 K36,79R)*
YJ567	set1Δ Set1-N1016Q H3 K4,36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW050 *(TRP1 ADH1 FLAG-set1-N1016Q)* pAM007 *(CEN6 ADE2 HHF2 hht2 K4,36,79R)*
YJ168	set1Δ Set1-C1068A H3	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW051 *(TRP1 ADH1 FLAG-set1-C1068A)* pJW028 *(CEN6 ADE2 HHT2 HHF2)*
YJ268	set1Δ Set1-C1068A H3 K36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW051 *(TRP1 ADH1 FLAG-set1-C1068A)* pAM003 *(CEN6 ADE2 HHF2 hht2 K36,79R)*
YJ568	set1Δ Set1-C1068A H3 K4,36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW051 *(TRP1 ADH1 FLAG-set1-C1068A)* pAM007 *(CEN6 ADE2 HHF2 hht2 K4,36,79R)*
YJ813	set1Δ Set1-RRMΔ H3	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW049 *(TRP1 ADH1 FLAG-set1-RRMΔ)* pJW028 *(CEN6 ADE2 HHT2 HHF2)*
YJ1113	set1Δ Set1-RRMΔ H3 K36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW049 *(TRP1 ADH1 FLAG-set1-RRMΔ)* pAM003 *(CEN6 ADE2 HHF2 hht2 K36,79R)*

Table 2-1 (continued): List of yeast strains and genotypes used in this study

Strain	Experiment	Genotype
YJ1413	set1Δ Set1-RRMΔ H3 K4,36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET1::KanMX4,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pJW049 *(TRP1 ADH1 FLAG-set1-RRMΔ)* pAM007 *(CEN6 ADE2 HHF2 hht2 K4,36,79R)*
YJ514	set2Δ H3 K4,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET2::TRP1,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM006 *(CEN6 ADE2 HHF2 hht2 K4,79R)*
YJ814	set2Δ H3 K4,36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SET2::TRP1,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM007 *(CEN6 ADE2 HHF2 hht2 K4,36,79R)*
YJ212	dot1Δ H3 K4,36R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 DOT1::TRP1,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM005 *(CEN6 ADE2 HHF2 hht2 K4,36R)*
YJ612	dot1Δ H3 K4,36,79R	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 DOT1::TRP1,* plus pJL001 *(CEN6 URA3 HHT2 HHF2)* pAM007 *(CEN6 ADE2 HHF2 hht2 K4,36,79R)*
YJ057	H3 Sir4-9xMyc	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SIR4-9xMyc::TRP1* plus pJW028 *(CEN6 ADE2 HHT2 HHF2)*
YJ058	H3 K4,79R Sir4-9xMyc	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SIR4-9xMyc::TRP1* plus pAM006 *(CEN6 ADE2 HHF2 hht2 K4,79R)*
YJ065	H3 Sir4-9xMyc	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SIR4-9xMyc::TRP1* plus pRM102 *(CEN4 URA3 pGAL10-HHT2 pGAL1-HHF2)* pJW028 *(CEN6 ADE2 HHT2 HHF2)*

Table 2-1 (continued): List of yeast strains and genotypes used in this study

Strain	Experiment	Genotype
YJ066	H3 K4,36,79R Sir4-9xMyc	*MAT**a** ade2-101 trp1Δ901 leu2-3,112 his3Δ200 ura3-52 lys2-801 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SIR4-9xMyc::TRP1* plus pRM102 *(CEN4 URA3 pGAL10-HHT2 pGAL1-HHF2)* pAM007 *(CEN6 ADE2 HHF2 hht2 K4,36,79R)*

Table 2-2: Oligonucleotides used in this study

Name	Sequence	Description
OJLW1B	CCACAATGGCCAGAACTAGACAAACAG CTAGAAAATC	Mutate H3 lysine 4 to arginine
OJW18	GATTTTCTAGCTGTTTGTCTAGTTCTGGC CATTGTGG	Mutate H3 lysine 4 to arginine
OJW13	CCGGTGGTGTTAGGAAGCCTCACAGATA TAAGC	Mutate H3 lysine 36 to arginine
OJW14	GCTTATATCTGTGAGGCTTCCTAACACC ACCGG	Mutate H3 lysine 36 to arginine
OJW15	CGCTCAAGATTTCAGGACCGACTTGAGA TTTCAATC	Mutate H3 lysine 79 to arginine
OJW16	GATTGAAATCTCAAGTCGGTCCTGAATC TTGAGCG	Mutate H3 lysine 79 to arginine
OJLW1	CTCCACAATGGCCAGAACTGGACAAAC AGCTAGAAAATCC	Mutate H3 lysine 4 to glycine
OJLW7	GGATTTTCTAGCTGTTTGTCCAGTTCTGG CCATTGTGGAG	Mutate H3 lysine 4 to glycine
OAM10	CATCTACCGGTGGTGTTGGGAAGCCTCA CAGATATAAG	Mutate H3 lysine 36 to glycine
OAM11	CTTATATCTGTGAGGCTTCCCAACACCA CCGGTAGATG	Mutate H3 lysine 36 to glycine
OAM8	GGTCAGAGAAATCGCTCAAGATTTCGGT ACCGACTTGAGATTTC	Mutate H3 lysine 79 to glycine
OAM9	GAAATCTCAAGTCGGTACCGAAATCTTG AGCGATTTCTCTGACC	Mutate H3 lysine 79 to glycine
OAM41	GACAAACAGCTAGAGGTGGCACTGGTG GTGGTGC	Mutate H3 lysine 9 to arginine
OAM42	GCACCACCACCAGTGCCACCTCTAGCTG TTTGTC	Mutate H3 lysine 9 to arginine
OJLW2	GTTCTTTTATATAGGACC	Sequence to confirm H3 mutations
OJLW3	AGATTGAAATCTCAAGTC	Sequence to confirm H3 mutations
OAM24	AAGGAGGTCACCAGTAATTGTGCGCTTT GGTTACATTTTGTTGTACAGTAAGATTG TACTGAGAGTGCAC	PCR amplify *HIS3* gene for *DOT1* deletion
OAM25	TATTTCTACTTAGTTATTCATACTCATCG TTAAAAGCCGTTCAAAGTGCCCTGTGCG GTATTTCACACCG	PCR amplify *HIS3* gene for *DOT1* deletion
OAM26	TAACATTCCTTATTTGTTGAATCTTTATA AGAGGTCTCTGCGTTTAGAGACGTACGC TGCAGGTCGAC	PCR amplify KanMX4 gene for *SET1* deletion

Table 2-2 (continued): Oligonucleotides used in this study

Name	Sequence	Description
OAM45	ACCTTTTGCTGGAAAGCAACGATATGTTAAATCAGGAAGCTCCAAACAAAATCGATGAATTCGAGCTCG	PCR amplify KanMX4 gene for *SET1* deletion
OAM28	CTTCCTTTGGGACAGAAAACGTGAAACAAGCCCCAAATATGCATGTCTGGAGATTGTACTGAGAGTGCAC	PCR amplify *LEU2* gene for *SET2* deletion
OAM29	AGTCGTGCTGTCAAACCTTTCTCCTTTCCTGGTTGTTGTTTTACGTGATCCTGTGCGGTATTTCACACCG	PCR amplify *LEU2* gene for *SET2* deletion
OAM30	CGGCTAAAGACCGTAGAGTGACTTT	Sequence to confirm *DOT1* deletion
OAM31	GGTGAGGAGAAATTATCTGCTCCTA	Sequence to confirm *DOT1* deletion
OAM32	CGATTTCTTACTAACGTAAACAACG	Sequence to confirm *SET1* deletion
OAM33	ATGCTGTCGGTAGCCTAAAGAA	Sequence to confirm *SET1* deletion
OAM34	TGTAAATTTCCCGATGCGGTAA	Sequence to confirm *SET2* deletion
OAM35	AAAATATGTGTAGAGGACGGTGAAT	Sequence to confirm *SET2* deletion
YJIN28	TAACATTCCTTATTTGTTGAATCTTTATAAGAGGTCTCTGCGTTTAGAGAAGATTGTACTGAGAGTGCAC	PCR amplify *TRP1* gene for *SET1* deletion
YJIN29	ACCTTTTGCTGGAAAGCAACGATATGTTAAATCAGGAAGCTCCAAACAAACTGTGCGGTATTTCACACCG	PCR amplify *TRP1* gene for *SET1* deletion
YJIN37	TCGGTAGACACATTCAAACCATTTTTCCCTCATCGGCACATTAAAGCTGGAGATTGTACTGAGAGTGCAC	PCR amplify *TRP1* gene for *SIR2* deletion
YJIN38	TGTAAATTGATATTAATTTGGCACTTTTAAATTATTAAATTGCCTTCTACCTGTGCGGTATTTCACACCG	PCR amplify *TRP1* gene for *SIR2* deletion
OAR69	CATGTGTACATAGGCATATCTATGGCGGAAGTGAAAATGAATGTTGGTGGAGATTGTACTGAGAGTGCAC	PCR amplify *TRP1* gene for *SIR3* deletion
OAR70	TTACAGGGGTTTAAGAAAGTTGTTTTGTTCTAACAATTGGATTAGCTAAACTGTGCGGTATTTCACACCG	PCR amplify *TRP1* gene for *SIR3* deletion
YJIN39	AAAAAAGGAAGCTTCAACCCACAATACCAAAAAAGCGAAGAAAACAGCCAAGATTGTACTGAGAGTGCAC	PCR amplify *TRP1* gene for *SIR4* deletion

Table 2-2 (continued): Oligonucleotides used in this study

Name	Sequence	Description
YJIN40	GAAAACGACAAAGAAAAACAGGGTACACTTCGTTACTGGTCTTTTGTAGACTGTGCGGTATTTCACACCG	PCR amplify *TRP1* gene for *SIR4* deletion
YJIN14	AAAGAATTCACATCTAGCACTCCTTCCAA	Sequence to confirm *SIR2* deletion
YJIN15	AAAGGTACCCCAACGCCTTCTAAATAGTTCA	Sequence to confirm *SIR2* deletion
YJIN16	AAAGAGCTCAAGTAGAGAGTTGACAGTGTTAAGC	Sequence to confirm *SIR3* deletion
YJIN17	AAAACTAGTTATTATCGCGCAGGTGAGAG	Sequence to confirm *SIR3* deletion
YJIN18	AAAGAATTCGTATGTGAGTACATATATCCGC	Sequence to confirm *SIR4* deletion
YJIN19	AAAGGTACCCAAATCTAATAGTGGCAAGG	Sequence to confirm *SIR4* deletion
YJIN47	CAGGGGTTTAAGAAAGTTGT	Sequence *SIR3* suppressor
YJIN48	CCAGAGTCACAATGGAATGA	Sequence *SIR3* suppressor
YJIN49	AACTCTTCAGAACAGGAATC	Sequence *SIR3* suppressor
YJIN50	TAACAGATTCCTCCCAATTG	Sequence *SIR3* suppressor
YJIN51	TACAAATGTCCCGAAAGCAA	Sequence *SIR3* suppressor
YJIN52	GTCTACAAAAGGGTAAATTG	Sequence *SIR3* suppressor
YJIN53	CGCTCTTATTTACTTCGTTG	Sequence *SIR3* suppressor
YJIN54	AGATGAAGAGCATGCTTTTC	Sequence *SIR3* suppressor
YJIN130	CCAATTGCTCGAGAGATTTC	ChIP primer pair for *ACT1*
YJIN131	CATGATACCTTGGTGTCTTG	ChIP primer pair for *ACT1*
YJIN134	GTACAGTGGTACCTTCGTGTTATC	ChIP primer pair 1
YJIN135	CAGATTGCGCTGGGAGTTACC	ChIP primer pair 1
YJIN136	TCATTAATATTACTGTAGCGAGGAAG	ChIP primer pair 2
YJIN137	GGTTTACAGTACGAAGGCAACAGGAG	ChIP primer pair 2

Table 2-2 (continued): Oligonucleotides used in this study

Name	Sequence	Description
YJIN138	GTTACATTTGCTATCTCATTTTCAGGTC	ChIP primer pair 3
YJIN139	CTTCATAACAGATTCAACTTTTCAGG	ChIP primer pair 3
YJIN140	CAGAACATACCCTAGCAACCATCGGC	ChIP primer pair 4
YJIN141	GCTTCTTTTCATAGTAACCCAATAGG	ChIP primer pair 4
YJIN158	GCTCCCGAGATTATCAATGA	ChIP primer pair 5
YJIN159	AACCTTGTCTTCAATCCAGG	ChIP primer pair 5
YJIN154	GGTGGTATAGCCCGTTTCATTCAGCATT GTTGTGATCC	Mutate Set1 asparagine 1016 to glutamine
YJIN155	GGATCACAACAATGCTGAATGAAACGG GCTATACCACC	Mutate Set1 asparagine 1016 to glutamine
YJIN160	GACGAGGAAAGACTTCCTGCTTTATGTG GAGCACCTAATTG	Mutate Set1 cysteine 1068 to alanine
YJIN161	CAATTAGGTGCTCCACATAAAGCAGGAA GTCTTTCCTCGTC	Mutate Set1 cysteine 1068 to alanine
YJIN126	CATAAAACAATTGCGCCATACAAAACAT TCGTGGCTACAACTCGATATCCCTGTGC GGTATTTCACACCG	PCR amplify *TRP1* gene for *RPD3* deletion
YJIN127	CTTTTCTTCTTTTGTTTCACATTATTTATA TTCGTATATACTTCCAACTCAGATTGTAC TGAGAGTGCAC	PCR amplify *TRP1* gene for *RPD3* deletion
YJIN128	CAACTCAGAGCGTATAGGTA	Sequence to confirm *RPD3* deletion
YJIN129	GGACGAGACGTTTAGATAGT	Sequence to confirm *RPD3* deletion
YJIN61	ACAAATTGATGGAAAAAGATTTTCAAGT GAATAAGGAGATAAAACCGTATTCCGGT TCTGCTGCTAG	PCR for tagging *SIR4* with 9xMyc
YJIN62	AGAAAAACAGGGTACACTTCGTTACTGG TCTTTTGTAGAATGATAAAAAGCCTCGA GGCCAGAAGAC	PCR for tagging *SIR4* with 9xMyc
YJIN63	CGGTCTTTGTCAGATGAAGT	Sequence to confirm *SIR4-9xMyc* tagging
YJIN64	TGTAGCCAAAAGCCCAACTA	Sequence to confirm *SIR4-9xMyc* tagging

RESULTS

Systematic mutagenesis of histone H3 methylated lysine residues. To investigate whether histone H3 methylated lysine residues have redundant functions in transcription elongation and chromatin organization, we systematically mutated H3K4, H3K36, and H3K79, both individually and in all possible combinations. The phenotype of each histone H3 mutant was tested by transforming a yeast strain (WY121) with a plasmid bearing the mutant H3 allele, and subsequently removing the endogenous plasmid carrying the sole wild-type H3 allele by negative selection using 5-fluoroorotic acid (5-FOA). The results showed that each of the H3 double mutants (K4,36R; K4,79R; and K36,79R) was viable, though some mutants exhibited a slow growth phenotype (Figure 2-1A). However, the triple mutant, in which all three methylated lysine residues are mutated to arginine (H3 K4,36,79R), was lethal (Figure 2-1A).

This lethal phenotype was observed only when all three methylated lysine residues were mutated to arginine. For example, the triple glycine mutant (H3 K4,36,79G) was viable (Figure 2-1A). The arginine mutant is thought to mimic the hypomethylated lysine side chain, while the glycine mutant eliminates the side chain entirely, suggesting that the lethal phenotype may be due to replacing the methylated lysine residue with a hypomethylated mimic. Similarly, the H3 K9,36,79R mutant, in which two methylated lysine residues and one acetylated lysine residue (H3K9) were mutated to arginine, was viable (Figure 2-1A). This result suggests that the lethal phenotype was likely to be specific to methylated H3 lysine residues.

To further investigate this lethal phenotype, we employed a histone depletion strain (WY151) in which the sole copies of the histone H3 and H4 genes were regulated

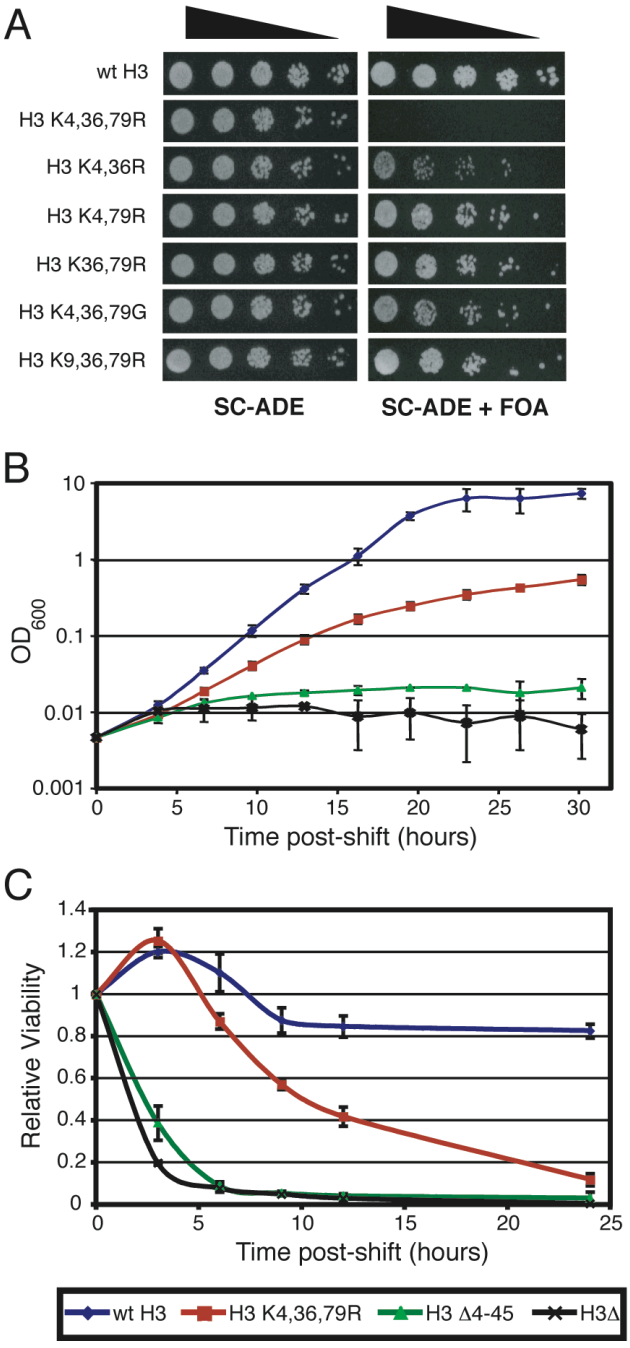

FIGURE 2-1: The histone H3 K4,36,79R mutant strain is inviable.

(**A**) The growth phenotypes of double and triple mutants in histone H3 methylated lysine residues were examined. 5-fold serial dilutions of each yeast strain (from about 10^4 cells)

were spotted on SC-ADE media or SC-ADE with 5-FOA as indicated. A wild-type histone H3 allele was present in each strain on SC-ADE plates, but was lost in SC-ADE plates with 5-FOA. (**B**) Growth rate of histone mutant strains. The cell density of strains containing the H3 K4,36,79R, H3 Δ4-45, H3Δ, or wild-type alleles of histone H3 was monitored by measuring the optical density at different time points following the depletion of wild-type histone H3. (**C**) Viability for each of the mutant strains shown in part B was determined by plating equal amounts of cells on galactose plates at different time points following the depletion of wild-type histone H3. Colonies formed on plates were scored after 72 hours incubation. Relative viability was calculated as the ratio of the colony number at certain time point to the 0 hour time point. Each error bar represents the standard deviation of three independent experiments.

by the GAL1-10 promoter (Durrin et al., 1992). Plasmids bearing the histone H3 mutant alleles (and wild-type histone H4) under the control of their endogenous promoters were transformed into the histone depletion strain, and the resulting growth defects were examined by shifting the cells to glucose media. The glucose shutdown represses the transcription of the wild-type histone H3 gene, leaving the H3 mutant as the sole source of histone H3 in the cell.

The growth curve of the histone H3 K4,36,79R mutant is shown in Figure 2-1B. The H3 K4,36,79R mutant displays a growth defect at 6 hours post-shift when compared to wild-type, and this growth defect is exacerbated over time, until the cells gradually cease growing by 25 - 30 hours post-shift. This phenotype is in sharp contrast with the rapid growth arrest observed in the H3 Δ4-45 mutant, which deletes part of the histone H3 core domain (Durrin et al., 1992), and in the histone H3 (and H4) depletion strain (H3Δ, see Figure 2-1B). We also measured the loss of viability of the histone H3 mutants by returning the glucose-treated cells to the permissive galactose media at various time points post-shift. Figure 2-1C shows that the H3 K4,36,79R mutant shows a gradual decrease in viability beginning at 6 - 9 hours post-shift, and is almost completely inviable by 24 hours. In contrast, the H3 Δ4-45 and H3 depletion mutants show rapid loss in cell viability 3 hours post-shift, and are almost completely inviable by 6 hours post-shift. Taken together, these results argue that the lethality of the H3 K4,36,79R mutant is not due to the loss or instability of histone H3 protein, as is the case in the H3 Δ4-45 and H3 depletion mutants, as the severity and kinetics of the H3 K4,36,79R growth defect are strikingly different from these other mutants.

The H3 K4,36,79R mutant is stable and competent for nucleosome assembly.

To directly test whether the H3 K4,36,79R mutant destabilizes the histone H3 protein, we measured H3 protein levels at consecutive time points following glucose shutdown of wild-type H3 expression. Western blot analysis of yeast cell extracts indicated that histone H3 protein levels were relatively constant during the time course in the wild-type and H3 K4,36,79R mutant strains (Figure 2-2A). In contrast, the H3 Δ4-45 and H3 depletion mutants showed a rapid loss in histone H3 protein. These data suggest that the H3 K4,36,79R mutant protein is stable *in vivo*; however, an alternative explanation for these data is that the wild-type H3 protein may continue to be expressed in the H3 K4,36,79R mutant strain, despite the presence of glucose in the media.

