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# ANALYSIS OF GCN5 FUNCTION IN *HIS3* EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

By

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## ABSTRACT

ANALYSIS OF GCN5 FUNCTION IN *HIS3* EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

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Histone acetylation plays an important role in the transcriptional regulation of several genes by an unknown mechanism. Because acetylation likely changes chromatin structure, histone mutations might mimic the downstream effects of acetylation. To test this hypothesis, a genetic screen in yeast was conducted to find histone H2A and H3 mutations that bypass the need for Gcn5-mediated acetylation in *HIS3* expression. Several Gcn5-independent (gin) candidates were identified based on resistance to 3-amino triazole, but none of these mutants increased *HIS3* expression.

Gcn5 is part of several multi-subunit complexes. Gcn5 functions in the SAGA complex to regulate activated *HIS3* expression and also promotes, in an unknown context, basal *HIS3* expression. The possibility that Gcn5 regulates the basal *HIS3* expression as a member of the ADA complex was tested. Results from these experiments suggest that the ADA complex is dispensable for basal *HIS3* expression, but may be required for activated *HIS3* expression.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 3-AT | 3-amino triazole |
| ADA | Adapter histone acetyltransferase complex |
| ChIP | Chromatin immunoprecipitation |
| Gcre | Gcn4 response element |
| gin | Gcn5-independent |
| HA | Hemagglutinin |
| HAT | Histone acetyltransferase |
| HDAC | Histone deacetylase |
| IgG | Immunoglobulin G |
| IP | Immunoprecipitation |
| lrs | loss of rDNA silencing |
| PCR | Polymerase chain reaction |
| RT | Reverse transcription |
| SAGA | Spt Ada Gcn5 Acetyltransferase complex |
| SD | Synthetic defined |
| sin | SWI/SNF-independent |
| YPD | Yeast extract peptone dextrose |

# CHAPTER 1

# LITERATURE REVIEW

## Eukaryotic genomes exist as chromatin

The linear length of DNA in eukaryotic genomes is much greater than that of the nucleus. Because of this, the DNA must be organized and compressed in a nucleoprotein structure called chromatin (1,2). Chromatin provides the essential compaction and regulatory properties required for mitotic segregation during the cell cycle. Although it provides these critical functions, several nuclear processes that require access to the DNA template are inhibited by the compact nature of chromatin (1-3). These opposing functions have been the focus of numerous studies involving transcriptional regulation.

The most fundamental repeating element of chromatin is the nucleosome. Nucleosomes are composed of approximately 146 base pairs of DNA wrapped in 1.8 turns around a complex made up of two copies of the histones H3, H4, H2A and H2B (4,5). The histone proteins are highly conserved across species and can be broadly characterized as having two domains, the tails (amino- and carboxy-terminal) and a globular core (6). The "core" region of the histone proteins is composed of three or more alpha helical structures separated by loop domains. This region of the histone proteins is responsible for histone-histone and histone-DNA interactions that are critical for the maintenance of nucleosome structure. All four core histone proteins contain amino-terminal extensions (tails) that are essential for cell viability (7,8). Histones H2A and H2B also have carboxy-terminal tails. The tails of the histone proteins are considered flexible or

unstructured based on X-ray crystallographic studies (9). The histone tails extend beyond the core of the nucleosome and many studies have suggested that they can interact with nearby nucleosomes and DNA (6,10-12). Additional biochemical and biophysical experiments show that the histone tails are essential for higher order folding of chromatin (13,14). Moreover, the tails of the histones are also known to interact with and to be post-translationally modified by multiple proteins, thus providing a platform for regulation of the genome.