To rule out this possibility, we measured the levels of H3K4 methylation in this strain during the glucose shutdown time course. As shown in Figure 2-2B, the anti-H3K4 dimethylation (me2) and trimethylation (me3) antibodies readily detected methylation of wild-type histone H3, but no signal was detected in the H3 K4R mutant strain, as expected. Both wild-type and K4R mutant histone H3 were detected by the anti-H3 antibody (Figure 2-2B). Western blot analysis of yeast cell extracts showed that while histone H3 is present at constant levels in the H3 K4,36,79R mutant in glucose shutdown time course, H3K4 monomethylation, dimethylation and trimethylation were almost completely absent by the 6 hour time point (Figure 2-2C, data not shown). We interpret this result to mean that the H3 K4,36,79R mutant protein had almost completely replaced the wild-type histone H3 by 6 hours post-shift. While it remains a formal possibility that the H3 wild-type histone is present but unmethylated at K4, this possibility seems unlikely, particularly since the wild-type H3 strain has normal H3K4 di- and

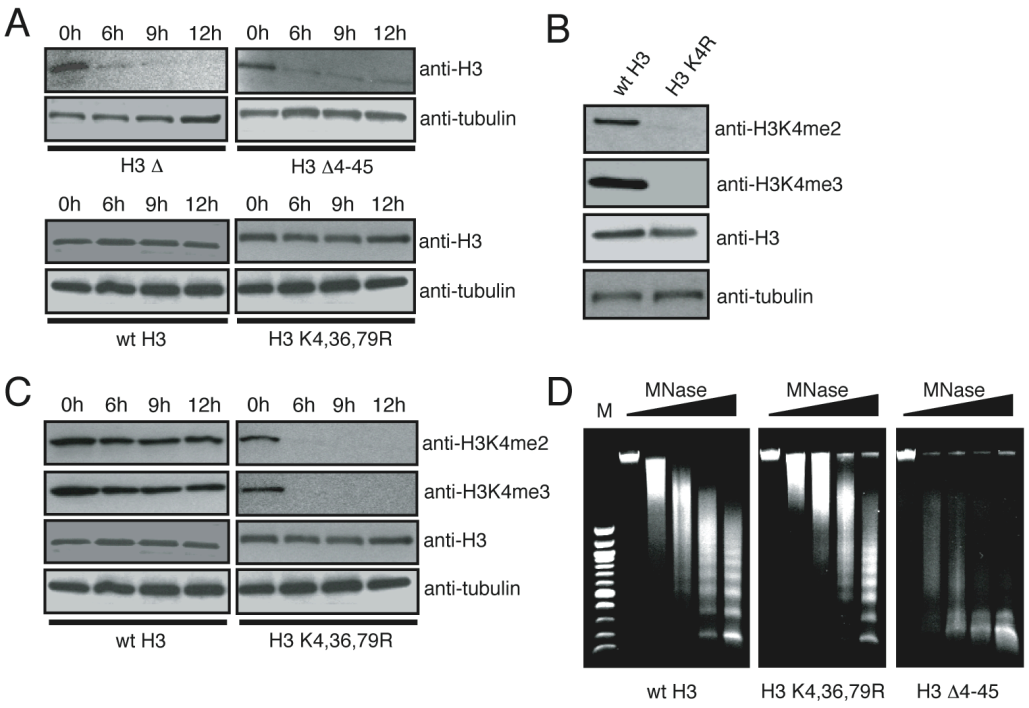

FIGURE 2-2: The histone H3 K4,36,79R mutant protein is stable and competent for nucleosome assembly.

(**A**) The protein stability of different histone mutants was measured by western blot analysis. Cell extracts from each of the mutants at four time points following the depletion of wild-type histone H3 were resolved by SDS-PAGE and probed with anti-H3 antibody. As an internal loading control, anti-α tubulin antibody was used. (**B**) Antibodies against di- and trimethylated histone H3K4 do not detect the histone H3 K4R mutant while antibody against histone H3 detects both wild-type histone H3 and H3 K4R mutant. Cell extracts from wild-type and histone H3 K4R mutant strains were analyzed

with antibodies as indicated. The level of α-tubulin subunit was used as an internal loading control. (**C**) Loss of histone H3K4 methylation at different time points following the depletion of wild-type histone H3. Cell extracts from wild-type histone H3 and the H3 K4,36,79R mutant at different time points following the depletion of wild-type histone H3 were examined by western blot analysis with appropriate antibodies, as in part B. (**D**) Genomic chromatin structure of the different histone mutants and wild-type strains was examined by MNase digestion assays. MNase digestion profiles for cells harvested at the 6 hour time point following the depletion of wild-type histone H3 are shown as a representative. Subsequent lanes had doubling concentration of MNase from 0 to 37.5 units/ml. M stands for the 100 bp DNA molecular weight ladder.

trimethylation levels throughout the glucose shutdown time course (Figure 2-2C).

To test whether the H3 K4,36,79R mutant can be assembled into the nucleosome, we used a Micrococcal nuclease (MNase) digestion assay to characterize the nucleosome content in the H3 mutant strain. MNase digestions were performed on the histone H3 K4,36,79R mutant at 6 hours following the shift to glucose media, when most of the cellular population of histone H3 is composed of the H3 K4,36,79R mutant protein (see Figure 2-2C). Comparison of the MNase digestion pattern of the H3 K4,36,79R mutant and wild-type strains showed no significant alterations in nucleosome content or bulk genomic chromatin structure (Figure 2-2D). In contrast, the H3 Δ4-45 mutant strain showed a general loss in nucleosome content (Figure 2-2D), presumably due to a defect in nucleosome assembly. In summary, these data strongly suggest that the histone H3 K4,36,79R mutant protein is stable and competent for proper nucleosome assembly. However, these results do not rule out the possibility that the H3 K4,36,79R mutant may have more subtle effects on nucleosome positioning and chromatin structure, which would not be detected by the genomic MNase digestion assay.

The H3 K4,36,79R mutant causes gene repression, beginning with genes located in telomeric heterochromatin. Previous studies have shown that methylation of H3K4, H3K36, and H3K79 plays an important role in transcriptional regulation (Sims et al., 2004). To characterize the effects of the H3 K4,36,79R mutant on genome-wide transcription, we isolated RNA from parallel cultures of wild-type and H3 K4,36,79R mutant strains at 0, 6, and 9 hours following the shift to glucose media. The resulting genome-wide changes in mRNA levels were profiled using Affymetrix oligonucleotide arrays, and are summarized in Table 2-3. The data for the 0 hour time point indicates that

the expression of many genes was altered prior to the shift to glucose media, when both the wild-type and mutant histone H3 proteins are expressed. This result suggests that even the partial replacement of wild-type histone H3 with the histone H3 K4,36,79R mutant can affect gene expression. Subsequent time points show a general trend of progressively greater numbers of genes down-regulated in the H3 K4,36,79R mutant, culminating in 361 genes with decreased expression levels by the 9 hour time point.

Chromosome plots of the genome-wide expression data showed that at each time point a large fraction of the genes down-regulated in the H3 K4,36,79R mutant were clustered near yeast telomeres (Figure 2-3). For example, 32% of genes located in telomere-proximal regions (defined as a location within 10 kb of a telomere) showed decreased mRNA levels in the H3 K4,36,79R strain at the 9 hour time point, compared to a genome-wide average of 6% ($P = 9.7 \times 10^{-13}$). Significant enrichment of telomere-proximal genes was also observed in the sets of down-regulated genes in the 0 hour ($P = 1.4 \times 10^{-7}$) and 6 hour time points ($P = 4.2 \times 10^{-16}$).

These data indicate that the H3 K4,36,79R mutant enhances telomeric gene silencing. Previous studies have shown that H3K4 and H3K79 methylation regulates telomeric silencing in yeast (Shilatifard, 2006; van Leeuwen and Gottschling, 2002), suggesting that the mutation of these residues alone may be responsible for the telomeric silencing effects observed in the H3 K4,36,79R mutant. To test this hypothesis, we used oligonucleotide arrays to profile the gene expression changes in a H3 K4,79R mutant. Analysis of the triplicate samples revealed that the mRNA levels of 61 genes were up-regulated and the mRNA levels of 26 genes were down-regulated in the H3 K4,79R mutant compared to wild-type. Further analysis shows that a significant fraction of the

Table 2-3: Genome-wide expression changes in the H3 K4,36,79R mutant time course

Time point (hrs)	Number of genes up-regulated	Number of genes down-regulated
0	13	33
6	5	74
9	30	361

genes down-regulated in the H3 K4,79R mutant were also down-regulated in the H3 K4,36,79R mutant at the 6 and 9 hour time points (data not shown). Nearly half of the genes down-regulated in the H3 K4,79R mutant are adjacent to yeast telomeres, a significant enrichment ($P = 7.1 \times 10^{-17}$). Indeed, the H3 K4,79R and H3 K4,36,79R mutants show strikingly similar effects on telomere-proximal gene expression (Figure 2-3). In contrast, the H3 K36R mutant has a modest effect on telomere-proximal gene expression (data not shown). This analysis indicates that the initial increase in telomere silencing observed in the H3 K4,36,79R time course is likely a consequence of mutating H3K4 and H3K79 to arginine.

In the 0 hour and 6 hour time points, relatively few genes outside of the telomere region are down-regulated in the H3 K4,36,79R mutant; however, in the 9 hour time point, many genes in euchromatin regions of the genome are down-regulated (Figure 2-3). We have also profiled the gene expression changes at 12 hour and 18 hour time points, and found that many more genes in euchromatin regions of yeast chromosomes are down-regulated at these later time points (data not shown). However, we are hesitant to draw conclusions from these data for two reasons. First, there is a considerable loss in the viability of the H3 K4,36,79R mutant at later time points (see Figure 2-1C). Hence, the gene expression changes we observe could be an indirect consequence of cell death. Second, there is considerable variability in the microarray data for these later time points between replicate experiments.

In summary, the genome-wide expression data show the following trend: at initial time points, the H3 K4,36,79R mutant represses the transcription of genes near telomere regions, while at later time points H3 K4,36,79R-mediated gene repression begins to

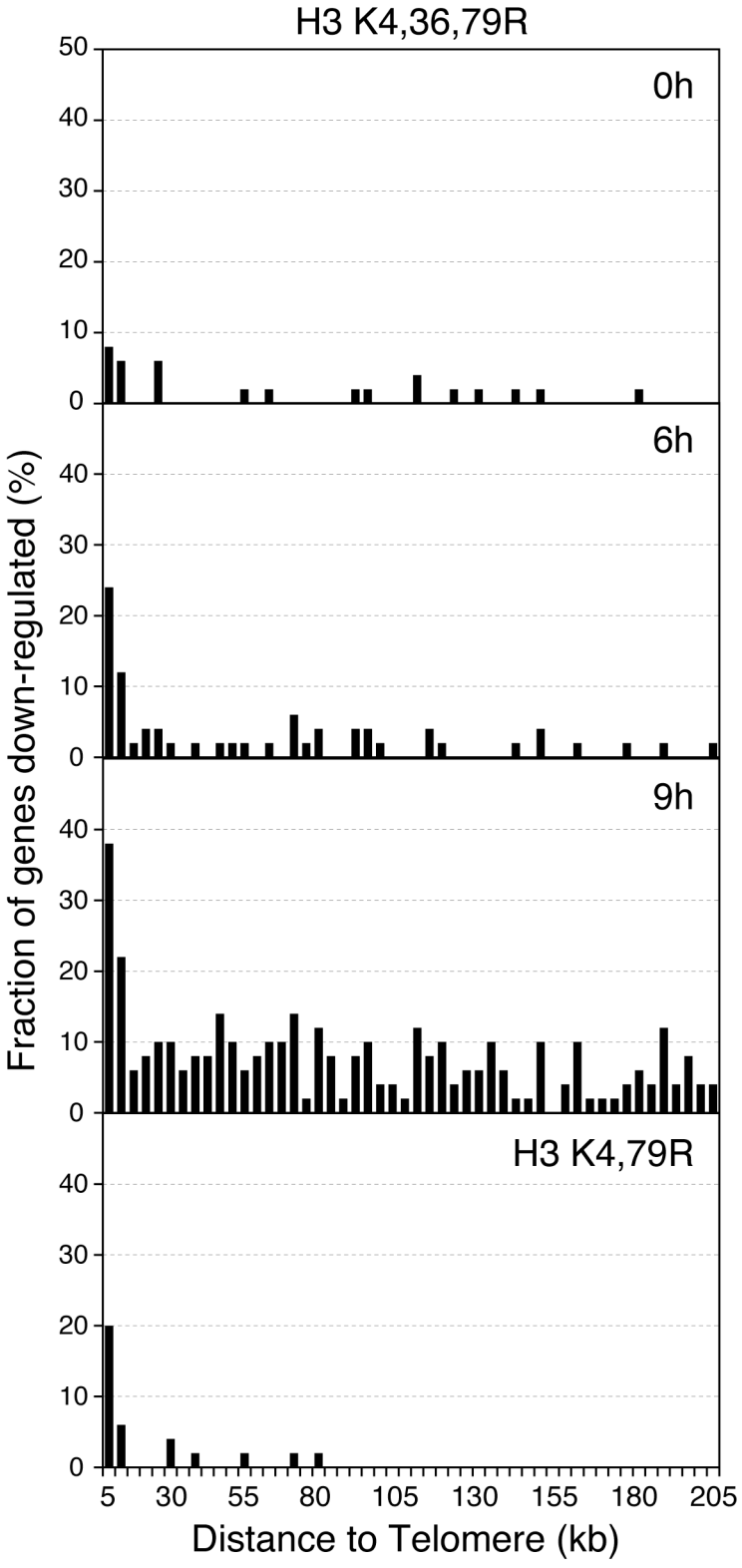
H3 K4,36,79R
0h
6h
9h
H3 K4,79R
Fraction of genes down-regulated (%)
0
10
20
30
40
50
5
30
55
80
105
130
155
180
205
Distance to Telomere (kb)

FIGURE 2-3: Effects of the histone H3 K4,36,79R mutant on genome-wide expression levels.

Chromosome plots of the microarray data from the H3 K4,36,79R mutant at different time points following the depletion of wild-type histone H3. For each time point, a histogram of the fraction of genes whose mRNA levels are down-regulated in H3 mutants is plotted as a function of their distance from a chromosome end. The microarray data for the H3 K4,79R mutant is also shown for comparison.

spread in the euchromatin regions of the chromosome. In contrast, the H3 K4,79R mutant represses gene transcription predominately at telomere proximal regions, and shows little effect on the expression of genes located elsewhere.

The H3 K4,36,79R mutant leads to aberrant cell cycle progression. Cell cycle display of the H3 K4,36,79R time course expression data revealed that a significant fraction of the genes down-regulated by the H3 K4,36,79R mutant are normally expressed during the G1 phase of the cell cycle (Figure 2-4A). This result suggested the possibility that the H3 K4,36,79R mutant may have a cell cycle defect resulting in fewer cells cycling into G1 phase. We tested this hypothesis by performing FACS analysis on propidium iodide stained H3 K4,36,79R mutant cells at consecutive time points following the shift to glucose media. The results indicated that there is a slight (~10%) but reproducible decrease in the number of unbudded (G1 phase) cells in the H3 K4,36,79R mutant compared to wild-type (Figure 2-4B), in accordance with the expression data. We also observed a broadening in the second (2N) peak in the H3 K4,36,79R mutant, which suggests slow progression through mitosis or cytokinesis in the mutant cells. The H3 Δ4-45 mutant, in contrast, shows an immediate G2/M arrest phenotype (Figure 2-4B), which is indicative of nucleosome depletion (Kim et al., 1988). Similar results were obtained from FACS analysis of Sytox Green-stained cells (data not shown).

At later time points (10-14 hours post-shift), there appeared to be significantly elevated numbers of rebudding cells in the H3 K4,36,79R mutant: we observed 11-16% multibudded cells in the H3 K4,36,79R mutant versus <2% multibudded cells in wild-type (Figure 2-5). We also observed more cells in anaphase in the H3 K4,36,79R mutant

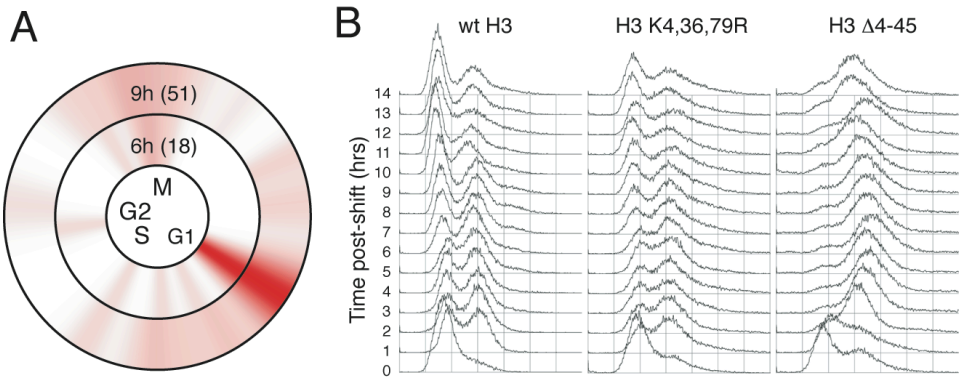

FIGURE 2-4: Cell cycle defects in the H3 K4,36,79R mutant strain.

(**A**) Cell cycle display (Simon et al., 2001) of the genes down-regulated during the H3 K4,36,79R mutant time course. The circles represent the cell cycle expression patterns of 800 cell cycle-regulated genes (Spellman et al., 1998). The intensity of red color indicates the number of genes that are normally expressed at that phase of the cell cycle which are down-regulated in the H3 K4,36,79R mutant strain. The total numbers of cell cycle genes that are down-regulated for each time point are listed in parenthesis. (**B**) FACS analysis was used to measure the cell cycle profile of the H3 K4,36,79R mutant strain following depletion of wild-type histone H3.

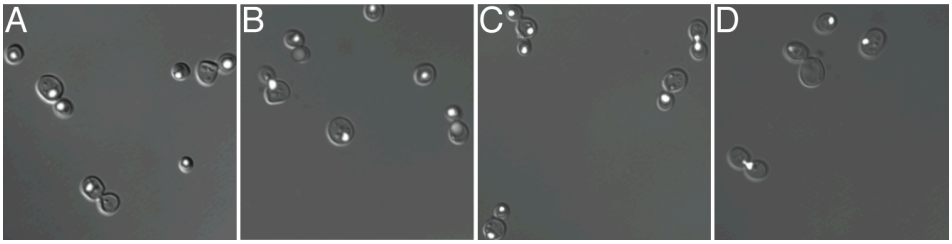

FIGURE 2-5: Cell cycle defects in the H3 K4,36,79R mutant strain.

(**A-B**) Representative images of DAPI stained wild-type cells at 12- and 14-hours post-shift, respectively. (**C-D**) Representative images of DAPI stained H3 K4,36,79R mutant cells at 12- and 14-hours post-shift, respectively.

(Figure 2-5). These data suggest that the H3 K4,36,79R mutation causes mitotic defects, especially anaphase delay, in a small but significant subpopulation of H3 K4,36,79R mutants.

The H3 K4,36,79R mutant enhances SIR complex binding to genomic regions adjacent to yeast telomeres. To gain insight into the mechanism underlying the H3 K4,36,79R lethality, we performed a genetic screen to identify extragenic mutations that suppressed the lethal phenotype (Figure 2-6A). We isolated and cloned one of the suppressors, which partially rescued the lethal phenotype, and found that the suppressing mutation resided in the *SIR3* gene. The suppressor mutant was a frame shift mutation caused by a single adenosine insertion into a stretch of 9 adenosines (nucleotides 1414 to 1422), resulting in the introduction of a premature stop codon in the Sir3 coding sequence following amino acid 485 (Figure 2-6B). The addition of a wild-type allele of the *SIR3* gene abolished suppression, demonstrating that the isolated *sir3* suppressor mutant is a recessive, loss-of-function mutation (data not shown). The Sir3 protein is a component of the SIR complex, which silences gene expression at yeast telomeres and at the silent mating loci. We systematically deleted each subunit of the SIR complex (*SIR2*, *SIR3*, and *SIR4*) in the H3 K4,36,79R mutant strain, and found that each of the deletions partially suppressed the H3 K4,36,79R lethal phenotype (Figure 2-7A). In contrast, deletion of *RPD3*, which encodes a histone deacetylase whose association with chromatin, is regulated by H3K36 methylation (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005), did not suppress the H3 K4,36,79R lethal phenotype. In summary, these results indicate that the SIR complex is required for the lethality of the H3 K4,36,79R mutant.

This finding suggests that the transcriptional repression observed in the H3

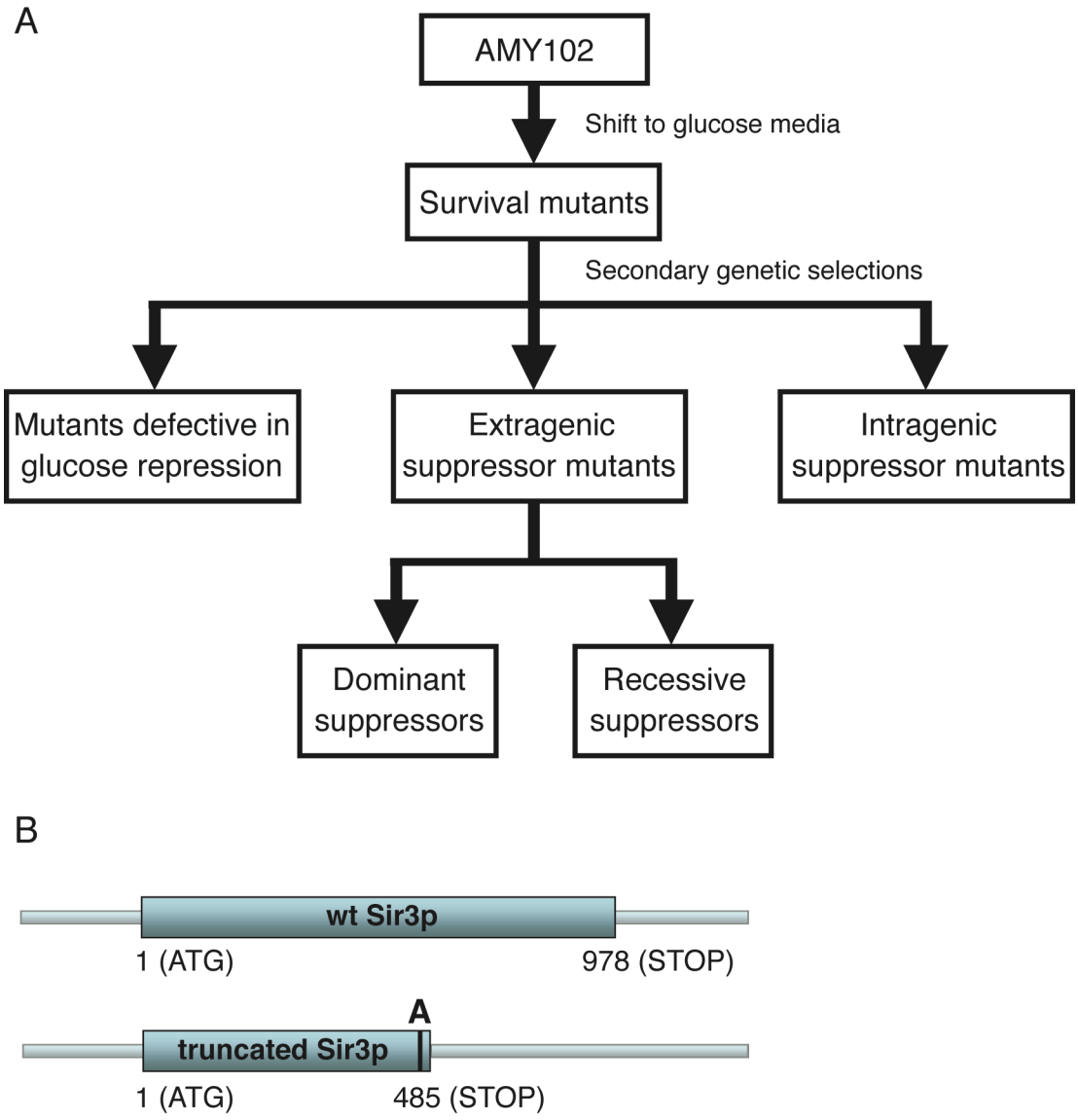

FIGURE 2-6: Mutated *sir3* was identified as an extragenic suppressor for the lethal phenotype of the histone H3 K4,36,79R mutant.

(A) A schematic of the genetic screen for identifying mutations that suppressed the lethal phenotype of the H3 K4,36,79R mutant. Extragenic suppressor mutants were further

analyzed. Mutated suppressor genes were isolated and sequenced. **(B)** Schematic representations of the *SIR3* gene and a recessive, loss-of function *sir3* suppressor mutant isolated from the proposed genetic screen.

K4,36,79R mutant may have been due, in part, to increased binding of the SIR silencing complex to genomic regions adjacent to telomeres. To test this possibility, we performed chromatin immunoprecipitation (ChIP) assays to measure the association of the Sir2 and Sir4 proteins with a series of DNA regions from 372 bp to 20,281 bp from telomere V-L (Figure 2-7B) (Krogan et al., 2003b). For the Sir4 experiments, Sir4 was tagged with a 9-myc epitope and anti-myc antibody was used for the ChIP assays (Knop et al., 1999); the Sir2 ChIP assays were performed using an anti-Sir2 antibody. ChIP assays were performed on wild-type and H3 K4,36,79R mutant strains at 0, 9, and 12 hours following the shift to glucose media. The results for the Sir4 ChIP experiments are shown in Figure 2-7C. In the strain containing wild-type histone H3, strong Sir4 binding was detected at the most telomere proximal DNA region (primer pair 1), but little if any binding was detected at DNA regions further from the telomere (primer pairs 2-5). In contrast, in the H3 K4,36,79R mutant strain, considerable Sir4 binding is observed at more distant DNA regions (primer pairs 2-4). We observed substantial Sir4 binding even at the 0 hour time point. This result is in accordance with the genome-wide expression data, which showed that a significant number of telomere proximal genes are repressed in the H3 K4,36,79R mutant at the 0 hour time point. The strength of the Sir4 interaction with these DNA regions increased at later time points. In addition, significant Sir4 binding was observed at the most distant DNA region assayed (primer pair 5) at the 9 and 12 hour time points, but not at the 0 hour time point. Similar results were obtained in the Sir2 ChIP experiments (Figure 2-8). The increase in Sir2 and Sir4 occupancy at these distant DNA regions was not due to an increase in SIR protein, as western blot analysis indicates that Sir2 and Sir4 protein levels are constant throughout the time course (Figure 2-8C).

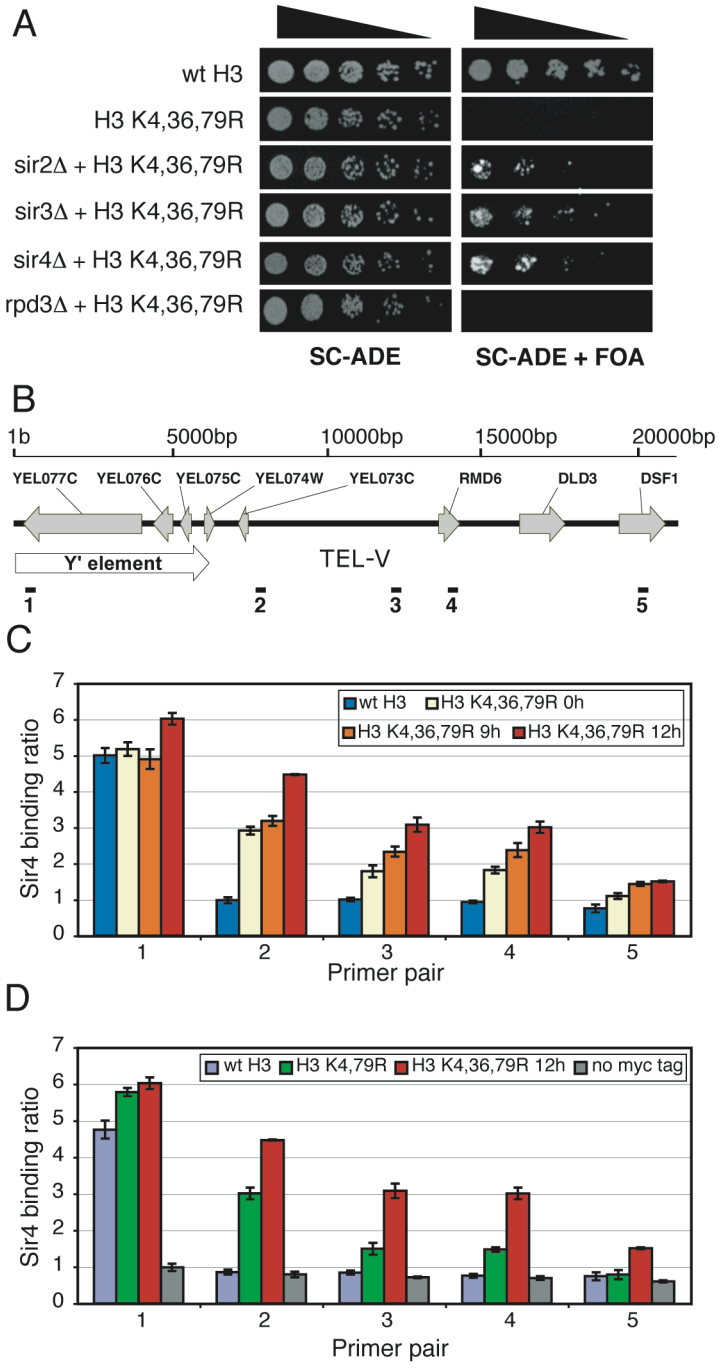
A
wt H3
H3 K4,36,79R
sir2Δ + H3 K4,36,79R
sir3Δ + H3 K4,36,79R
sir4Δ + H3 K4,36,79R
rpd3Δ + H3 K4,36,79R
SC-ADE
SC-ADE + FOA
B
1b
5000bp
10000bp
15000bp
20000bp
YEL077C
YEL076C
YEL075C
YEL074W
YEL073C
RMD6
DLD3
DSF1
Y' element
TEL-V
1
2
3
4
5
C
wt H3
H3 K4,36,79R 0h
H3 K4,36,79R 9h
H3 K4,36,79R 12h
Sir4 binding ratio
Primer pair
D
wt H3
H3 K4,79R
H3 K4,36,79R 12h
no myc tag
Sir4 binding ratio
Primer pair

FIGURE 2-7: SIR silencing complex is required for the lethal phenotype of the histone H3 K4,36,79R mutant.

(**A**) The growth of histone H3 K4,36,79R mutant strains lacking *SIR2*, *SIR3*, *SIR4*, or *RPD3* was tested on SC-ADE plates with or without 5-FOA, as in Fig. 1A. (**B**) A schematic representation of the chromosome V-L telomere (TEL V-L) region. The location of Y' element, coding sequences, and ChIP primer pairs are depicted. (**C**) and (**D**) Increased binding by the SIR silencing complex near the chromosome V-L telomere region. The abundance of Sir4 at TEL V-L region in wild-type strains and H3 mutants (and an untagged strain as control) were determined by ChIP analysis. Sir4 binding ratio is shown as the ratio of each primer pair to *ACT1* primer pair. Each error bar represents the standard deviation of three independent experiments.

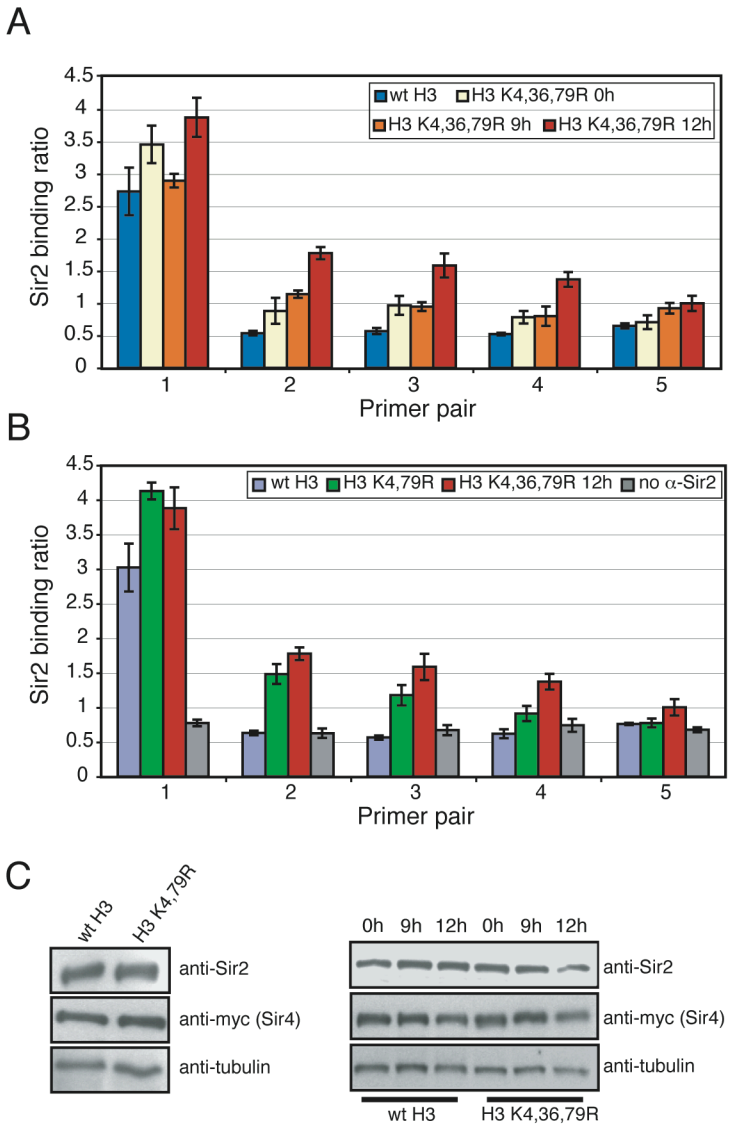

FIGURE 2-8: SIR silencing complex is required for the lethal phenotype of the histone H3 K4,36,79R mutant.

(**A**) and (**B**) Increased binding of the SIR silencing complex near the chromosome V-L telomere region. The abundance of Sir2 at TEL V-L region in wild-type strains and H3 mutants (and magnetic beads without anti-Sir2 antibody bound as control) were

determined by ChIP analysis. Sir2 binding ratio is shown as the ratio of each primer pair (Figure 2-7B) to *ACT1* primer pair. Each error bar represents the standard deviation of three independent experiments. (**C**) Western blot analysis was employed to measure the relative level of Sir2 and Sir4 in wild-type stains and H3 mutants.

We also compared the binding of the SIR complex in the H3 K4,79R mutant. As shown in Figure 2-7D, the binding of Sir4 to DNA regions was considerably stronger in the H3 K4,36,79R mutant than in the H3 K4,79R mutant strain. This was particularly apparent at the more distant DNA regions (primer pairs 3-5). These data indicated that mutating all three methylated lysine residues in histone H3 causes considerably greater binding of the SIR complex to adjacent genomic regions than is observed when only K4 and K79 are mutated.

Deletion of the histone methyltransferase genes suppresses the H3 K4,36,79R lethal phenotype. Because the arginine mutations are thought to mimic an unmethylated lysine side chain, we hypothesized that the H3 K4,36,79R mutant was lethal because it abolished histone H3 methylation. To test this hypothesis, we systematically deleted all three histone H3 lysine methyltransferases, and characterized the phenotypes of the resulting *set1*Δ*set2*Δ*dot1*Δ mutant strain. Surprisingly, unlike the H3 K4,36,79R mutant, the triple methyltransferase mutant was viable (Figure 2-9A). Phenotypic analysis of the *set1*Δ*set2*Δ*dot1*Δ mutant strain indicated that it grew normally under most growth conditions, though it showed caffeine sensitivity (Figure 2-10). This result calls into question whether the lethality of the H3 K4,36,79R mutant was due to the loss of lysine methylation.

To test this finding further, we constructed yeast strains containing combinations of methyltransferase deletions and histone H3 mutants. For example, deletion of *SET1*, which is required for H3K4 methylation, was not lethal in a H3 K36,79R mutant strain (Figure 2-9B), as would be expected if the loss of H3 methylation were the cause of lethality in the H3 K4,36,79R mutant. Similar mutant combinations were constructed for

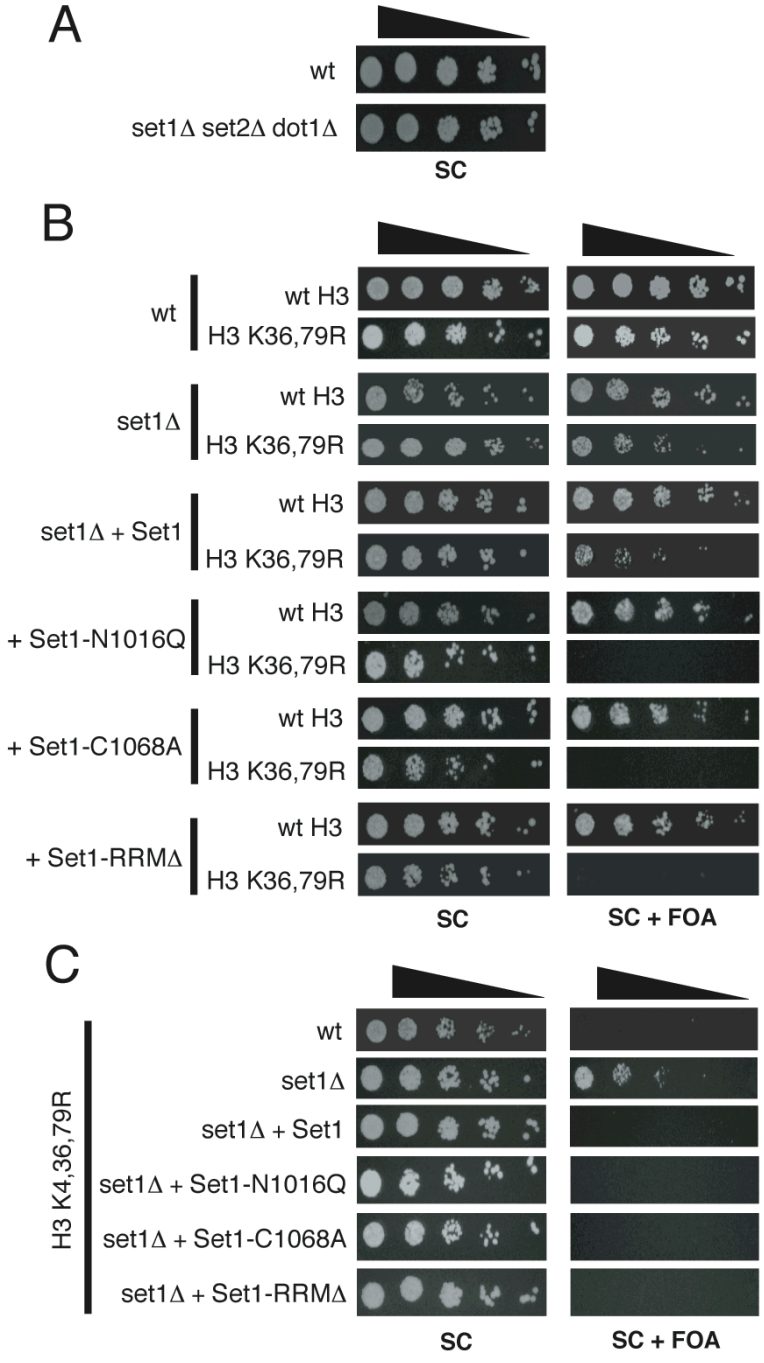
A
wt
set1Δ set2Δ dot1Δ
SC
B
wt
wt H3
H3 K36,79R
set1Δ
wt H3
H3 K36,79R
set1Δ + Set1
wt H3
H3 K36,79R
+ Set1-N1016Q
wt H3
H3 K36,79R
+ Set1-C1068A
wt H3
H3 K36,79R
+ Set1-RRMΔ
wt H3
H3 K36,79R
SC
SC + FOA
C
H3 K4,36,79R
wt
set1Δ
set1Δ + Set1
set1Δ + Set1-N1016Q
set1Δ + Set1-C1068A
set1Δ + Set1-RRMΔ
SC
SC + FOA

FIGURE 2-9: Deletion of the histone methyltransferase genes suppresses the H3 K4,36,79R lethal phenotype.

(**A**) Growth ability of the *set1*Δ*set2*Δ*dot1*Δ mutant strain. (**B**) and (**C**) Histone H3 mutant strains lacking Set1 methyltransferase were transformed with the wild-type *SET1* or different *set1* mutant alleles and then tested for viability, as in Figure 2-1A.

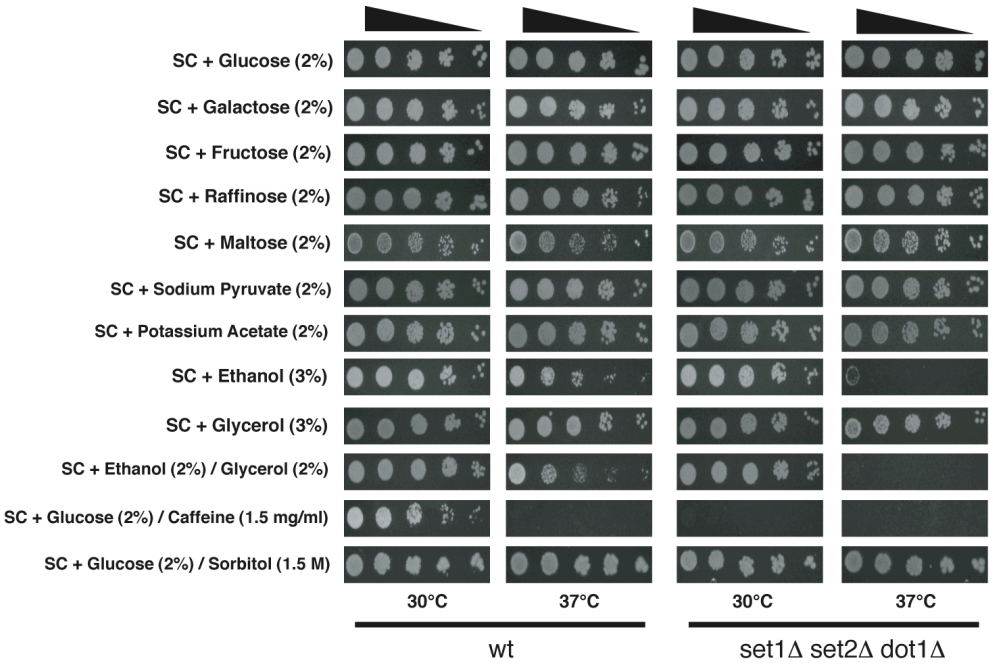

FIGURE 2-10: Phenotypic analysis of the *set1Δset2Δdot1Δ* mutant strain.

5-fold serial dilutions of each yeast strain were spotted on solid medium containing the indicated compounds and incubated at indicated temperatures for 3-5 days.

the *SET2* and *DOT1* deletion strains, but none recapitulated the lethal phenotype of the H3 K4,36,79R mutant (data not shown).

In addition to the *set1* deletion, we tested two different mutations in the Set1 active site (*set1-N1016Q* and *set1-C1068A*), which have been previously shown to render Set1 catalytically inactive (Santos-Rosa et al., 2004; Santos-Rosa et al., 2003). Surprisingly, we found that both *set1-N1016Q* and *set1-C1068A* were lethal in combination with the H3 K36,79R mutant (Figure 2-9B). The lethal phenotype of these *set1* point mutants was specific to the H3 K36,79R mutant; the *set1* point mutants were not lethal in combination with the H3 K4,79R or H3 K4,36R mutants, nor with the H3 K4,36,79G mutant (data not shown). We also tested the viability of strains containing the *set1-RRM*Δ mutant allele, in which the RNA Recognition Motif domain of Set1 (amino acids 230-335) had been deleted. Unlike the *set1-N1016Q* and *set1-C1068A* mutants, which eliminated all methylation of H3K4 (Figure 2-11A), the *set1-RRM*Δ mutant only abolished H3K4 trimethylation and reduced H3K4 dimethylation, leaving H3K4 monomethylation intact (Figure 2-11B), in accordance with previous studies (Fingerman et al., 2005; Schlichter and Cairns, 2005). The H3 K36,79R *set1-RRM*Δ was also inviable (Figure 2-9B). Again, the *set1-RRM*Δ mutant was not lethal in combination with the H3 K4,79R or H3 K4,36R mutants, nor with the H3 K4,36,79G mutant (data not shown).

Perhaps the simplest interpretation of these results is that the histone methyltransferase proteins (e.g., Set1) must be present in the cell for the loss of histone methylation to be lethal. If this hypothesis was correct, then we would expect that deleting the genes encoding the histone methyltransferase enzymes would rescue the H3 K4,36,79R lethal phenotype. To test this possibility, we measured the viability of the H3

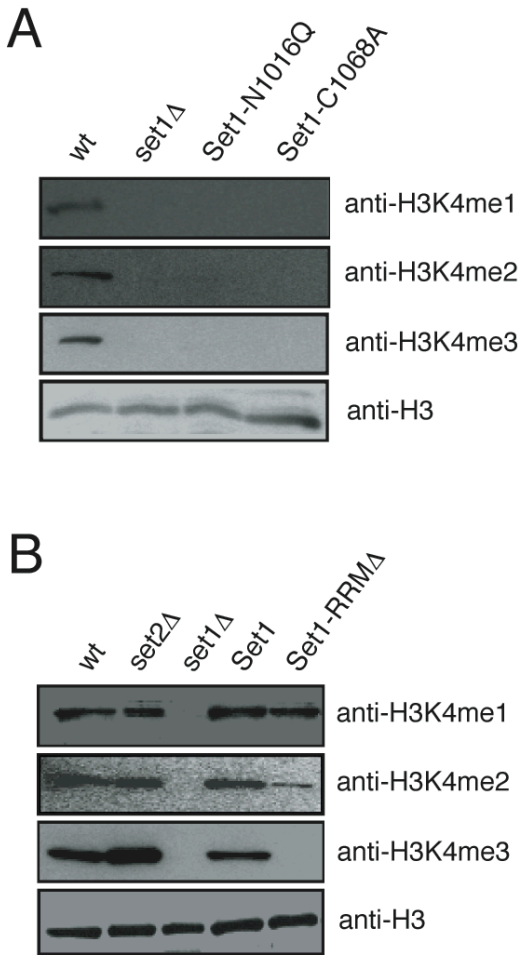

FIGURE 2-11: Western blot analysis of methylated histone H3K4 in various *set1* mutants.

Western blot analysis of mono-, di-, and trimethylated H3K4 in various *set1* mutants. The level of histone H3 was used as an internal loading control.

K4,36,79R yeast strain in which the *SET1* gene was deleted. As shown in Figure 2-9C, deletion of *SET1* rescued the lethal phenotype of the H3 K4,36,79R mutant, but this lethality was restored if a wild-type copy of the *SET1* gene was added. Intriguingly, addition of any of the set1 mutants that abolished or disabled its methyltransferase activity (i.e., *set1-N1016Q*, *set1-C1068A* or *set1-RRM*Δ mutants) also restored lethality (Figure 2-9C). This result indicates that the Set1 protein, but not its enzymatic activity, was required for the H3 K4,36,79R lethality. Deletion of the *SET2* or *DOT1* genes also rescued the lethal phenotype of the H3 K4,36,79R mutant, though the H3 K4,36,79R *dot1*Δ mutant strain was very slow growing (data not shown).

DISCUSSION

Understanding how methylated histone lysine residues act in concert to regulate gene expression requires the systematic genetic analysis of each lysine mutant combination. Here, we demonstrate that while single or double lysine-to-arginine mutations in H3K4, H3K36, and H3K79 have relatively modest effects on yeast cell viability, the H3 K4,36,76R triple mutant is inviable. This lethal phenotype does not appear to be the consequence of a structural defect in the histone H3 protein, as the H3 K4,36,79R mutant protein is stable and appears to be incorporated into nucleosomes at normal levels. Instead, we find that the H3 K4,36,79R mutant triggers a progressive gene transcription defect, which originates in yeast telomeric heterochromatin, and then spreads into the euchromatin regions of the genome. The H3 K4,36,79R mutant also causes cell cycle delays that are characteristic of defects in mitosis and cytokinesis. We show that the lethal phenotype requires a functional SIR silencing complex, and that the SIR complex displays increased binding to genomic regions adjacent to yeast telomeres in the H3 K4,36,79R mutant strain. Intriguingly, we also show that presence of intact histone methyltransferase proteins is required for lethality of the H3 K4,36,79R mutant.

Phenotype of the histone H3 K4,36,79R mutant. Genome-wide expression profiling of the H3 K4,36,79R mutant strain following depletion of wild-type histone H3 revealed an initial decline in the transcription of genes proximal to telomere regions. At later time points, even genes located in euchromatin regions of yeast chromosomes suffer a decline in transcription. These data support a model in which the decrease in the transcription of essential genes in the H3 K4,36,79R mutant strain causes cell lethality. The genome-wide expression data alone do not distinguish whether the decline in

transcriptional activity is due to a defect in transcriptional activation or elongation, or due to an enhancement in transcriptional repression. The isolation of loss-of-function suppressor mutations in the SIR transcriptional silencing complex and our observation that the SIR complex binds to adjacent euchromatin regions in the mutant strain, however, indicates that the transcriptional defect is at least in part due to SIR-mediated transcriptional repression.

The H3 K4,36,79R mutant strain also displays defects in cell cycle progression, particularly in mitosis. While a fairly small subset of mutant cells (11-16%) show cell cycle irregularities during the time course, the observed defects are significantly enriched in the mutant strain as compared to wild-type, and could be a contributing factor to the H3 K4,36,79R lethal phenotype. Previous studies have shown that Set1 methylation of Dam1 plays an important role in regulating chromosome segregation during mitosis (Zhang et al., 2005). Our data indicate that Set1-catalyzed methylation of histone H3, in parallel with Set2- and Dot1-catalyzed methylation, is also vital for proper mitotic progression.

It is important to note that previous studies have linked the spreading of the SIR complex with yeast growth and cell-cycle defects. Deletion of the *GCN5* and *ELP3* histone acetyltransferases, which caused the spreading of the SIR complex into subtelomeric heterochromatin, resulted in a yeast strain with multiple growth defects (Kristjuhan et al., 2003). A second study showed that over-expression of the SIR complex led to cell cycle defects in mitosis and a decrease in chromosome stability (Holmes et al., 1997). Hence, it is possible that the cell cycle defects observed in the H3 K4,36,79R

mutant may be a consequence of the aberrant spreading of SIR-mediated heterochromatin.

Role of the SIR complex in the H3 K4,36,79R lethal phenotype. We have isolated two distinct classes of mutants that suppress the H3 K4,36,79R lethal phenotype. These classes of suppressor mutants provide insight into the mechanism underlying the H3 K4,36,79R lethal phenotype. The first class of suppressor mutants occurs in components of the SIR silencing complex.

Previous studies have shown that the association of the SIR silencing complex with chromatin is regulated by the methylation of H3K4 and H3K79. Trimethylation of H3K4 and H3K79 blocks SIR association (Ng et al., 2003a; Santos-Rosa et al., 2004), while hypomethylated H3K4 and H3K79 favor SIR association (Ng et al., 2003a; Santos-Rosa et al., 2004). This model fits our data, as the lysine-to-arginine mutations in the H3 K4,36,79R mutant, which elicit SIR binding (see Figure 2-7 and 2-8), are thought to mimic hypomethylated lysine residues. It is also possible, however, that the arginine mutants bind more strongly to the SIR complex than would hypomethylated lysine residues, and thus cause an artificially high degree of SIR binding.

We find that the H3 K4,79R mutant alone causes only a modest increase in gene silencing, primarily confined to genes located within 10 kb of a telomere. Only when H3K36 is also mutated to arginine is the silencing of euchromatin genes observed. This conclusion is supported by the SIR binding data, which clearly demonstrate more extensive binding of the SIR complex further into the chromosome in the H3 K4,36,79R mutant as compared to the H3 K4,79R mutant. These data suggest that H3K36 plays an important role in regulating telomeric silencing. Previous studies have shown that

mutants in the Paf1 complex, which regulates H3K4 and H3K36 methylation, have silencing phenotypes (Krogan et al., 2003a; Landry et al., 2003; Ng et al., 2003b). This finding is also in accordance with a recent study, which showed that Set2 regulated SIR complex spreading (Tompa and Madhani, 2007). Our microarray data indicate the H3 K36R mutant has a significant but modest effect on telomeric gene expression (data not shown).

Role of histone methyltransferase proteins in the H3 K4,36,79R lethal phenotype. The second class of suppressor mutants we have identified are in the histone lysine methyltransferase genes themselves. Deletion of either *SET1*, *SET2*, or *DOT1* rescued the H3 K4,36,79R lethal phenotype (though the *dot1* suppressor mutant was very slow growing). In light of this finding, it is not surprising that the *set1*Δ *set2*Δ *dot1*Δ triple deletion also yields a viable yeast strain. However, Set1 mutants that eliminate or disable its methylation activity are lethal in combination with either the H3 K4,36,79R or H3 K36,79R histone mutants. It is important to note that while the *set1-N1016Q* and *set1-C1068A* mutations disrupt Set1 catalytic activity, they do not affect the stability of the Set1 protein or the integrity of the Set1-associated COMPASS complex (Santos-Rosa et al., 2003), unlike the *SET1* deletion mutant (Roguev et al., 2001).

We have yet to test the phenotypes of catalytically inactive mutations in Set2 and Dot1, as inactivating mutations in these methyltransferases have not been as well as studied as those in Set1. However, we have found that deletion of *CTK1*, which is required for Set2-catalyzed methylation of H3K36 (Xiao et al., 2003), is lethal in combination with H3 K4,79R mutant (data not shown). The *CTK1* deletion disrupts Set2

methyltransferase activity, but not the integrity of the Set2 protein; hence, this result mirrors the effects seen for the Set1 catalytically inactive mutants.

In summary, we have shown that the histone methyltransferases are required for the lethality of the H3 K4,36,79R mutant. Perhaps the simplest explanation of this observation is what we call the 'stymied methyltransferase' model. This model asserts that the lethal phenotype arises, in part, due to the association of histone methyltransferase enzymes with a histone substrate that cannot be methylated. Thus, this model would predict that the histone H3 lysine-to-arginine mutants and the catalytically inactive methyltransferase mutants are lethal because they prevent lysine methylation without disrupting the association of the methyltransferase with its substrate. On the other hand, mutants that disrupted this association (e.g., methyltransferase deletion or histone lysine-to-glycine mutants) would not be expected to be lethal, in accordance with our results. While it is not clear why a 'stymied methyltransferase' would necessarily be lethal, it is intriguing to speculate that the Set1 protein may have a direct inhibitory function, as is the case for the yeast Kss1 MAPK (Bardwell et al., 1998; Cook et al., 1997; Madhani et al., 1997; Schwartz and Madhani, 2004). For example, Set1 binding to histone substrates in the absence of histone methylation could inhibit gene transcription. Alternatively, Set1 could exert its inhibitory effect through its interaction with other substrates, such as Dam1 (Zhang et al., 2005).

A second possible explanation for these genetic data is that proteins associated with the histone methyltransferases may have additional functions that are required for lethality. Deletion of the histone methyltransferase would disrupt these functions, and suppress the lethal phenotype. For example, it is possible that other components of the

COMPASS complex have additional functions, which may be compromised by the deletion of *SET1*. In any case, future studies are needed to test these hypotheses.

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CHAPTER THREE

GENETIC AND GENOME-WIDE ANALYSIS OF SIMULTANEOUS MUTATIONS IN ACETYLATED AND METHYLATED LYSINE RESIDUES IN HISTONE H3 IN *SACCHAROMYCES CEREVISIAE*

[Jin, Y., Rodriguez, A. M., and Wyrick, J. J. (2008) Genetic and Genome-wide Analysis of Simultaneous Mutations in Acetylated and Methylated Lysine Residues in Histone H3 in *Saccharomyces cerevisiae*. Genetics, *Under revision*]

ABSTRACT

Acetylated and methylated lysine residues in histone H3 play important roles in regulating yeast gene expression and other cellular processes. Previous studies have suggested that histone H3 acetylated and methylated lysine residues may functionally interact through interdependent pathways to regulate gene transcription. A common genetic test for functional interdependence is to characterize the phenotype of a double mutant. Using this strategy, we tested the genetic interaction between histone H3 mutant alleles that simultaneously eliminate acetylated or methylated lysine residues. Our results indicate that mutation of histone H3 acetylated lysine residues alleviates growth phenotypes exhibited by the H3 methylated lysine mutant. In contrast, histone H3 acetylated and methylated lysine mutants display largely independent effects on yeast gene expression. Intriguingly, these expression changes are preferentially associated with chromosomal regions in which histone H3 lysine residues are hypo-acetylated and hypo-methylated. Finally, we show that the acetylated and methylated lysine mutants have strikingly different effects on the binding of Sir4 to yeast telomeres, suggesting that histone H3 acetylated lysine residues regulate yeast silencing through a mechanism independent of SIR binding.

My contribution to this project:

This project was began by Amy Rodriguez generating yeast mutants lacking acetylated lysine residues (H3 K9,14,18,23,27G), methylated lysine residue (H3 K4,36,79G), or both acetylated and methylated lysine residues (H3 K4,9,14,18,23,27,36,79G). She also generated the RNA from the histone H3 mutants for performing DNA microarray. I focused on characterizing growth phenotypes of these mutants under different growth conditions and various chemical treatments. I also further analyzed genome-wide expression profiles of these three histone mutants and aided in modeling the genetic interaction between acetylated and methylated histone H3 lysine residues. In addition, I was responsible for the telomeric silencing assays, the RT-PCR analysis, and the SIR silencing complex binding analysis. Finally, I aided in profiling histone modification status of genes regulated by histone H3 lysine residues. The data from these experiments are described in this chapter.

INTRODUCTION

Post-translationally modified residues in histone proteins, such as acetylated or methylated lysine residues, play critical roles in the regulation of gene transcription and silencing (Shahbazian and Grunstein, 2007; Shilatifard, 2006). Acetylation of the e-amino group of lysine residues in histone proteins is catalyzed by histone acetyltransferases (HATs) (Lee and Workman, 2007) and expunged by histone deacetylases (HDACs) (Yang and Seto, 2008). In the N-terminal domain of histone H3 the lysine residues K9, K14, K18, K23, and K27 are acetylated. These residues are acetylated predominately by Gcn5 and Sas3 (Howe et al., 2001; Suka et al., 2001), and are deacetylated by Hda1, Hos2, and Rpd3 (Millar and Grunstein, 2006). Histone H3 acetylation occurs both in the promoter and coding region of actively transcribed genes (Kurdistani and Grunstein, 2003; Millar and Grunstein, 2006). The functional consequences of eliminating histone H3 acetylation are varied: mutation or deletion of histone H3 acetylated lysine residues causes a significant increase in the transcription of many genes (Sabet et al., 2003), including *GAL1* (Durrin et al., 1992; Grunstein et al., 1995) and genes located in telomeric and subtelomeric heterochromatin (Martin et al., 2004; Thompson et al., 1994). In contrast, deletion of Gcn5 causes a reduction in the transcription of many genes (Durant and Pugh, 2006; Holstege et al., 1998; Lee et al., 2000). Histone acetylation also regulates other DNA metabolic processes, including DNA replication (Aparicio et al., 2004; Vogelauer et al., 2002) and repair (Escargueil et al., 2008; Groth et al., 2007).

Histone lysine residues are also methylated by histone methyltransferase enzymes. In *S. cerevisiae*, histone H3 K4, K36, and K79 are methylated by Set1, Set2,

and Dot1, respectively. Histone methylation occurs predominately in transcriptionally active chromosomal regions, and the methylation marks are deposited on chromatin during the transcription cycle through the recruitment of the cognate histone methyltransferase (Hampsey and Reinberg, 2003; Li et al., 2007a; Shilatifard, 2006). Mutations in methylated lysine residues or histone methyltransferase enzymes disrupt epigenetic silencing in yeast (Briggs et al., 2001; Bryk et al., 2002; Krogan et al., 2002; Ng et al., 2002a; Tompa and Madhani, 2007; van Leeuwen and Gottschling, 2002b), and can affect yeast viability (Jin et al., 2007).

Accumulating evidence suggests there is considerable interplay between acetylated and methylated lysine residues in histone H3. For example, methylation of H3K4 is strongly correlated with acetylation of histone H3 N-terminal lysine residues (Millar and Grunstein, 2006; Taverna et al., 2007; Zhang et al., 2004). Methylation of H3K4 recruits the Gcn5 and Sas3 histone acetyltransferase complexes, as these complexes contain subunits (Chd1 and Yng1, respectively) that interact with methylated H3K4 (Lee and Workman, 2007; Martin et al., 2006a; Martin et al., 2006b; Pray-Grant et al., 2005; Taverna et al., 2006). Hence, abrogation of H3K4 methylation alters the acetylation pattern of adjacent histone H3 lysine residues (Jiang et al., 2007; Taverna et al., 2006). Intriguingly, acetylation of histone H3 N-terminal lysine residues also affects the methylation state of H3K4 (Jiang et al., 2007). There is evidence that H3K36 methylation also directs the recruitment of the H3-specific Sas3 complex (Martin et al., 2006b). Set2-catalyzed histone H3K36 methylation also recruits the Rpd3S histone deacetylase complex, which deacetylates histones in the coding regions of actively transcribed genes (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005).

Taken together, these studies argue that histone H3 acetylated and methylated lysine residues often function in interdependent molecular pathways to regulate gene transcription (Lee and Workman, 2007).

There is considerable genetic evidence, however, suggesting that histone H3 acetylated and methylated lysine residues may regulate gene expression via redundant molecular mechanisms. In a previous study, we found that a significantly greater number of genes were differentially expressed when both a methylated (H3K4) and acetylated (H3 K9, K14, K18, K23, K27) lysine residues were mutated in combination as opposed to when these residues were mutated individually (Martin et al., 2004). These results suggest that acetylated and methylated lysine residues may act in a redundant manner to regulate gene transcription.

In this study, we have employed a genetic approach to investigate the functional interplay between histone H3 acetylated and methylated lysine residues. We have constructed yeast mutants lacking acetylated lysine residues (H3 K9,14,18,23,27G), methylated lysine residue (H3 K4,36,79G), or both acetylated and methylated lysine residues (H3 K4,9,14,18,23,27,36,79G). Surprisingly, yeast strains harboring each of these mutants were viable and grew normally. Indeed, mutations in histone H3 acetylated lysine residues rescued the slow growth and other phenotypes (e.g., hydroxyurea sensitivity) exhibited by the H3 methylated lysine mutants. To investigate the interplay between histone H3 acetylation and methylation in gene expression, we used DNA microarrays to profile the changes in transcription in each histone H3 mutant strain. Surprisingly, our results do not support either the interdependent or redundant models, but instead indicate that mutations in acetylated and methylated lysine residues have

independent and additive effects on yeast gene expression. As many of the gene expression changes occurred adjacent to yeast telomeres, we also characterized the effects of eliminating histone H3 acetylated and/or methylated lysine residues on the binding of the SIR complex to telomere proximal DNA.

MATERIALS AND METHODS

Yeast strain and growth conditions. The yeast strains used in this study are listed in Table 3-1. For genome-wide expression analysis of the H3 mutants, duplicate cultures of the H3 mutant strains and wild-type strain were grown in parallel to a final OD_{600} of 0.5 - 0.7 in yeast extract/peptone/dextrose (YPD) media, and then harvested as described previously (Holstege et al., 1998). For the spotting assay, yeast cells were grown to mid-log phase, normalized by OD_{600}, 5-fold serially diluted, and spotted on appropriate SC plates containing various carbon sources and indicated chemicals. All spotted plates were incubated at 30°C. Plates were photographed after 2-4 days incubation. All spotting assays were repeated to ensure reproducibility.

Plasmid construction and site-directed mutagenesis. The QuikChange kit (QuikChange Kit, Stratagene) was used to mutate histone H3 lysine residue to glycine, using plasmid pJL001 or pJL004 (Martin et al., 2004) as a template. Plasmid pJL001 is derived from pRS316 and contains wild-type histone H3 (*HHT2*) and H4 (*HHF2*) genes. Plasmid pJL004 contains mutant histone H3 (*hht2-K9,14,18,23,27G*) and wild-type histone H4 (*HHF2*) genes. The complete list of mutagenic primer sequences is listed in Table 3-2. The resulting mutant histone H3 alleles from site-directed mutagenesis were subsequently cloned into the pRS412 shuttle vector (Brachmann et al., 1998) using the *NotI* and *XhoI* restriction enzymes. All mutants were confirmed by DNA sequencing.

Western blot analysis. Yeast cultures were grown in YPD media to a final OD_{600} of 0.8 – 1.0 and harvested by centrifugation. Cell pellets were resuspended in lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate) containing fresh complete protease inhibitors cocktail (Roche

Diagnostics), and then lysed by vigorous agitation with acid-washed glass beads (425 – 600 μm, Sigma). The crude extract was briefly sonicated and further clarified by centrifugation. The supernatant was heated with Laemmli loading buffer at 95°C for 10 min. The protein extracts were resolved on a Tris-HCL 4 – 20% gradient gel (BioRad) and transferred to Hybond-ECL nitrocellulose membrane (GE Healthcare). Membranes were then probed with appropriate antibodies (anti-histone H3 and anti-tubulin, Abcam). The signals of target proteins were quantified using GelDoc EQ imager with Quantity One software (Bio-Rad).

Genome-wide expression profiling. RNA isolation, cDNA amplification, and biotin-cRNA synthesis were performed as described previously (Martin et al., 2004). The cRNA was hybridized to a single S98 yeast genome oligonucleotide array and scanned following standard protocols (Affymetrix). Fluorescence intensities were determined using GeneChip software (Affymetrix) and a single raw expression level for each gene was calculated. The data from each microarray were normalized by the GeneChip software using a median global intensity scaling method. Differentially expressed genes were identified using previously published criteria: the average change in mRNA levels (up or down) was greater than 2-fold, the change in mRNA levels in each replicate experiment was greater than 1.5-fold, and the absolute intensity change was above background levels. See the reports by Jin et al. (Jin et al., 2007) and Martin et al. (Martin et al., 2004) for more details. Microarray data for the H3 K[Ac]G mutant are from a previous study (Martin et al., 2004). Microarray data are available at the following website: http://wyrick.sbs.wsu.edu/histoneH3AcetylMethyl/

Microarray data analysis. An additive model was used to test whether the

histone H3 acetylated and methylated lysine mutants had independent effects on yeast gene expression. According to the additive model (Mani et al., 2008), the expected fold change in the H3 K[AcMe]G double mutant is given by the following equation:

$$E(F_{AM}) = F_A + F_M - 1 \quad (1)$$

where F_A is the fold change measured in the H3 K[Ac]G mutant, F_M is the fold change measured in the H3 K[Me]G mutant, and $E(F_{AM})$ is the expected fold change in the H3 K[AcMe]G mutant. However, the standard additive model has difficulties with down-regulated genes. For example, if $F_A = 1/3$ and $F_M = 1/3$, then according to equation 1, $E(F_{AM}) = -1/3$. This is a nonsensical result, as a negative fold change in mRNA levels cannot be interpreted biologically. In order to use the additive model to analyze microarray data, we made following modification: for genes down-regulated (fold change < 1), we first converted the fold change to a fold decrease (e.g., a 0.5-fold change was converted to a 2-fold decrease) prior to using equation 1. The calculated fold decrease was then converted to fold change by taking its inverse.

We also tested for genetic interaction using the Log model (Mani et al., 2008), which is given by the following equation:

$$E(F_{AM}) = \log_2[(2^{F_A} - 1)(2^{F_M} - 1) + 1] \quad (2)$$

For genes that are significantly up-regulated (F_A and $F_M >> 1$), the following approximations hold for equation 2:

$$E(F_{AM}) \sim \log_2[(2^{F_A})(2^{F_M}) + 1] \sim \log_2[(2^{F_A+F_M})]$$

$$E(F_{AM}) \sim F_A + F_M$$

Hence, for up-regulated genes, the Log model approximates the result obtained from the

additive model.

Telomere-proximal gene analysis and statistics. Chromosome plots were conducted as described previously (Jin et al., 2007), except that genes flagged as dubious were included in the analysis, and yeast genes were divided into consecutive 100-gene bins based on their distance from a chromosome end. The fraction of genes up-regulated and down-regulated in the histone mutant and the average distance of the genes from the telomere were plotted for each bin. Only those genes within ~ 200 kb of a telomere were plotted. The significance of enrichment of up-regulated genes in telomeric (0 - 10 kb from a telomere end) or subtelomeric regions (10 - 20 kb from a telomere end) was determined using a hypergeometric probability distribution.

A Wilcoxon rank sum test was used as an alternative method to test for a bias of up-regulated genes adjacent to yeast telomeres. Genes in each histone H3 mutant data set were ranked according to the magnitude of their fold-change in mRNA levels. Genes that were called absent, or had inconsistent or background intensity levels were excluded from this analysis. The Wilcoxon rank sum test was employed to test whether genes located adjacent to a telomere end showed a bias in their distribution of ranks in any of the histone H3 mutant strains.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were performed as described previously (Jin et al., 2007). Briefly, 50 ml of yeast cells were grown to a final OD_{600} of 0.8 – 1.0 in YPD media and cross-linked with 1% formaldehyde. Cells were then lysed by glass beads and sonicated to shear the chromatin to fragments sizes of 150 to 400 bp. Cross-linked chromatin fragments were immunoprecipitated with anti-Myc antibody (Biosource, AHO0052) bound to magnetic beads (Dynal Biotech). After the

cross-links were reversed, the DNA fragments were extracted for PCR analysis. Taq DNA polymerase (New England Biolabs) and appropriate primer pairs were used in the PCR amplification reactions. PCR products were subjected to gel electrophoresis, stained with ethidium bromide and quantified using GelDoc EQ imager with Quantity One software (Bio-Rad). Primer sequences are listed in Table 3-2.

Telomeric silencing assays. The yeast strains for telomeric silencing assays were derived from UCC1369 [*MATa, ade2::hisG his3-Δ200, leu2-Δ0, lys2-Δ0, met15-Δ0, trp1-Δ63, ura3-Δ0, adh4::URA3-TEL (VIIL), ADE2-TEL (VR), hhf2-hht2::MET15, hhf1-hht1::LEU2,* pMP9 *(CEN LYS2 HHF2 HHT2)*] (van Leeuwen and Gottschling, 2002a). The plasmid pMP9 was then replaced by the histone H3 mutant plasmids to make following strains YJ118, YJ119, YJ120, and YJ121. Silencing assays were performed as previously described (van Leeuwen and Gottschling, 2002a). Yeast strains for telomeric silencing assays expressing the indicated histone H3 mutant were grown to mid-log phase, normalized by OD_{600}, 5-fold serially diluted, and spotted on SC-TRP plates or SC-TRP plates containing 5-fluoroorotic acid (5-FOA) (Zymo Research). All spotted plates were incubated at 30°C. SC-TRP plates were photographed after 48 hours incubation and 5-FOA plates were photographed after 72 hours. All spotting assays were repeated to ensure reproducibility.

Reverse transcription-PCR (RT-PCR). Yeast growth condition and RNA isolation were performed as described in microarray experiments. RNase-free DNase I (Roche) was used to remove any genomic DNA contamination in RNA samples. CDNA was synthesized using a First strand cDNA synthesis kit (Roche) following the manufacturer's manual. The resulting cDNA was used as a template for multiplex PCR.

Primer sequences for amplifying *YEL077C* and *ACT1* can be found in Table 2-2. The following PCR cycling parameter were used: 30 s at 94°C, 1 min at 50°C, and 1 min at 72°C with a 10-min extension at 72°C after 30 cycles. PCR products were resolved on 2% agarose TBE gels stained with ethidium bromide and quantified using a GelDoc EQ imager with Quantity One software (Bio-Rad). *YEL077C* expression levels were normalized using the *ACT1* intensity as an internal loading control.

Microarray data accession number. The data discussed in this report have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession numbers GSE10930 and GSE10933.

Table 3-1: List of yeast strains and genotypes used in this study

Strain	Experiment	Genotype
WY121	Wild-type	*MAT**a** ade2-101 his3Δ200 lys2-801 trp1Δ901 ura3-52 hht1,hhf1::LEU2 hht2,hhf2::HIS3* plus pJL001 *(CEN URA3 HHF2 HHT2)*
WY139	Wild-type	Isogenic to WY121, plus pJW028 *(CEN ADE2 HHF2 HHT2)*
WY124	H3 K9,14,18,23,27G	Isogenic to WY121, plus pJL004 *(CEN URA3 HHF2 hht2-K9,14,18,23,27G)*
YJ107	H3 K9,14,18,23,27G	Isogenic to WY121, plus pYJ028 *(CEN ADE2 HHF2 hht2-K9,14,18,23,27G)*
AMY018	H3 K4,36,79G	Isogenic to WY121, plus pAM018 *(CEN ADE2 HHF2 hht2-K4,36,79G)*
AMY025	H3 K4,9,14,18,23,27,36,79G	Isogenic to WY121, plus pAM025 (*CEN ADE2 HHF2 hht2-K4,9,14,18,23,27,36,79G)*
YJ057	Wild-type Sir4-9xMyc	*MAT**a** ade2-101 his3Δ200 lys2-801 trp1Δ901 ura3-52 hht1,hhf1::LEU2 hht2,hhf2::HIS3 SIR4-9xMyc::TRP1* plus pJW028 *(CEN ADE2 HHF2 HHT2)*
YJ108	H3 K9,14,18,23,27G Sir4-9xMyc	Isogenic to YJ057, plus pYJ028 *(CEN ADE2 HHF2 hht2-K9,14,18,23,27G)*
YJ099	H3 K4,36,76G Sir4-9xMyc	Isogenic to YJ057, plus pAM018 *(CEN ADE2 HHF2 hht2-K4,36,79G)*
YJ098	H3 K4,9,14,18,23,27,36,79G Sir4-9xMyc	Isogenic to YJ057, plus pAM025 *(CEN ADE2 HHF2 hht2-K4,9,14,18,23,27,36,79G)*
YJ118	Wild-type	*MAT**a**, ade2::hisG his3Δ200, leu2Δ0, lys2Δ0, met15Δ0, trp1Δ63, ura3Δ0, adh4::URA3-TEL (VIIL), ADE2-TEL (VR), hhf2,hht2::MET15, hhf1,hht1::LEU2,* plus pYJ029 *(CEN TRP1 HHF2 HHT2)*
YJ119	H3 K4,36,79G	Isogenic to YJ118, plus pYJ030 *(CEN TRP1 HHF2 hht2-K4,36,79G)*
YJ120	H3 K9,14,18,23,27G	Isogenic to YJ118, plus pYJ031 *(CEN ADE2 HHF2 hht2-K9,14,18,23,27G)*
YJ121	H3 K4,9,14,18,23,27,36,79G	Isogenic to YJ118, plus pYJ032 *(CEN ADE2 HHF2 hht2-K4,9,14,18,23,27,36,79G)*

Table 3-2: Oligonucleotides used in this study

Name	Sequence	Description
OJLW1	CTCCACAATGGCCAGAACTGGACAAAC AGCTAGAAAATCC	Mutate H3 lysine 4 to glycine
OJLW7	GGATTTTCTAGCTGTTTGTCCAGTTCTGG CCATTGTGGAG	Mutate H3 lysine 4 to glycine
OAM10	CATCTACCGGTGGTGTTGGGAAGCCTCA CAGATATAAG	Mutate H3 lysine 36 to glycine
OAM11	CTTATATCTGTGAGGCTTCCCAACACCA CCGGTAGATG	Mutate H3 lysine 36 to glycine
OAM8	GGTCAGAGAAATCGCTCAAGATTTCGGT ACCGACTTGAGATTTC	Mutate H3 lysine 79 to glycine
OAM9	GAAATCTCAAGTCGGTACCGAAATCTTG AGCGATTTCTCTGACC	Mutate H3 lysine 79 to glycine
OJLW18	CCGGTGCTGCCAGAGGATCCGCCCCATC TA	Mutate H3 lysine 27 to glycine
OJLW9	TAGATGGGGCGGATCCTCTGGCAGCACC GG	Mutate H3 lysine 27 to glycine
OJLW2	GTTCTTTTATATAGGACC	Sequence to confirm H3 mutations
OJLW3	AGATTGAAATCTCAAGTC	Sequence to confirm H3 mutations
T3	AATTAACCCTCACTAAAGGG	Sequence to confirm H3 mutations
T7	TAATACGACTCACTATAGGG	Sequence to confirm H3 mutations
YJIN130	CCAATTGCTCGAGAGATTTC	ChIP primer pair for *ACT1*
YJIN131	CATGATACCTTGGTGTCTTG	ChIP primer pair for *ACT1*
YJIN134	GTACAGTGGTACCTTCGTGTTATC	ChIP primer pair for Chr V-L telomere
YJIN135	CAGATTGCGCTGGGAGTTACC	ChIP primer pair for Chr V-L telomere
YJIN204	ACTGTCCCGTCATTGATGCGTCTC	ChIP primer pair for *SPS2*
YJIN205	GGGATCGTTGCATTAGTGTTAACC	ChIP primer pair for *SPS2*
YJIN202	GCGTAACAAAGCCATAATGCCTCC	ChIP primer pair for Chr VI-R telomere
YJIN203	CTCGTTAGGATCACGTTCGAATCC	ChIP primer pair for Chr VI-R telomere
OMP94	GCAATGATCTTGACCTTCATG	RT-PCR primer pair for *ACT1*

Table 3-2 (continued): Oligonucleotides used in this study

Name	Sequence	Description
OMP95	CCTACGTTGGTGATGAAGCT	RT-PCR primer pair for *ACT1*
YJIN134	GTACAGTGGTACCTTCGTGTTATC	RT-PCR primer pair for *YEL077C*
YJIN135	CAGATTGCGCTGGGAGTTACC	RT-PCR primer pair for *YEL077C*

RESULTS

Phenotypes of the histone H3 acetylated and methylated lysine mutants.

Previous studies have suggested that acetylation and methylation of histone H3 lysine residues play important and interconnected roles in regulating yeast gene expression and other cellular processes. To further test these hypotheses, we constructed yeast strains containing one of three different histone H3 alleles: H3 K[Ac]G, in which the five histone H3 N-terminal acetylated lysine residues were mutated to glycine (i.e., *hht2-K9,14,18,23,27G*); H3 K[Me]G, in which the three methylated lysine residues were mutated to glycine (i.e., *hht2-K4,36,79G*); and H3 K[AcMe]G, in which the acetylated and methylated lysine residues were mutated to glycine (i.e., *hht2-K4,9,14,18,23,27,36,79G*). Each of these histone H3 mutant strains was viable. While the H3 K[Me]G mutant was not lethal, as was the case for the H3 K[Me]R (i.e., histone H3 K4,36,79R) mutant (Jin et al., 2007), it did exhibit a slower doubling time compared to wild-type in synthetic complete (SC) media (Figure 3-1A). This result is in accordance with previous studies, which showed that mutating H3K4 in yeast caused a significant growth defect (Briggs et al., 2001). Intriguingly, this phenotype was suppressed in the H3 K[AcMe]G mutant (Figure 3-1A), indicating that acetylated lysine mutations rescued the growth defect caused by the methylated lysine mutations.

The histone H3 mutant alleles displayed phenotypes in various growth conditions (Figure 3-1B and 3-1C). These include growth defects in certain carbon sources (e.g., maltose, glycerol, and ethanol), in high salt (i.e., 0.5 M NaCl), and upon treatment with specific chemicals (e.g., caffeine). In many conditions, the phenotype of the H3 K[AcMe]G mutant reflected the phenotypes of the H3 K[Ac]G and H3 K[Me]G mutants.

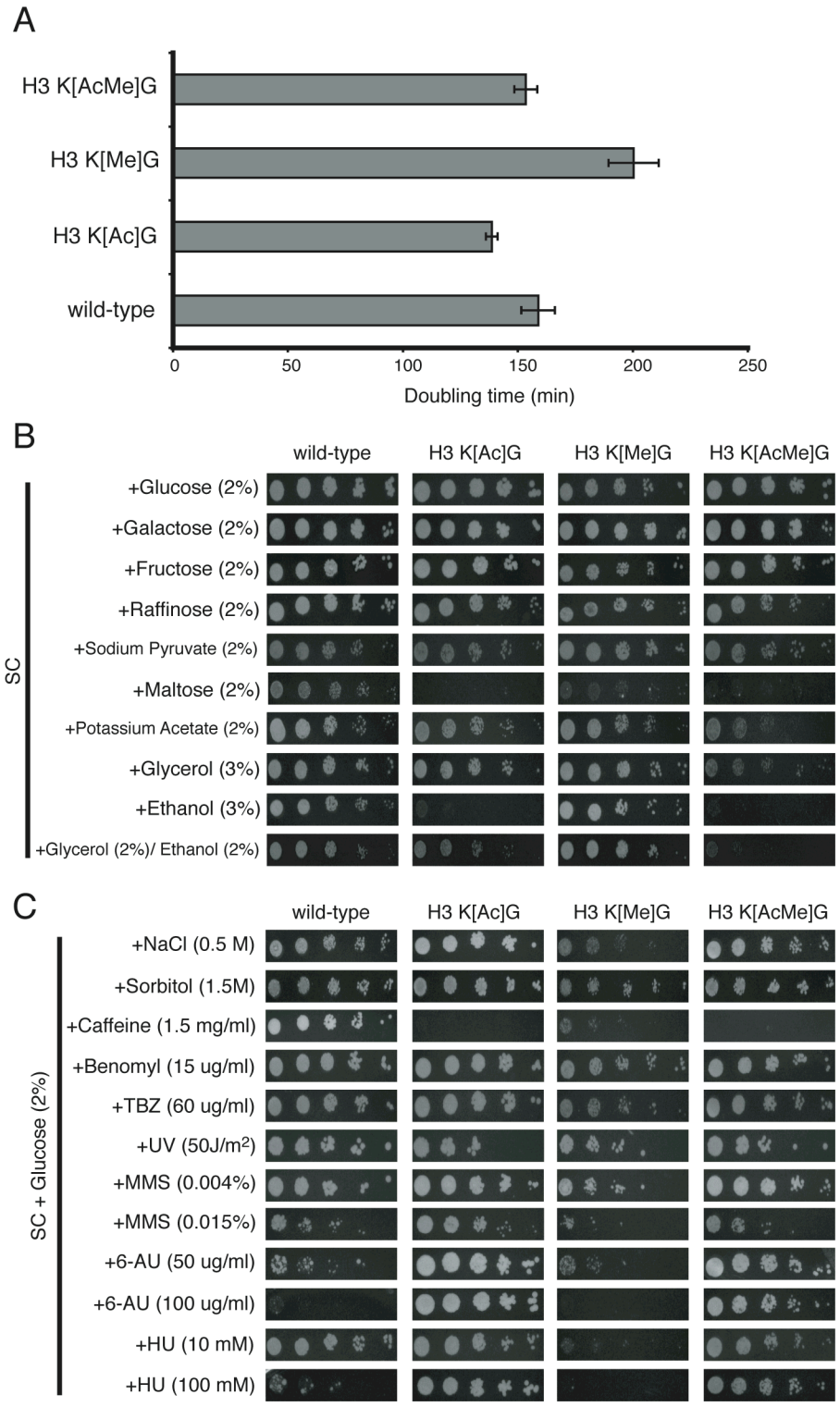
A
H3 K[AcMe]G
H3 K[Me]G
H3 K[Ac]G
wild-type
0
50
100
150
200
250
Doubling time (min)
B
wild-type
H3 K[Ac]G
H3 K[Me]G
H3 K[AcMe]G
SC
+Glucose (2%)
+Galactose (2%)
+Fructose (2%)
+Raffinose (2%)
+Sodium Pyruvate (2%)
+Maltose (2%)
+Potassium Acetate (2%)
+Glycerol (3%)
+Ethanol (3%)
+Glycerol (2%)/ Ethanol (2%)
C
wild-type
H3 K[Ac]G
H3 K[Me]G
H3 K[AcMe]G
SC + Glucose (2%)
+NaCl (0.5 M)
+Sorbitol (1.5M)
+Caffeine (1.5 mg/ml)
+Benomyl (15 ug/ml)
+TBZ (60 ug/ml)
+UV (50J/m2)
+MMS (0.004%)
+MMS (0.015%)
+6-AU (50 ug/ml)
+6-AU (100 ug/ml)
+HU (10 mM)
+HU (100 mM)

FIGURE 3-1: Phenotypic analysis of the histone H3 acetylated and methylated lysine mutants.

(A) Wild-type, H3 K[Ac]G, H3 K[Me]G, and H3 K[AcMe]G yeast strains were grown in SC media at 30°C. Cell density was monitored by OD_{600} at consecutive time points. The doubling time was measured based on the growth curve. Each error bar represents the standard deviation of four independent experiments. **(B)** and **(C)** 5-fold serial dilutions of each yeast strain from about 10^4 cells were spotted on SC media containing various carbon sources and indicated chemicals.

There were four notable exceptions. The high salt (NaCl), methyl methane sulfonate (MMS), 6-azauracil (6-AU), and hydroxyurea (HU) sensitivities exhibited by the H3 K[Me]G mutant were alleviated in the H3 K[AcMe]G mutant (Figure 3-1C). Indeed, the H3 K[Ac]G mutant was more resistant to treatment with HU, a DNA replication inhibitor, than the wild-type strain (Figure 3-1C). Hence, these phenotypes of the histone methylated lysine mutant were rescued upon mutating acetylated lysine residues in histone H3.

It is possible that the mutant phenotypes could be an indirect consequence of changes in the stability of the histone H3 protein. We tested whether the histone H3 mutant alleles affected the cellular levels of histone H3 protein by performing western blot analysis of yeast extracts using an anti-histone H3 antibody. As shown in Figure 3-2, the mutant alleles did not affect the levels of histone H3 protein.

Histone H3 acetylated and methylated lysine mutants have independent effects on gene transcription. We used Affymetrix oligonucleotide microarrays to profile the changes in gene transcription in each histone mutant strain. The results indicate that the H3 K[AcMe]G mutant caused an increase in the mRNA levels of 250 genes and a decrease in the mRNA levels of 81 genes (Figure 3-3A and 3-3B). The differentially expressed genes function in a variety of cellular processes, including vitamin metabolism ($P = 6.3 \times 10^{-5}$). The microarray data sets for the H3 K[Ac]G (Martin et al., 2004) and H3 K[Me]G affected the expression of similar but smaller sets of genes (Figure 3-3A and 3-3B).

Comparison of the data using Venn diagrams indicated that there were significant overlaps in the sets of genes regulated in each mutant (Figure 3-3A and 3-3B). Many of

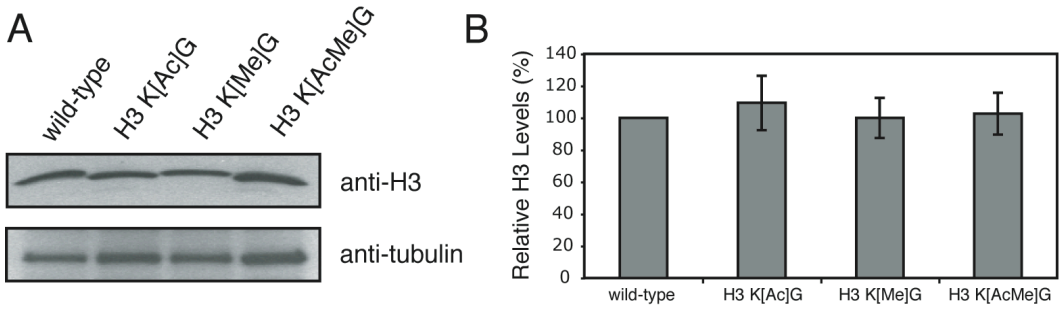

FIGURE 3-2: The stability of histone H3 protein from different histone mutants was measured by western blot analysis.

Cell extracts from each strain were resolved by SDS-PAGE and probed with anti-H3 antibody. The level of α-tubulin subunit was used as an internal loading control. (B) Quantification of histone H3 protein level in histone H3 mutants. The signal of histone H3 protein was normalized by the tubulin signal. The calculated signal of wild-type histone H3 protein was set to 100%. Each error bar represents the standard deviation of three independent experiments.

the genes up-regulated in the H3 K[AcMe]G mutant were also affected in the H3 K[Ac]G or H3 K[Me]G mutants (or both). Significant overlaps were also observed between the sets of down-regulated genes, though to a lesser extent (Figure 3-3B). It is important to note, however, that a few genes showed altered expression only in the H3 K[Ac]G or H3 K[Me]G. For example, 34 genes were up-regulated and 20 genes were down-regulated only in the H3 K[Ac]G mutant (Figure 3-3A - 3-3C). Presumably, the expression of these genes was not altered in the H3 K[AcMe]G double mutant due to epistatic effects exerted by the H3 K[Me]G mutant. We also observed that a significant number of genes were up- or down-regulated only in the H3 K[AcMe]G mutant (123 and 51 genes, respectively). However, inspection of the microarray data revealed that most of these genes were also affected in the H3 K[Ac]G and H3 K[Me]G mutants, though not to the level of the 2-fold threshold (Figure 3-3C). Hence, the greater number of genes differentially expressed in the H3 K[AcMe]G mutant may simply be due to the cumulative effects of the H3 K[Ac]G and H3 K[Me]G mutants.

The microarray data allow us to test which model of genetic interaction best explains the gene expression changes observed in the H3 K[AcMe]G double mutant. The null hypothesis is that the H3 K[Ac]G and H3 K[Me]G mutants make independent, and thus additive, contributions to the gene expression changes in the H3 K[AcMe]G double mutant. This additive model has been commonly used to test for genetic interactions between transcription factors and other genes (Carey et al., 1990; Hertel et al., 1997; Lin et al., 1990; Mani et al., 2008). Significant deviations from the additive model would support either the interdependence model or the redundancy model, depending upon the direction of the deviation (see Discussion section for more details).

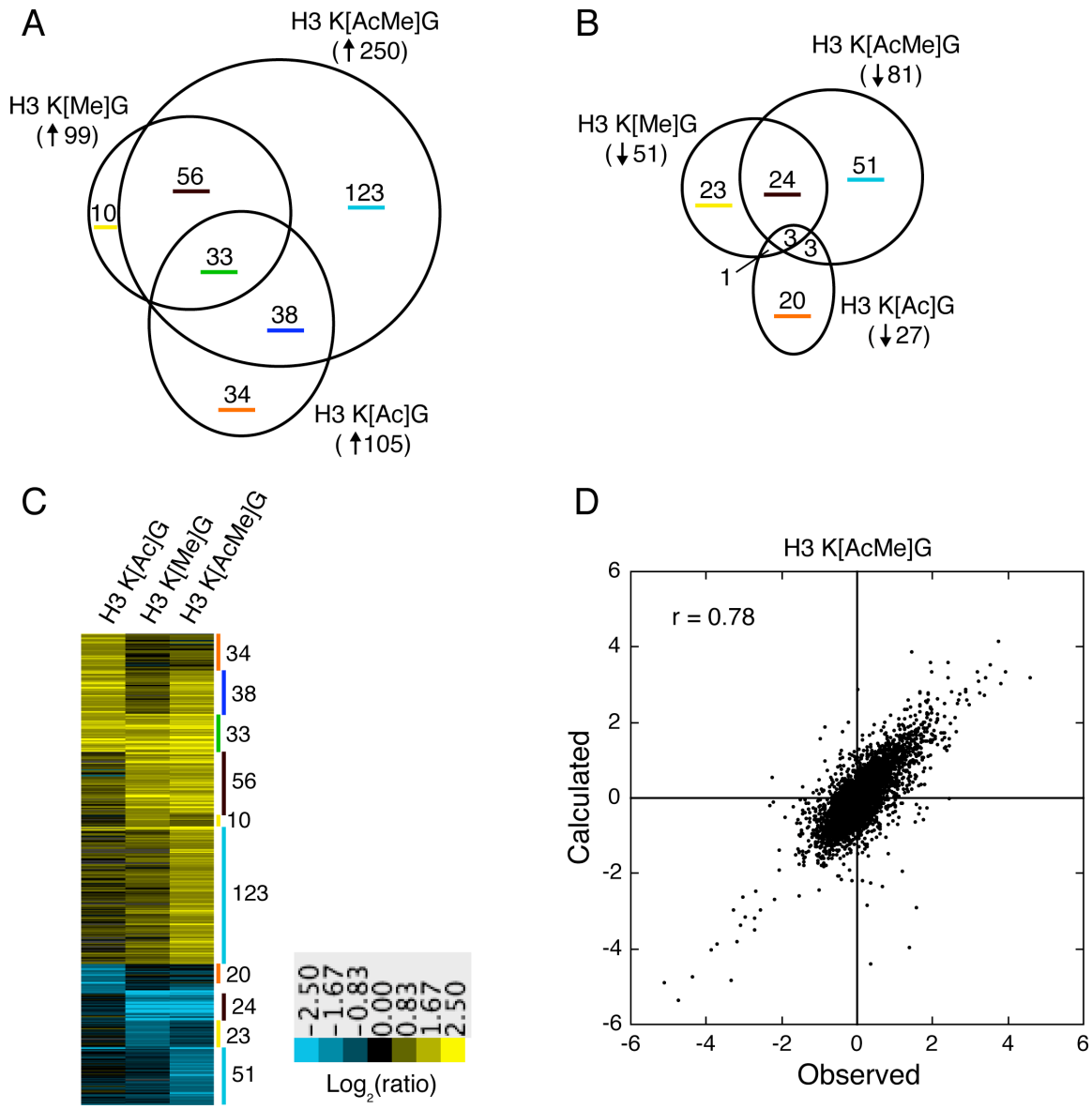

FIGURE 3-3: Effects of histone H3 acetylated and methylated lysine mutants on gene transcription.

Venn diagrams were used to compare the sets of **(A)** up-regulated genes and **(B)** down-regulated genes among the histone H3 mutants. The circle size is proportionate to the

number of genes in a data set. The degree of overlap between two circles is proportionate to the number of genes shared between data sets. Data for histone H3 K[Ac]G mutant is from Martin et al. (Martin et al., 2004). **(C)** The microarray data were displayed as a $\log_2$ ratio of mRNA fold change (mutant/wild-type) using the Treeview software. The data sets in each column correspond to the indicated histone H3 mutant. Each row represents a single gene; the $\log_2$ ratio of the fold change is represented by a color (see color scale). Genes that are differentially expressed among the histone H3 mutants are displayed. Each vertical color bar indicates the corresponding subset of genes in the Venn diagrams above. **(D)** The histone H3 acetylated and methylated lysine residues make independent contributions to gene transcription. The observed gene expression changes in the H3 K[AcMe]G mutant were compared with the calculated gene expression changes based the H3 K[Ac]G and H3 K[Me]G data sets using a modified additive model. The comparison is viewed as a scatter plot of the $\log_2$ ratios for all yeast genes.

We compared the observed gene expression changes in the H3 K[AcMe]G mutant with the expected changes based on an additive (i.e., independent) model. It is important to note that the standard additive model cannot be readily fit to microarray expression data that includes down-regulated genes (see Materials and Methods for more details). For this reason, we used a modified version of the additive model to fit the data, as described in the Materials and Methods section. The results shown in Figure 3-3D indicates that calculated and observed gene expression changes were highly correlated ($r = 0.78$; $P < 10^{-100}$). This correlation was even higher if just the differentially expressed genes were examined ($r = 0.90$; $P < 10^{-100}$). Indeed, the calculated and observed gene expression changes among the differentially expressed genes displayed a linear relationship (slope and R^2 are 0.96 and 0.81, respectively). This analysis indicates that the gene expression changes in the H3 K[AcMe]G mutant can largely be explained by a model assuming that the histone H3 acetylated and methylated lysine residues make independent and additive contributions to gene transcription.

As an alternative method to test the independence model, we used a Log model to test for genetic interaction. For significantly up-regulated genes, the Log model closely approximates the predictions of the additive model (see Materials and Methods for more details), but unlike the standard additive model, the Log model does not encounter difficulties handling data for down-regulated genes. Thus, the Log model can be readily applied to microarray data, and has been previously validated for genetic interaction testing (Mani et al., 2008). We find that calculated expression changes based on the Log model were highly correlated with the observed expression changes in the H3 K[AcMe]G mutant, both for differentially expressed genes ($r = 0.90$; $P < 10^{-100}$), and for the entire

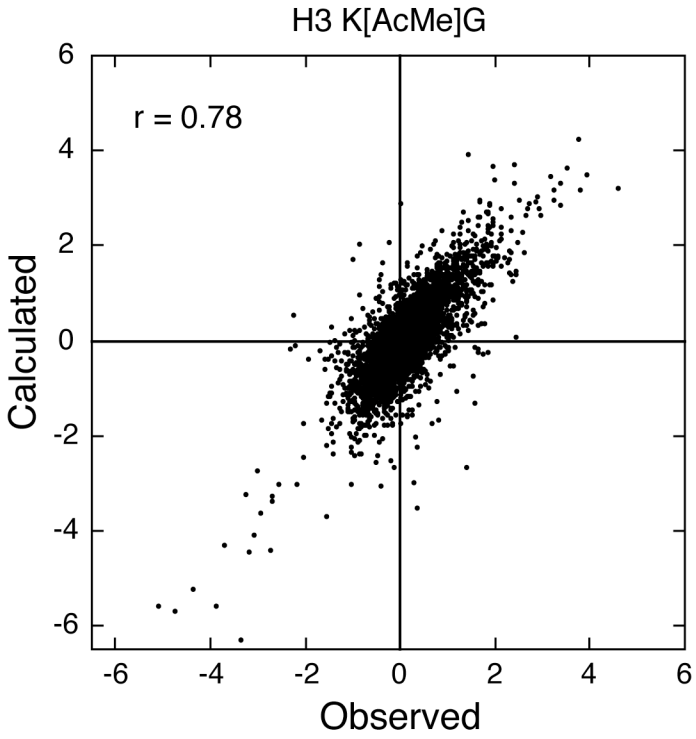

FIGURE 3-4: The histone H3 acetylated and methylated lysine residues make independent contributions to gene transcription.

The observed gene expression changes in the H3 K[AcMe]G mutant were compared with the calculated gene expression changes based the H3 K[Ac]G and H3 K[Me]G data sets using a LOG model. The comparison is viewed as a scatter plot for all yeast genes.

genome (Figure 3-4). Taken together, these results do not support the alternative hypotheses of interdependence or redundancy, but instead support the model that histone H3 acetylated and methylated lysine residues make independent contributions to transcription regulation.

Histone H3 lysine mutants preferentially affect telomere-proximal gene expression. Both methylated and acetylated lysine residues in histone H3 have been implicated in telomeric silencing in yeast. Hence, we examined whether the histone H3 mutants had a particular effect on telomeric gene expression, which we defined as genes located from 0 - 10 kb from a chromosomal end (Martin et al., 2004; Wyrick et al., 1999). Chromosome plots of the genome-wide expression data for the histone H3 acetylated and methylated lysine mutants are shown in Figure 3-5. We observed a significant cluster of up-regulated genes in telomeric regions (0 - 10 kb) in the H3 K[Me]G mutant ($P = 9.5 \times 10^{-9}$), but not in the H3 K[Ac]G mutant ($P = 0.1$). In contrast, both the H3 K[Ac]G ($P = 1.8 \times 10^{-4}$) and H3 K[Me]G ($P = 1.2 \times 10^{-4}$) mutants significantly up-regulate the expression of subtelomeric genes (defined as genes located 10 - 20 kb from a telomere end (Martin et al., 2004; Wyrick et al., 1999)). The H3 K[AcMe]G mutant shows a striking increase in telomeric gene expression (see Figure 3-5), likely due to the cumulative effects of mutating the acetylated and methylated lysine residues in combination.

It is possible that the H3 K[Ac]G mutant might affect the expression of telomeric genes, but not to the level of the 2-fold threshold used to analyze the microarray data. To test this possibility, we used a rank sum statistical test to measure whether telomeric genes showed a quantitative increase in expression in the H3 K[Ac]G mutant. The results

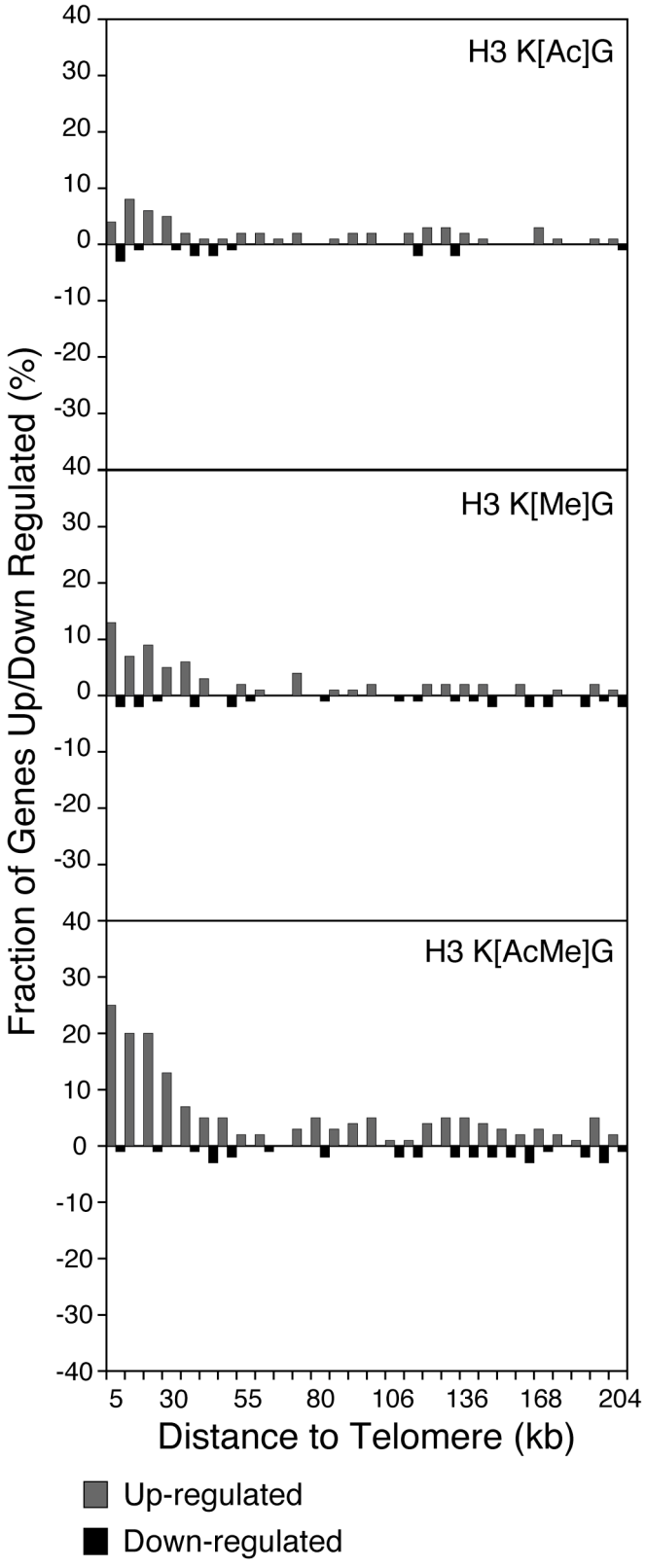
H3 K[Ac]G
H3 K[Me]G
H3 K[AcMe]G
Fraction of Genes Up/Down Regulated (%)
40
30
20
10
0
-10
-20
-30
-40
5
30
55
80
106
136
168
204
Distance to Telomere (kb)
Up-regulated
Down-regulated

FIGURE 3-5: Histone H3 mutants preferentially affect telomere-proximal gene expression.

Chromosome plots of the genome-wide expression data for the H3 K[Ac]G, H3 K[Me]G, and H3 K[AcMe]G mutants. For each histone H3 mutant, a histogram of the fraction of genes whose mRNA levels are up/down-regulated is plotted as a function of their distance from a chromosome end.

shown in Table 3-3 and 3-4 indicate that there is a significant increase in the expression of telomeric genes in the H3 K[Ac]G mutant, although to a lesser extent than the H3 K[Me]G mutant. This conclusion is supported by data from telomeric silencing assays. The histone H3 K[Ac]G mutant significantly disrupts the silencing of a telomere-located *URA3* reporter gene (Figure 3-6A), in accordance with the results from a previous study (Thompson et al., 1994).

We next examined how these mutants affected the binding of the SIR silencing complex to DNA regions adjacent to telomeres. We used ChIP assays to measure the binding of the Sir4 protein to genomic regions adjacent to telomere V-L (372 bp away) and telomere VI-R (525 bp away; see (Xu et al., 2007)) in the histone H3 mutant strains (Figure 3-6B and 3-6C). The H3 K[Me]G mutant caused a striking decrease in Sir4 binding at each telomere proximal region relative to wild-type ($P < 0.001$). In contrast, the H3 K[Ac]G mutant caused only a slight, insignificant decrease in Sir4 binding ($P > 0.05$). The decrease in Sir4 binding in the H3 K[AcMe]G mutant was similar to the decrease observed in the H3 K[Me]G mutant.

For comparison, we used RT-PCR to examine the changes in the mRNA levels of the *YEL077C* gene in the histone H3 mutants. *YEL077C* is located directly adjacent to telomere V-L and overlaps with the PCR probes used for the Sir4 ChIP experiments. The results shown in Figure 3-7 indicate that the mRNA levels of *YEL077C* showed a small but significant increase in the H3 K[Me]G and H3 K[Ac]G mutants, although the expression change in the H3 K[Ac]G mutant was somewhat marginal ($P < 0.05$). Importantly, although relatively little if any Sir4 binding was observed in either the H3 K[Me]G or H3 K[AcMe]G mutants (see Figure 3-6), the expression of *YEL077C* was

Table 3-3: Effects of histone H3 mutant strains on telomere proximal gene expression

Mutants	Telomeric genes*		Random gene set	
	Rank Percentile	*P*-Value	Rank Percentile	*P*-Value
H3 K[Ac]G	65%	$< 10^{-5}$	51%	0.74
H3 K[Me]G	78%	$< 10^{-16}$	50%	0.92
H3 K[AcMe]G	82%	$< 10^{-21}$	50%	0.90

*** Genes located less than 10 kb from a telomere**

Table 3-4: Effects of histone H3 mutant strains on telomere proximal gene expression

Mutants	Telomere genes*		Random gene set	
	Rank Percentile	*P*-Value	Rank Percentile	*P*-Value
H3 K[Ac]G	65%	0.0056	53%	0.60
H3 K[Me]G	74%	$< 10^{-4}$	47%	0.54
H3 K[AcMe]G	80%	$< 10^{-7}$	49%	0.77

*** Genes located less than 5 kb from a telomere**

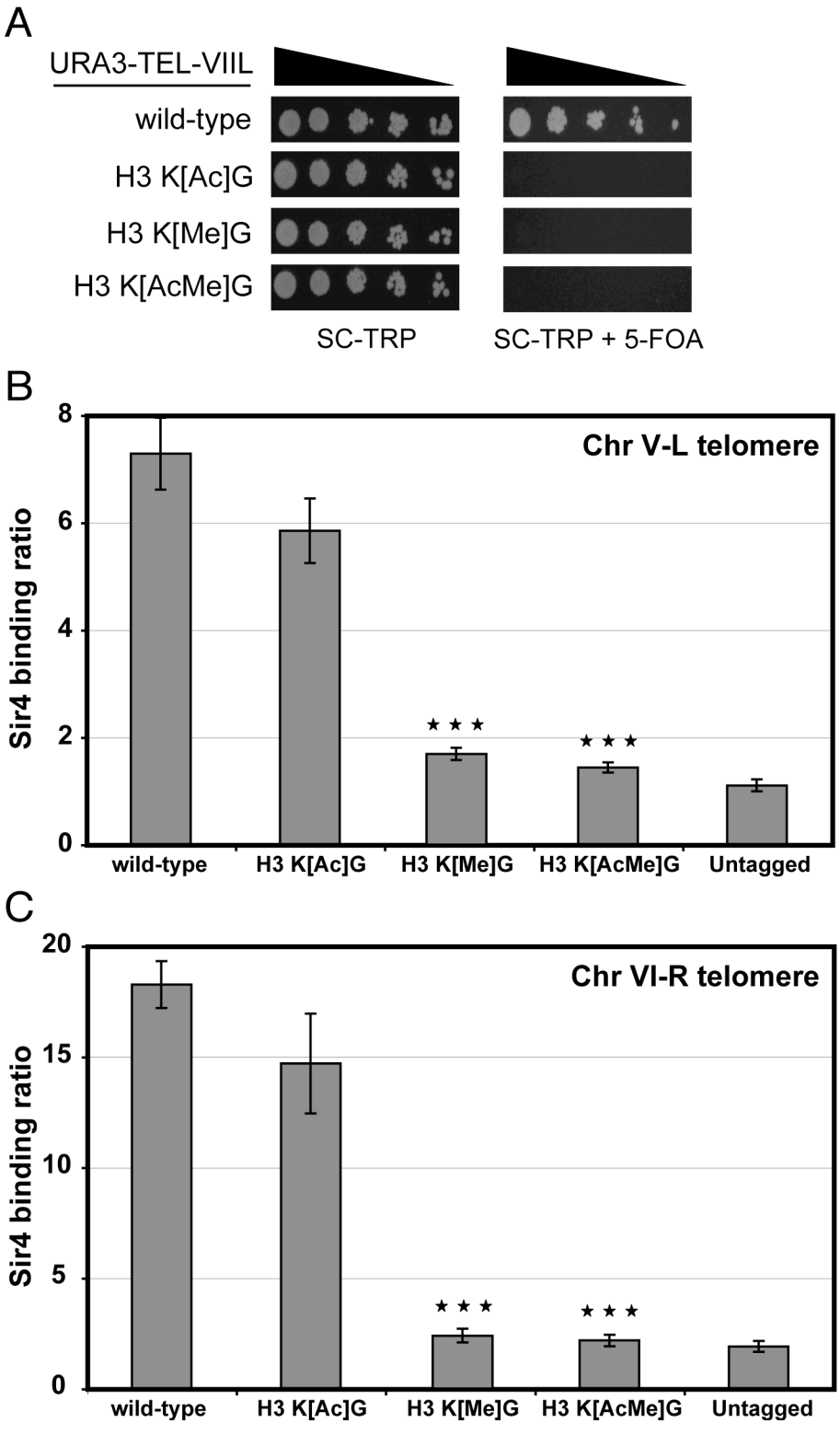
A
URA3-TEL-VIIL
wild-type
H3 K[Ac]G
H3 K[Me]G
H3 K[AcMe]G
SC-TRP
SC-TRP + 5-FOA
B
Chr V-L telomere
Sir4 binding ratio
0
2
4
6
8
wild-type
H3 K[Ac]G
H3 K[Me]G
H3 K[AcMe]G
Untagged
C
Chr VI-R telomere
Sir4 binding ratio
0
5
10
15
20
wild-type
H3 K[Ac]G
H3 K[Me]G
H3 K[AcMe]G
Untagged

FIGURE 3-6: Histone H3 lysine mutants disrupt telomere silencing.

(A) Telomeric silencing of a *URA3* reporter gene was measured in histone H3 mutant strains. 5-fold serial dilutions of each yeast strain were spotted on SC-TRP media or SC-TRP with 5-FOA as indicated. **(B)** and **(C)** Effects of the histone H3 acetylated and methylated lysine mutants on the binding of the SIR silencing complex near the chromosome telomere regions. The abundance of Sir4 at chromosome V-L (Chr V-L) and chromosome VI-R (Chr VI-R) telomere regions in wild-type strains and H3 mutant strains were determined by ChIP analysis. An untagged strain was used as control. Sir4 binding ratio at Chr V-L is normalized to an internal control (*ACT1*) and the input DNA. Sir4 binding ratio at Chr VI-R is normalized to an internal control (*SPS2*) and the input DNA. Each error bar represents the standard deviation of three independent ChIP experiments. A *t*-test was applied to generate the *p*-values. Three asterisks indicate $p < 0.001$ compared to wild-type.

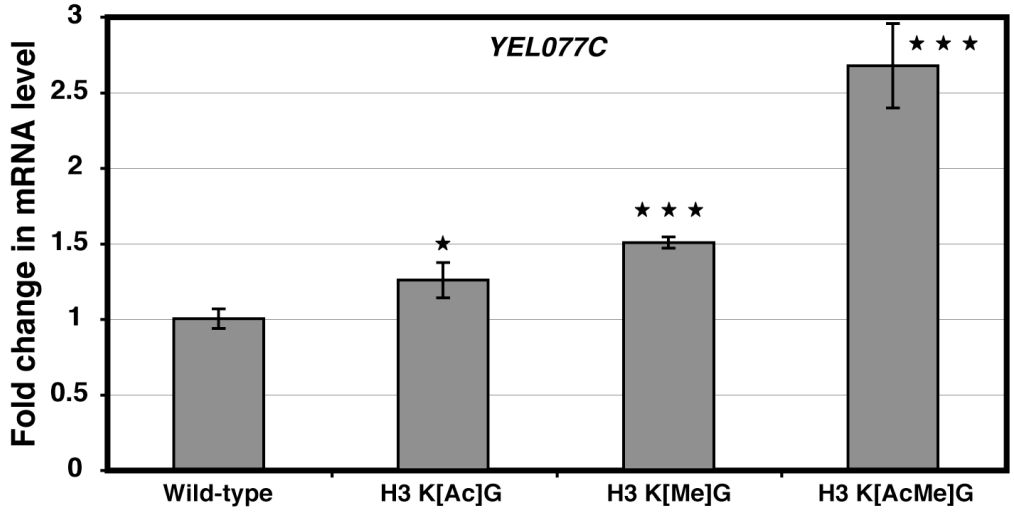

FIGURE 3-7: *YEL077C* transcript levels in wild-type and histone H3 mutants were compared using RT-PCR analysis.

The signal of *YEL077C* transcript was normalized by *ACT1* signal. The calculated signal of *YEL077C* transcript in wild-type was set to one. Each error bar represents the standard deviation of three independent experiments. A *t*-test was applied to generate the *p*-values. Single asterisk indicates $p < 0.05$ and three asterisks indicate $p < 0.001$ compared to wild-type.

significantly greater in the H3 K[AcMe]G mutant than in the H3 K[Me]G mutant (Figure 3-7). Hence, the transcriptional induction observed in telomeric genes upon mutating the histone H3 acetylated lysine residues appears largely independent of any effect on Sir4 binding. In contrast, the histone H3 methylated lysine residues regulate telomere silencing principally through their effects on the binding of the SIR complex.

Histone modification status of genes regulated by histone H3 lysine residues. The histone H3 K[Ac]G, H3 K[Me]G, and H3 K[AcMe]G mutants eliminate both modified and unmodified lysine residues in genomic chromatin. Hence, the transcriptional changes in these mutants could theoretically be due to the loss of the modified version of the histone H3 lysine residues or the unmodified version. To distinguish between these two possibilities, we used ChromatinDB (O'Connor and Wyrick, 2007) to compare the gene expression changes in the histone H3 mutants with published ChIP-chip data (Bernstein et al., 2002; Kurdistani et al., 2004; Rao et al., 2005) for histone acetylation and methylation.

We first compared ChIP-chip data for acetylation of H3 K9, K14, K18, K23, and K27 (Kurdistani et al., 2004) with the expression data for the H3 K[Ac]G and H3 K[AcMe]G mutant strains. For simplicity, we analyzed the average acetylation level for the five acetylated H3 lysine residues. Figure 3-8A indicates that the genes up-regulated in the H3 K[Ac]G mutant were predominately those that originally had low levels of promoter histone acetylation in wild-type cells. This trend was even more pronounced in the H3 K[AcMe]G mutant (Figure 3-8A). Each of these trends was statistically significant, as judged using a Wilcoxon rank sum test ($P < 10^{-3}$ and $P < 10^{-10}$ for the H3 K[Ac]G and H3 K[AcMe]G data sets, respectively). A similar result was obtained when

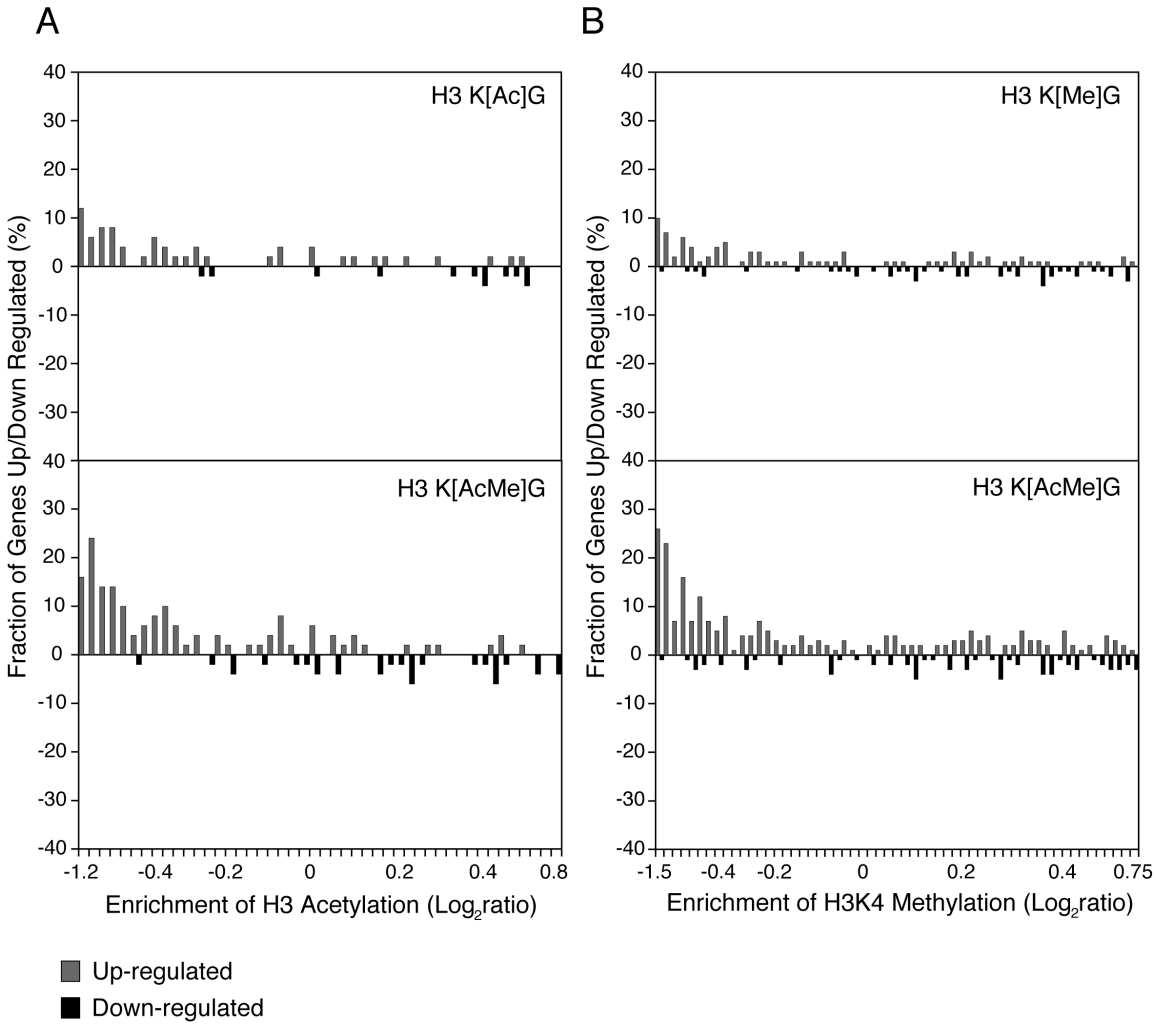

FIGURE 3-8: Correlation between histone modification levels and gene expression changes in the histone H3 mutants.

(A) For the H3 K[Ac]G and H3 K[AcMe]G mutants, the fraction of genes whose mRNA levels are up/down-regulated is plotted against their average promoter H3 acetylation levels in wild-type cells. The ChIP-chip data has been normalized for nucleosome levels, as described (O'Connor and Wyrick, 2007). **(B)** For the H3 K[Me]G and H3 K[AcMe]G

mutants, the fraction of genes whose mRNA levels were up/down-regulated is plotted against their average level of nucleosome-normalized histone H3K4 di-methylation in wild-type cells.

we tested the acetylation levels of each histone H3 lysine residue individually (data not shown). However, this trend was not observed in the coding regions of the up-regulated genes. In summary, disruption of hypoacetylated chromatin was correlated with de-repression in the H3 K[Ac]G mutant.

A similar pattern was observed when we compared ChIP-chip data for methylation of H3K4 and H3K36 (Bernstein et al., 2002; Rao et al., 2005) with the expression data for the H3 K[Me]G and H3 K[AcMe]G mutant strains. Figure 3-8B shows that genes up-regulated in the H3 K[Me]G mutant were biased towards those genes that had low levels of H3K4 di-methylation in their promoter regions ($P < 10^{-8}$). Again, this effect was exaggerated in the H3 K[AcMe]G mutant ($P < 10^{-22}$). Low levels of K36 di-methylation in the coding regions of genes was also correlated with gene expression changes in the H3 K[Me]G and H3 K[AcMe]G mutant strains ($P < 10^{-4}$ and $P < 10^{-23}$, respectively), as was H3K4 di-methylation (data not shown).

Previous studies have shown that histone H3 acetylated and methylated lysine residues are depleted of these modifications at telomeric and subtelomeric genomic regions (Bernstein et al., 2002; Millar and Grunstein, 2006; Robyr et al., 2002). Hence, we classified the genes up-regulated in the histone H3 mutants into telomere-proximal (i.e., gene located in telomeric or subtelomeric genomic regions) and non-telomere proximal (i.e., genes located further than 20 kb from a telomere end) groups and repeated the analysis described above. This analysis indicated that the non-telomere proximal groups of up-regulated genes were also significantly associated with low levels of histone H3 acetylation and H3K4 methylation ($P < 10^{-3}$ and $P < 10^{-5}$, respectively). It is important to note that these unmodified, up-regulated genes were distributed throughout

the genome (based on visual inspection, data not shown), and did not appear to be an indirect effect of the loss of telomeric silencing in these mutants, based on our analysis of a *sir2*Δ microarray data set (Martin et al., 2004; Wyrick et al., 1999). Hence, the correlation with unmodified histone H3 lysine residues was not limited to silenced genes, but was a feature of genes located throughout the genome.

DISCUSSION

Previous studies have suggested that acetylated and methylated lysine residues in histone H3 functionally interact to regulate gene expression and other cellular processes. In this study, we have used a genetic approach to characterize the functional interactions between acetylated and methylated lysine residues in histone H3. We constructed histone H3 mutant alleles that simultaneously eliminate acetylated lysine residues (H3 K[Ac]G), methylated lysine residues (H3 K[Me]G), or both acetylated and methylated lysine residues (H3 K[AcMe]G). Analysis of the growth phenotypes and gene expression changes exhibited in these mutant strains argues against the models of either functional interdependence or redundancy; instead our results are consistent with the model that acetylated and methylated histone lysine residues function through distinct mechanisms to make independent contributions to yeast gene expression and silencing.

For the purposes of this paper, we have classified the histone H3 lysine residues into a group that is predominately acetylated (H3 K9, K14, K18, K23, K27) and a group that is predominately methylated (H3 K4, K36, K79). It should be noted, however, that a recent report suggests that histone H3 K36 is also acetylated (Morris et al., 2007).

Mutations in histone H3 methylated lysine residues (e.g., H3K4) or histone methyltransferase enzymes (e.g., Set1) have been previously shown to exhibit various growth phenotypes, including slow growth defects and sensitivity to the DNA replication inhibitor hydroxyurea (HU) (Briggs et al., 2001; Fingerman et al., 2005; Miller et al., 2001). We observed similar growth defects in the H3 K4,36,79G mutant strain (H3 K[Me]G, see Figure 3-1), including a slowed doubling time in SC media, and striking sensitivities to the transcription elongation inhibitor 6-AU, and the DNA replication

inhibitor and damaging agent HU and MMS. Strikingly, the simultaneous mutation of H3 acetylated lysine residues rescues these growth phenotypes in the H3 methylated lysine mutant strain. Indeed, the H3 K[Ac]G mutant suppresses the HU sensitivity of a *SET1* deletion mutant (data not shown), and is more resistant to HU than wild-type. Previous studies have shown that histone acetylation can regulate the firing of replication origins (Aparicio et al., 2004; Vogelauer et al., 2002); hence, it is possible that the H3 K[Ac]G mutant directly modulates the process of DNA replication.

Alternatively, the different phenotypes in the H3 K[Ac]G or H3 K[Me]G mutants could be due to differing effects on yeast gene expression. While the H3 K[Ac]G and H3 K[Me]G mutants have fairly similar effects on gene expression, there are a number of genes whose expression change is different in the mutant strains. These genes are listed in Table 3-5. Inspection of the gene lists suggests a number of potential candidate genes involved in DNA repair (e.g., *MRE11*, *FYV6*, and *REV1*), whose expression change could be responsible for the differing HU and MMS phenotypes in the histone mutant strains.

Comparison of the H3 K[Ac]G, H3 K[Me]G, and H3 K[AcMe]G microarray data sets enabled us to test these histone residues genetically interact to regulate transcription. As our null hypothesis (i.e., neutrality function), we used a modified version of the additive model. If the measured expression data for the H3 K[AcMe]G mutant significantly deviated from this model, this would indicate a genetic interaction. For example, if histone H3 acetylated and methylated lysine residues were functionally redundant, then the change in transcription in the H3 K[AcMe]G mutant should be significantly greater than the sum of the effects observed in the H3 K[Ac]G and H3 K[Me]G mutants. On the other hand, if the interdependence model were correct, then the

Table 3-5: Differentially expressed genes in histone H3 mutant strains

Genes with altered expression only in the H3 K[Me]G mutant		
Gene	Fold Change	Protein Function
IME4	3.5	Activate IME1 in response to cell-type and nutritional signals; Regulate meiosis
HSP30	2.5	Induced by heat shock, ethanol treatment, and entry into stationary phase
HVG1	2.4	Homologous to VRG4
YDR543C	2.3	Dubious ORF
XRS2	2.2	DNA repair protein, component of MRX complex
STR3	2.1	Cystathionine beta-lyase
ICY2	2.1	Potential Cdc28 substrate
PEX21	2.1	Peroxin required for targeting of peroxisomal matrix proteins
ATR1	2.0	Multidrug efflux pump of the major facilitator superfamily
TOS8	2.0	Homeodomain-containing transcription factor, regulating genes in G1/S phase
UBP8	-2.9	Deubiquitinating enzyme, component of SAGA complex
AXL1	-2.6	Haploid specific endoprotease; Required for axial budding pattern
YNL203C	-2.5	Dubious ORF
CNE1	-2.5	Calnexin; Integral membrane ER chaperone
PPM2	-2.4	tRNA methyltransferase
BNA1	-2.3	3-hydroxyanthranilic acid dioxygenase
YLR040C	-2.3	Up-regulated by Mcm1-Alpha1 transcription factor
ARA2	-2.3	NAD-dependent arabinose dehydrogenase
TAZ1	-2.3	Lyso-phosphatidylcholine acyltransferase
BNA6	-2.2	Quinolinate phosphoribosyl transferase
YLR462W	-2.2	Similar to helicases
TRI1	-2.2	Similar to components of human SWI/SNF complex
SNO2	-2.2	Induced before the diauxic shift or in the absence of thiamin
YGL193C	-2.1	Haploid-specific gene; Turned off in a *sir3*Δ mutant
OST6	-2.1	Subunit of the oligosaccharyltransferase complex of the ER lumen
YRF1-6	-2.1	Helicase encoded by the Y' element of subtelomeric regions
MRE11	-2.1	DNA repair protein, component of MRX complex
DSL1	-2.1	Peripheral membrane protein required for Golgi-to-ER retrograde traffic
FYV6	-2.1	Proposed to regulate double-strand break repair via NHEJ
NRK1	-2.1	Nicotinamide riboside kinase
BSC1	-2.0	Similar to Muc1
REV1	-2.0	Deoxycytidyl transferase; Involved in DNA repair
Genes with altered expression only in the H3 K[Ac]G mutant		
Gene	Fold Change	Protein Function
YPR078C	4.0	Hypothetical protein; Possible role in DNA metabolism and in genome stability
YKL070W	3.9	Hypothetical protein
FLR1	3.9	Plasma membrane multidrug transporter
AAD4	3.7	Putative aryl-alcohol dehydrogenase
RIM4	3.5	Putative RNA-binding protein required for the expression of sporulation genes
YDR215C	3.4	Dubious ORF
FMP16	3.0	Hypothetical protein
YLR346C	2.9	Hypothetical protein
PDC6	2.5	Minor isoform of pyruvate decarboxylase
YPS3	2.5	GPI-anchored aspartic protease
STF1	2.4	Regulate the mitochondrial F1F0-ATP synthase
SRL3	2.4	Over-expression suppresses the lethality of a *rad53*Δ mutant

Table 3-5 (Continue): Differentially expressed genes in histone H3 mutant strains

Gene	Fold Change	Protein Function
KRE11	2.2	Involved in biosynthesis of cell wall beta-glucans; Subunit of TRAPP complex
PAU13	2.2	Hypothetical protein
PFK26	2.2	6-phosphofructo-2-kinase
ECM4	2.2	Omega class glutathione transferase
SSK22	2.2	MAP kinase kinase kinase of the HOG1 mitogen-activated signaling pathway
YDL027C	2.2	Hypothetical protein
HSP31	2.2	Possible chaperone and cysteine protease
GLC3	2.2	1,4-glucan-6-(1,4-glucano)-transferase
SAM3	2.2	High-affinity S-adenosylmethionine permease
ZTA1	2.2	Zeta-crystallin homolog
AIM3	2.1	Potentially involved in maintaining mitochondria genome
SCC4	2.1	Subunit of cohesin loading factor; Involved in DNA double-strand break repair
TAM41	2.1	Mitochondrial protein involved in protein import; Maintain TIM23 function
RTA1	2.1	Involved in 7-aminocholesterol resistance
YGR250C	2.1	Hypothetical protein
UBI4	2.1	Ubiquitin
COS3	2.1	Involved in salt resistance; Interact with sodium:hydrogen antiporter Nha1
PAU5	2.0	Member of the seripauperin gene family mainly in subtelomeric regions
PAU20	2.0	Hypothetical protein
YLR414C	2.0	Hypothetical protein
LCB5	2.0	Minor sphingoid long-chain base kinase
FMN1	2.0	Riboflavin kinase
IRC7	-21.3	Putative cystathionine beta-lyase
AAD15	-6.9	Putative aryl-alcohol dehydrogenase
RDS1	-4.1	Zinc cluster transcription factor
YCR102C	-2.7	Hypothetical protein; Possible role in copper metabolism
YDR222W	-2.7	Hypothetical protein
POL30	-2.5	Proliferating cell nuclear antigen; Possible role in DNA replication and repair
ECM3	-2.4	Involved in signal transduction and the genotoxic response
GPI14	-2.4	Glycosylphosphatidylinositol-alpha 1,4 mannosyltransferase I
YER071C	-2.4	Hypothetical protein
YML019W	-2.3	Hypothetical protein
FRE7	-2.3	Putative ferric reductase
BDH1	-2.2	NAD-dependent (R,R)-butanediol dehydrogenase
YGL204C	-2.1	Dubious ORF
FUR1	-2.1	Uracil phosphoribosyltransferase
UBX6	-2.1	UBX (ubiquitin regulatory X) domain-containing protein
STP4	-2.1	Containing a Kruppel-type zinc-finger domain
PLP1	-2.1	Interact with CCT complex and has a role in actin and tubulin folding
CTP1	-2.1	Mitochondrial inner membrane citrate transporter
YLR101C	-2.0	Dubious ORF
IZH4	-2.0	Involved in zinc metabolism; Possible role in sterol metabolism

expression changes in the H3 K[AcMe]G should be significantly smaller than predicted by the additive model, as mutating either the acetylated or methylated lysine residues would disrupt their interdependent functions.

Our analysis revealed that the gene expression changes in the H3 K[AcMe]G "double" mutant could be accurately modeled as the sum of the effects of the H3 K[Ac]G and H3 K[Me]G mutants, and thus fit the additive model. There were some exceptions: for a few genes, the increase in mRNA levels observed in the H3 K[Ac]G mutant was suppressed upon mutating the histone H3 methylated lysine residues, suggesting an epistatic interaction. Despite these exceptions, the data as a whole supported the additive model. In summary, our data suggest that the acetylated and methylated H3 lysine mutants make independent contributions to yeast gene expression. This finding is in accordance with the conclusion of a previous study, which showed that mutations in histone H4 acetylated lysine residues (H4 K5, K8, K12) had a cumulative effect on yeast gene expression (Dion et al., 2005).

Previous studies have shown that mutations that affect H3K36 di-methylation can stimulate aberrant internal transcription to generate cryptic transcripts (Carrozza et al., 2005; Li et al., 2007b). Comparison of our microarray data with a profile of cryptic transcripts generated in a *set2*Δ mutant (Li et al., 2007b) revealed a significant overlap with each of the histone mutants, particularly the H3 K[Me]G mutant data set (Figure 3-9). This suggests that a subset of the up-regulated genes observed in the histone mutants may be due to an increase in internally initiated cryptic sense transcripts, as expected from previous studies.

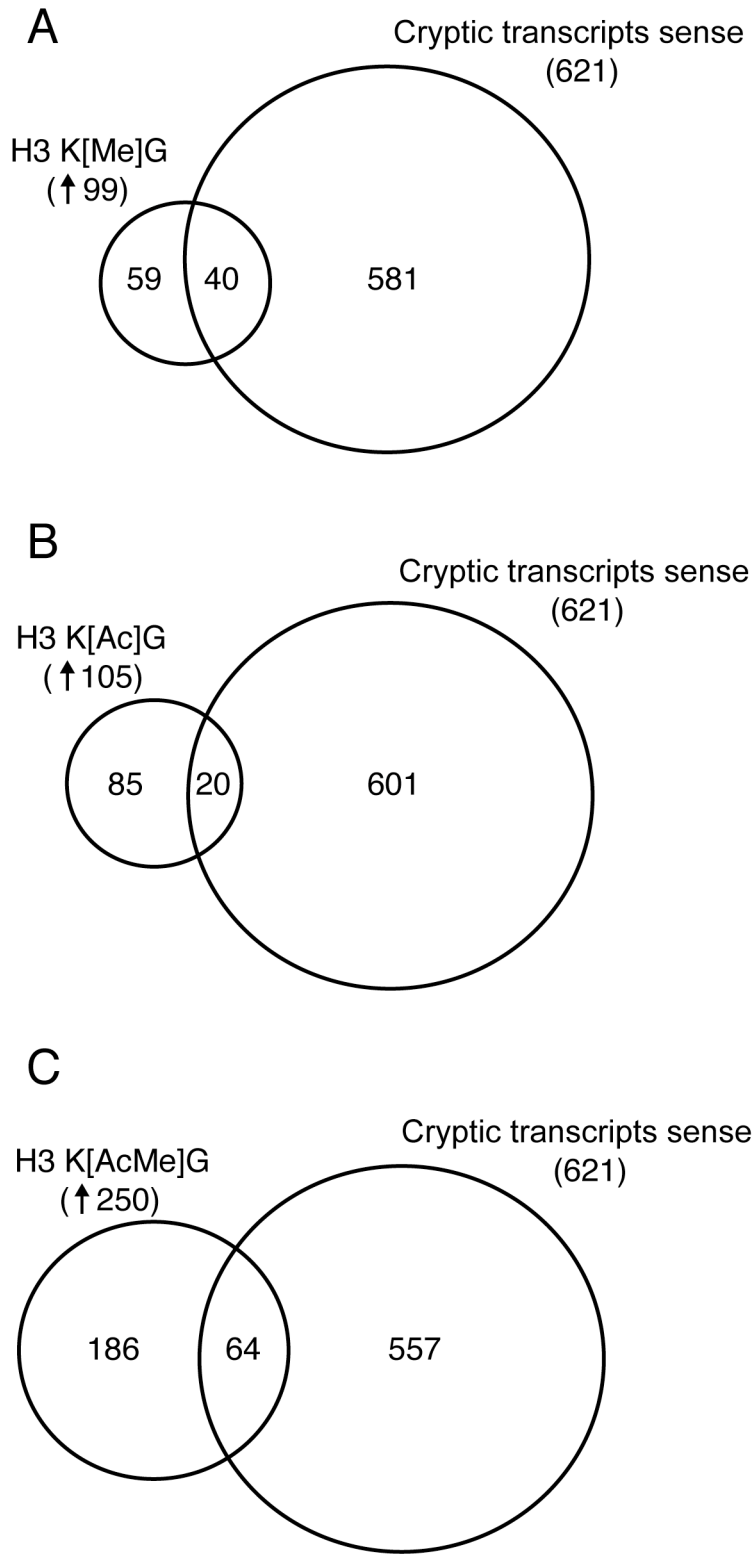
A
Cryptic transcripts sense
(621)
H3 K[Me]G
(↑99)
59
40
581
B
Cryptic transcripts sense
(621)
H3 K[Ac]G
(↑105)
85
20
601
C
Cryptic transcripts sense
(621)
H3 K[AcMe]G
(↑250)
186
64
557

FIGURE 3-9: Effects of histone H3 acetylated and methylated lysine mutants on cryptic transcripts.

Venn diagrams were used to compare the sets of up-regulated genes in each of the histone H3 mutants with a profile of cryptic internal transcripts generated in a *set2*Δ mutant (Li et al., 2007b).

The functional independence of the histone H3 acetylated and methylated lysine residues is highlighted in their effects on telomeric silencing. Previous studies have indicated that both methylated and acetylated lysine residues in histone H3 regulate telomeric silencing. While we find that both the methylated and acetylated lysine mutants affect telomeric gene expression, they appear to do so through independent mechanisms. The H3 K[Me]G mutant causes a significant increase in telomeric gene transcription, and a significant decrease in Sir4 binding to telomere regions. In contrast, the H3 K[Ac]G mutant causes a small but significant increase in telomeric gene expression and disrupts the silencing of a telomere-located reporter gene, but has only a slight, insignificant effect on Sir4 binding. While it is formally possible that the H3 K[Ac]G mutant could affect the binding of other members of the SIR complex (e.g., Sir2 or Sir3), we do not favor this model. Our results show that binding of Sir4 (and presumably the SIR complex) is similarly disrupted in the H3 K[Me]G and H3 K[AcMe]G mutants, yet the H3 K[AcMe]G mutant has a significantly greater effect on telomeric gene expression than the H3 K[Me]G mutant. Taken together, these results suggest that acetylated lysine residues in histone H3 regulate telomeric silencing through a mechanism independent of SIR binding.

The histone H3 acetylated and methylated lysine mutants also affect the expression of genes located elsewhere in the yeast genome. Intriguingly, we observed that each of the histone H3 mutants up-regulates a significant fraction of genes located in HAST chromosomal domains ($P < 0.001$). These domains were previously identified as subtelomeric regions that were depleted for histone acetylation by the Hda1 histone deacetylase (Robyr et al., 2002). This analysis suggests that acetylated and methylated

lysine residues in histone H3 may play a direct role in regulating the expression of these genes.

Finally, our analysis indicates that the transcriptional repression exerted by histone H3 acetylated and methylated lysine residues is associated with the unmodified versions of these lysine residues. Hence, the genetic interactions we describe cannot necessarily be attributed to the loss of histone acetylation or methylation. Previous studies have shown that the SIR silencing proteins bind preferentially to the unmethylated forms of histone lysine residues (Ng et al., 2003; Santos-Rosa et al., 2004; van Leeuwen and Gottschling, 2002b). This observation could explain why telomeric genes are up-regulated in the histone H3 lysine-to-glycine mutants, as this mutant eliminates the unmethylated lysine side chain, and thus should disrupt SIR binding. It is intriguing to speculate that the mutation of unacetylated lysine residues in histone H3 could disrupt the binding of other repressor proteins. Likely possibilities include the Tup1 repressor (Malave and Dent, 2006) or SANT domain-containing proteins (Boyer et al., 2004), as these proteins bind preferentially to unacetylated chromatin (Boyer et al., 2004; Davie et al., 2002; Edmondson et al., 1996; Malave and Dent, 2006).

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CHAPTER FOUR

SUMMARY AND GENERAL DISCUSSION

The studies in this thesis focused on investigating the functional interplay among the three methylated lysine residues in histone H3 and between methylated and acetylated histone H3 lysine residues in *Saccharomyces cerevisiae*.

Exploring the Genetic Interaction of Histone H3 Methylated Lysine Residues

It is surprising that while histone H3 methylation play important roles in various biological processes, such as transcription regulation, individual mutations in H3K4, H3K36, or H3K79 have little effect on cell growth or viability. It seems more likely that there is a high degree of redundancy among histone H3 methylated lysine residues in biological processes. To investigate this possible redundancy, we systematically mutated each of the three methylated lysine residues (H3K4, H3K36, and H3K79) to an arginine residue, both individually and in all possible combinations. Mutations in multiple methylated lysine residues showed a variety of synthetic growth defects, the most of striking of which is the lethal phenotype of the H3 K4,36,79R mutant. Experimental evidence also confirmed that the lethal phenotype is not due to a structural defect in the histone H3 mutant protein or a nucleosome assembly defect. This surprising discovery of the lethal H3 K4,36,79R mutant strongly suggests that the three methylated lysine residues have redundant functions on yeast cell growth.

Further genome-wide transcription profiling experiments revealed that the H3 K4,36,79R mutant causes a genome-wide progressive gene transcription defect, which initiates in telomeric heterochromatin regions, and then spreads further into the chromosome. Additionally, the SIR silencing complex was identified to be required for the lethal phenotype of the H3 K4,36,79R mutant and displays increased binding to

genomic regions adjacent to yeast telomeres in the H3 K4,36,79R mutant. This inappropriate binding of the SIR silencing complex contributes, in part, to the spreading of gene silencing observed in the H3 K4,36,79R mutant and leads to its lethal phenotype. More intriguingly, we also found that the H3 K4,79R mutant causes only a modest increase in gene silencing, primarily confined to genes located within 10 kb of a telomere. Only when H3K36 is also mutated to arginine is a global defect in gene silencing observed. The SIR binding data demonstrates more spreading of the SIR silencing complex further into the euchromatin regions in the H3 K4,36,79R mutant as compared to the H3 K4,79R mutant, in accordance with the expression data. These data together suggest that H3K4, H3K36, and H3K79 act in a redundant manner to limit the spreading of the SIR silencing complex. In addition to the transcription defect, defects were also observed in the cell cycle stage of mitosis and cytokinesis in the H3 K4,36,79R mutant. A previous study showed that over-expression of the Sir2 or Sir3 protein led to a significant decrease in chromosome stability, suggesting that the SIR silencing complex interferes with the mitotic transmission of chromosomes (Holmes et al., 1997). We speculate that the cell cycle defects in the H3 K4,36,79R mutant could be a result of the abnormal spreading of heterochromatin mediated by the SIR silencing complex.

Although we found the extensive spreading of the SIR silencing complex near the telomere proximal regions (~20 kb away from a telomere end), it is not clear that whether the SIR complex misplacement also occurs in some other regions of the genome (e.g., the silent mating loci, or rDNA loci). In addition, previous studies argued that the pool of Sir proteins in cells are limited (Hecht et al., 1996; Renauld et al., 1993), and an extensive spreading of the SIR silencing complex would cost the relocation of Sir proteins from

somewhere else in chromatin (Kristjuhan et al., 2003; Suka et al., 2002). In the future, a ChIP-chip analysis of the SIR silencing complex distribution in genomic chromatin should be performed in the H3 K4,36,79R mutant. The comprehensive profile of the SIR silencing complex distribution in the H3 K4,36,79R mutant will not only help us to more accurately determine the extent of the SIR spreading, but also address that whether the spreading of the SIR complex is at the expense of losing Sir proteins elsewhere in genomic chromatin.

It is worth noting that the deletion of the SIR silencing complex only partially suppressed the lethality of the H3 K4,36,79R mutant, suggesting other regulators also contribute to the lethal phenotype of the H3 K4,36,79R mutant. Indeed, we have found that over-expression of the *MAD1* gene suppressed the lethal phenotype of the H3 K4,36,79R mutant. Yeast Mad1 is a nuclear protein with predominantly coiled-coil structure and becomes hyperphosphorylated during normal cell cycle and when spindle assembly is interfered (Hardwick and Murray, 1995). Previous studies suggested that the Mad1 protein interacts with the Mad2 protein and delivers the Mad2 protein to unattached kinetochores (Lew and Burke, 2003). Later, the Cdc20 protein substitutes the Mad1 protein in the Mad1/Mad2 complex to form the Cdc20/Mad2 complex, subsequently initiating the spindle checkpoint process (De Antoni et al., 2005; Lew and Burke, 2003). It has been proposed that the ratio among the Mad1, Mad2, and Cdc20 proteins in cells is critical for appropriate spindle assembly checkpoint function (De Antoni et al., 2005; Hwang et al., 1998; Lew and Burke, 2003; Luo et al., 2002). The finding that the Mad1 protein is involved in the lethality of the H3 K4,36.79R mutant

suggests a possible novel function role of methylated histone H3 lysine residues in the regulation of spindle assembly.

In addition to the proposed genetic screening (Figure 2-6), an alternative genetic screen using systematic yeast over-expression library will also be carried out to isolate regulators, over-expression of which will suppress the lethal phenotype of the H3 K4,36,79R mutant. The identification of other important regulators using genetic screenings will significantly advance our understanding of the molecular mechanism underneath the observed lethal phenotype.

Considering lysine and arginine residues are similar in size and charge, and arginine is not methylated by HKMTs *in vivo*, arginine residue has been widely used as a mimic of an unmethylated lysine residue. It is possible that the cause of the lethality of the H3 K4,36,79R mutant is due to the loss of histone H3 methylation throughout the genome. However, a *set1*Δ *set2*Δ *dot1*Δ triple deletion yields a viable yeast strain and fails to recapitulate the lethal phenotype of the H3 K4,36,79R mutant. This conflicting observation was explained by the discovery that a deletion of the Set1 HKMT suppressed the lethal phenotype of the H3 K4,36,79R mutant, which suggested that the Set1 protein is required for the lethality. Our data further indicated that the Set1 protein, but not its histone lysine methyltransferase activity, was required for the lethal phenotype of the H3 K4,36,79R mutant. The simple interpretation of this intriguing observation is that the lethal phenotype is, in part, due to the association of HKMTs with a histone substrate that cannot be methylated. The histone H3 lysine-to-arginine mutants and the catalytically inactive HKMT mutants are lethal because they prevent histone H3 lysine methylation without disrupting the association of the methyltransferase with its substrate. In this case,

mutants that disrupted this association (e.g., the *set1*Δ *set2*Δ *dot1*Δ mutant or the H3 K[Me]G) would not be expected to be lethal, in accordance with our data. Currently, we don't know why a so-called 'stymied methyltransferase' would necessarily be lethal. However, it is possible that that the Set1 protein may have a direct inhibitory function. For example, the Set1 binding to histone substrates in the absence of histone methylation could inhibit gene transcription. To investigate a global effect of the catalytically inactive methyltransferase mutants in gene transcription using DNA microarray technology will help to address this possibility. A previous study also showed that the Set1 HKMT has another substrate (i.e., the Dam1 protein) in addition to histone proteins (Zhang et al., 2005a). It is intriguing to speculate that the Set1 HKMT could exert its inhibitory effect through its interaction with other substrates. Alternatively, proteins associated with HKMTs may have additional functions that are required for the lethality of the H3 K4,36,79R mutant. It is important to note that deletion of the Set1 protein disrupts the larger Set1-containning COMPASS complex (Roguev et al., 2001). It is possible that other components of the COMPASS complex, which may be compromised by the deletion of the Set1 protein, are required for the lethal phenotype of the H3 K4,36,79R mutant. Nonetheless, future studies are needed to investigate these possibilities.

In conclusion, we have proposed a model in which H3K4, H3K36, and H3K79 act in a redundant manner to regulate various biological processes in yeast, such as cell growth, gene transcription and cell cycle progression. Indeed, hypomethylation of all three histone H3 lysine residues in concert across the whole genome, mimicked by the lysine to arginine mutation, elicits the spreading of the SIR silencing complex (and may

affect the binding of some other factors too), causing the inappropriate silencing of gene expression, the disruption of cell cycle, and eventual cell death.

Genetic Interaction of Histone H3 Acetylated and Methylated Lysine Residues

The work described in Chapter Three focused on the functional interplay between acetylated and methylated lysine residues in histone H3. Eight modified lysine residues are divided into two groups, one group includes predominately acetylated lysine residues (H3 K9,14,18,23,27), each of which is located at the histone H3 N-terminal domain, and the other is predominately methylated (H3 K4,36,79). Acetylated lysine residues, methylated lysine residues, or both acetylated and methylated lysine residues were simultaneously mutated to glycine. The advantage of using glycine mutants is to allow us to observe the potential effects caused by loss of both the modified and unmodified versions of the histone H3 lysine residues. Comprehensive phenotypical characterizations of three histone mutants revealed various growth phenotypes under different conditions. Indeed, the H3 K[Me]G mutant exhibits a slow growth defect in SC media and sensitivity to damaging reagents (i.e., 6-AU, HU, and MMS). 6-AU is a common used transcription inhibitor and inhibits RNAPII elongation. HU generally inhibits DNA replication process and MMS is an alkylating agent that causes DNA double-strand breaks in cells. Increased sensitivity to these three damaging reagents suggests that the H3 K[Me]G mutant is defective in transcription elongation, DNA replication, and DNA repair processes. Our data is in accordance with previous observations that a *set1*Δ mutant is sensitive to 6-AU, HU, or MMS treatment (Miller et al., 2001; Wysocki et al., 2005; Zhang et al., 2005b). Intriguingly, mutation of acetylated lysine residues suppressed these growth defects in the

H3 K[Me]G mutant, indicating that the H3 K[Ac]G and H3 K[Me]G mutants have opposing effects on cell growth, DNA replication, and DNA repair processes. The molecular mechanism beneath this striking interplay is unclear.

One possible connection between histone acetylation and DNA replication is that histone acetylation might be involved in the regulation of replication origins firing (Aparicio et al., 2004; Vogelauer et al., 2002). Evidence indicated that histone deacetylation predominately delays the activation of many late-replicating origins (Aparicio et al., 2004). The strong HU insensitivity phenotype of the H3 K[Ac]G mutant suggests that histone H3 acetylated lysine residues might affect chromosomal replication timing, potentially through regulating late-firing origins. One of intriguing hypotheses is that histone acetylation level may affect local chromatin structures, and subsequently alter the interaction of origins with replication initiation factors (Aparicio et al., 2004), but the mechanism still remains unknown. Future studies will be focused on addressing which acetylated lysine residue(s) in histone H3 plays a key role in regulating DNA replication dynamics and isolating the unknown replication initiation factors, binding of which is regulated by histone H3 acetylation in the DNA replication process. However, it is important to note that we cannot rule out the possibility that the different phenotypes in the H3 K[Ac]G or H3 K[Me]G mutants could be due to differing effects on gene expression. Indeed, we identified a few candidate genes (e.g., *MRE11*, *FYV6*, and *REV1*), and their expression change could be responsible for the differing HU and MMS phenotypes in the histone lysine mutants.

Genome-wide gene transcription changes in these three histone mutants were also investigated by using DNA microarray technology. Overall, we found that the gene

expression changes in the H3 K[AcMe]G mutant could be accurately modeled as the sum of the gene expression changes of the H3 K[Ac]G and H3 K[Me]G mutants using an additive model. This interesting result suggested that acetylated and methylated lysine mutants generally make independent and additive contributions to yeast gene expression.

It is important to acknowledge that for a subset of yeast genes, acetylated and methylated histone H3 lysine residues do show epistatic interactions in transcription regulation. Also, our analysis did not distinguish between the contributions of individual acetylated or methylated histone H3 lysine residues in regulating gene expression. However, comparison of the histone H3 K[Me]G data set with the previously published H3 K4G data set (Martin et al., 2004) indicates that the H3 K4G mutant accounts for the expression changes of 13 of the 99 genes up-regulated and 2 of the 51 genes down-regulated in the H3 K[Me]G mutant (Figure 4-1). Presumably, the combination of the H3 K36G and H3 K79G mutants account for the remaining genes that are differentially expressed in the H3 K[Me]G mutant strain. Future studies will examine the contributions of individual acetylated histone H3 lysine residues in transcription regulation, in turn to better understand the level of uniqueness and redundancy of these acetylated lysine residues.

Our data supports the model from previous studies that both acetylated and methylated lysine residues in histone H3 regulate telomeric silencing, in accordance to previous studies (Ng et al., 2003; Santos-Rosa et al., 2004; Thompson et al., 1994). However, our data also suggests that the H3 K[Ac]G and H3 K[Me]G mutants affect telomere proximal gene transcription through independent mechanisms. Indeed, the H3 K[Me]G mutant almost eliminated the SIR silencing complex binding in the telomeric

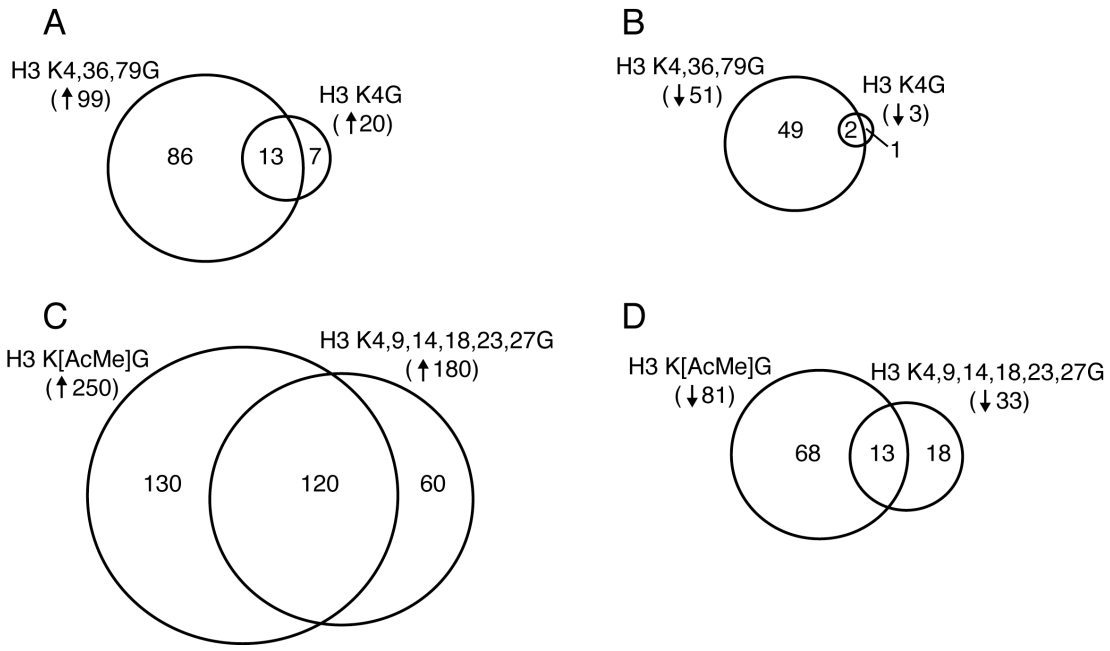


Figure 4-1: Effects of histone H3 acetylated and methylated lysine mutants on gene transcription.

Venn diagrams were used to compare the sets of **(A, C)** up-regulated genes and **(B, D)** down-regulated genes among the histone H3 mutants. Data for histone H3 K4G mutant and H3 K4,9,14,18,23,27G mutant are from Martin *et al*. (Martin et al., 2004).

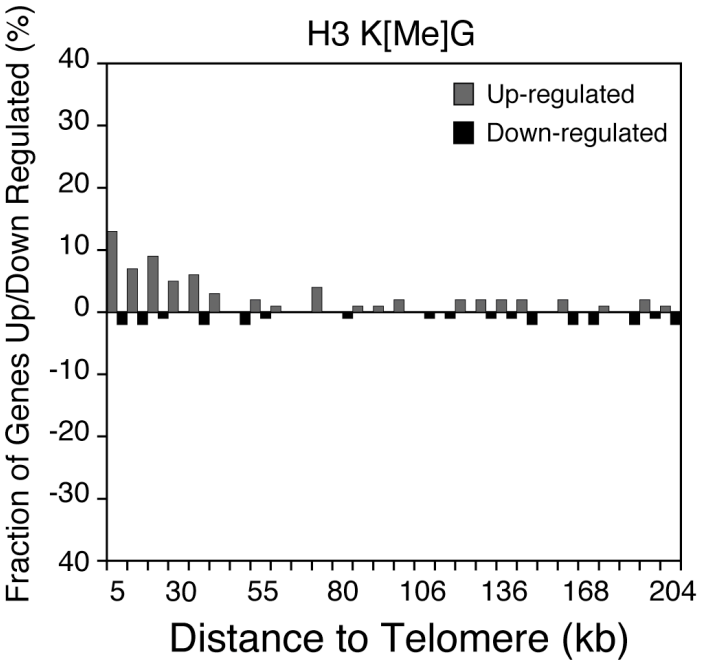
heterochromatin regions (e.g., Chr V-L and Chr VI-R). We believe that the loss of the SIR silencing complex is largely due to the disruption of unmethylated histone H3 lysine residues, which is in accordance with the model that Sir proteins preferentially bind to hypomethylated histone H3 lysine residues (Ng et al., 2003; Santos-Rosa et al., 2004; van Leeuwen and Gottschling, 2002). On the other hand, the H3 K[Ac]G mutant did not significantly change the SIR silencing complex binding in the same heterochromatin regions. Compared to the well-studied mechanism by which methylated histone H3 lysine residues regulating telomeric silencing, the nature of histone H3 acetylated lysine residues regulating telomere proximal gene silencing is unclear. We speculate that as yet unknown repressor protein, which is likely to bind hypoacetylated lysine residues in histone H3, is involved. The Tup1 protein is one of those potential repressors. Tup1 is known as a general transcription repressor with various molecular mechanisms (Courey and Jia, 2001; Malave and Dent, 2006; Smith and Johnson, 2000). Previous studies suggested that the Tup1 protein can recruit HDACs (Edmondson et al., 1996; Watson et al., 2000; Wu et al., 2001) and interact with the hypoacetylated N-terminal tails of histone H3 and H4 (Davie et al., 2002; Edmondson et al., 1996). It has been proposed that the Tup1 protein might be involved in formation of subtelomeric heterochromatin regions (Robyr et al., 2002). Alternatively, a number of repressors with the SANT domain could be involved. Previous data showed that the SANT domain preferentially binds unacetylated histone proteins (Boyer et al., 2004; de la Cruz et al., 2005; Hartman et al., 2005; Yu et al., 2003). In the future, more work is needed to characterize these potential repressors and to better understand the mechanism of how acetylated lysine residues regulate telomeric and subtelomeric gene silencing.

Lastly, we found that the lysine-to-glycine mutations of acetylated and methylated histone H3 lysine residues significantly up-regulated transcription of genes originally associated with the unmodified version of these lysine residues throughout the genome. The data strongly indicated that transcriptional repression is carried out by unmodified versions of histone H3 acetylated and methylated lysine residues. A similar repressive function of methylated histone H3 lysine residues has been previously proposed to regulate telomeric heterochromatin silencing, as the SIR silencing complex binds preferentially to the hypomethylated histone H3 lysine residues (Ng et al., 2003; Santos-Rosa et al., 2004; van Leeuwen and Gottschling, 2002). However, we believe that the mechanism of repression is different in telomeric regions compared to elsewhere in the genome, due to a couple of reasons. First, Sir proteins are predominantly found in silencing regions (i.e., telomere proximal regions, silent mating loci, and rDNA repeats) (Moazed, 2001). Second, we compared our histone lysine mutant data sets with the previously published data set of a *sir2*Δ mutant (Martin et al., 2004; Wyrick et al., 1999), which eliminates silencing in yeast. We found only a minimal overlap between the non-telomeric genes up-regulated in the *sir2*Δ mutant and histone H3 lysine mutant data sets (0%, 3.9%, and 2.5% of up-regulated genes for the H3 K[Ac]G, K[Me]G, and K[AcMe]G mutants, respectively). Third, we also examined the genomic distribution of the hypo-modified genes that are up-regulated in the histone H3 lysine mutants. These genes appear to be fairly randomly distributed on chromosomes, and are not associated with telomere regions, silent mating loci, or rDNA repeats. Taken together, these multiple lines of evidence indicate that the transcription repression mechanism(s) for those non-telomere proximal genes, up-regulated in the histone H3 lysine mutants is independent of

the Sir proteins-mediated silencing. Future studies should focus on exploring these novel repression mechanisms.

Differences Between Arginine and Glycine Substitutions for Methylated Histone H3 Lysine Residues

We noticed that the lysine-to-arginine and lysine-to-glycine mutations of methylated histone H3 lysine residues showed radically different growth phenotypes, transcription defects, and SIR binding profiles. The H3 K[Me]R (H3 K4,36,79R) mutant is lethal, while the H3 K[Me]G mutant is viable with a mild growth defect. In terms of transcription defects, the H3 K[Me]R mutant enhances telomeric silencing and causes gene silencing elsewhere in the genome; the H3 K[Me]G mutant largely disrupts telomeric gene silencing, and up-regulates the transcription of other genes located outside of telomeric proximal regions (Figure 4-2). The opposite effects on telomeric gene expression are reflected in the opposite effects on SIR silencing complex binding in the different histone mutants. The H3 K[Me]R mutant causes increased binding of the SIR silencing complex in the telomeric heterochromatin regions and then further binding into adjacent euchromatin regions. In contrast, the H3 K[Me]G mutant disrupts SIR binding in telomere regions. Previous studies suggested that histone H3 methylation limits spreading of the SIR silencing complex, largely by limiting the binding and spreading of the Sir3 protein (Bernstein et al., 2002; Fingerman et al., 2005; Ng et al., 2002; Santos-Rosa et al., 2004; van Leeuwen et al., 2002). Indeed, the conserved bromo-adjacent homology (BAH) domain of the Sir3 protein has been shown to preferentially bind unmodified lysine residues in both histone H3 and H4, and is critical for silent chromatin assembly


H3 K[Me]G
Up-regulated
Down-regulated
Fraction of Genes Up/Down Regulated (%)
40
30
20
10
0
-10
-20
-30
40
5
30
55
80
106
136
168
204
Distance to Telomere (kb)

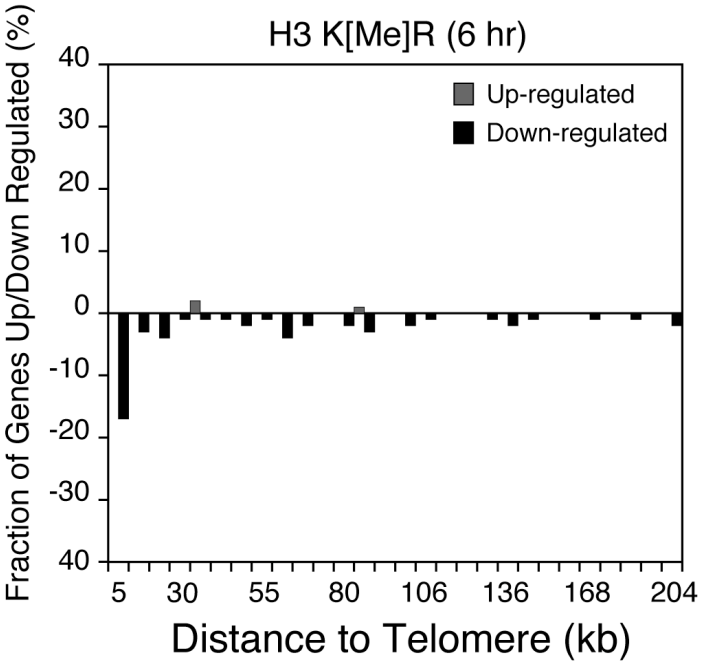

H3 K[Me]R (6 hr)
Up-regulated
Down-regulated
Fraction of Genes Up/Down Regulated (%)
40
30
20
10
0
-10
-20
-30
40
5
30
55
80
106
136
168
204
Distance to Telomere (kb)

FIGURE 4-2: Effects of the histone H3 K[Me]G and H3 K[Me]R mutants on genome-wide expression levels.

Chromosome plots of the microarray data for the H3 K[Me]G mutant. A histogram of the fraction of genes whose mRNA levels are up/down-regulated in H3 mutants is plotted as a function of their distance from a chromosome end. The microarray data from the H3 K[Me]R mutant at the 6 hour time point (including dubious genes) following the depletion of wild-type histone H3 is also shown for comparison.

(Buchberger et al., 2008; Connelly et al., 2006; Onishi et al., 2007; van Welsem et al., 2008). It is possible that the lysine-to-arginine mutations in the H3 K[Me]R mutant, which are thought to mimic hypomethylated lysine residues, would promote the inappropriate SIR binding. This would lead to extensive gene silencing defects, causing cell death. On the other hand, the lysine-to-glycine mutations in the H3 K[Me]G mutant disrupt SIR binding because they eliminate the hypomethylated lysine side-chain, which potentially serves as the binding dock for the BAH domain of the Sir3 protein. This explains why the H3 K[Me]G mutant is viable, while the H3 K[Me]R mutant is lethal.

Histone proteins and their post-translational modifications have been the subject of intense interest in the frontier of biology research since the histone code hypothesis was proposed. Numerous studies have demonstrated that histone modifications are undoubtedly involved in various important biological processes. Nowadays, in addition to characterizations of individual histone modifications and their functions, many current studies focus on investigating how different histone modifications work together to regulate DNA-templated processes and complicated interplays among histone modifications. The work described in this thesis sheds some lights on the functional redundancy of histone H3 methylation, and reviews interesting genetic interactions between histone H3 acetylated and methylated lysine residues in *Saccharomyces cerevisiae*. The objective of our work provides information describing connections between histone codes. It is likely, however, that the histone code is much more complicated than we now appreciate, and a wealth of molecular mechanisms underlying its complexity has yet to be discovered. In the future, further elucidation of the complex

interaction of histone codes will advance our current understanding and give us a more comprehensive view of histone modifications and their functions.

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