## Chromatin modification transcriptional regulation

Chromatin structure is generally repressive to several processes that require access to the DNA template. Several mechanisms exist to modulate the chromatin architecture to allow activities like transcription and DNA repair to occur. ATP-dependent chromatin remodeling by complexes such as SWI/SNF use the energy created by ATP-hydrolysis to physically alter the path of DNA on the nucleosome (15). This activity is known to increase access to DNA by transcription factors (16). The SWI/SNF complex can be recruited to gene promoters by DNA-bound transcription activators and its function is important for the proper expression of multiple genes (16). Several different ATP-dependent chromatin remodeling complexes exist in yeast, such as SWI/SNF, RSC, Ino80, and CHRAC. Of these complexes SWI/SNF was the first to be identified. Initially, SWI/SNF components were discovered by separate genetic screens for defects in mating type switching and sucrose fermentation (17,18). Later, many

of the SWI and SNF genes were shown to be part of a multi-subunit co-activator complex for certain genes (19,20). Additional studies showed SWI/SNF could modulate chromatin structure. The most compelling evidence was shown when mutations in histones H3 and H4 could partially bypass SWI/SNF transcription co-activator function (21). Further study of the SWI/SNF-independent (sin) histone alleles showed that these mutations most likely regulate histone-DNA contacts and higher-order chromatin folding (22,23). These histone mutations studies were important for characterization of SWI/SNF function and therefore multiple studies have targeted the histone genes to analyze the function of other aspects of chromatin structure and transcriptional regulation.

Other recently described mechanisms of chromatin modification include histone removal and histone variant replacement. The FACT complex was recently shown to facilitate transcription elongation by histone removal and replacement (24). Additionally, several groups have shown that the SWR1 complex can replace histone H2A containing dimers with those containing the H2A.Z variant, which is known to be positively correlated with transcriptional activity (25-27). The main focus of chromatin modification described will be on the ATP-dependent remodeling and histone acetylation.

## Histone acetylation and transcriptional activation

The post-translational modification of the histone proteins is another well-known mechanism of chromatin modification. Most modifications of the histones occur

on the N-terminal tails, but more recent studies have shown that the histones are also subject to modification within the core domain and the C-termini (28,29). Enzymes can modify histones by acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and other reactions. The histone proteins can have several different modifications at the same time and most of these modifications are dynamic in nature and therefore can be reversed. For example, histone acetylation and ubiquitination can be reversed by the activity of histone deacetylases and deubiquitinases, respectively (30,31). In addition, some modifications can positively and negatively affect the modification status of residues in *cis* or in *trans* (32,33). Of the known histone modifications, acetylation is the best described post-translational modification.

Histone acetylation was the first described modification of the histones (34). In general, histone acetylation is positively correlated with transcriptionally active genes, whereas the lack of acetylation is correlated with inactive or silent genes. Histone acetyltransferases (HATs) use acetyl coenzyme A to transfer the acetyl group to the epsilon amino group of lysine residues. Acetylation can be reversed by the enzymatic action of histone deacetylases (HDACs). Because of the positive correlation between histone acetylation and transcriptional activity, it is no surprise that many HATs function as transcriptional co-activators (30). On the other hand, many HDACs are known to be involved in transcriptional repression and silencing (35). How acetylation leads to transcriptional activation is unknown. One of the first hypotheses suggests that since acetylation neutralizes

the positive charge of the lysine side chain, it reduces the affinity of the tail for nearby nucleosomes or DNA (36,37). Because of these changes, the local chromatin structure would then become more permissive for transcription factor access (38). Another more recent hypothesis suggests that histone acetylation and other modifications produce a "histone code" which is in turn interpreted by other downstream effectors (32). This hypothesis is supported by the finding that many transcription regulatory proteins contain binding domains that specifically recognize modified versions of the histone tails. For example, the bromodomain recognizes acetyl lysines (39,40) and is found in many transcription factors.

## Yeast histone acetyltransferases

Histone acetyltransferases can be broadly categorized by the location of their activity. Type A HATs are located in the nucleus and type B HATs are located in the cytoplasm. The type B HATs acetylate the newly synthesized histones in the cytoplasm for transport to the nucleus and subsequent incorporation into the chromatin. B-type HATs are not known to regulate gene expression, therefore will not be discussed further. Type A HATs acetylate histones in the context of chromatin and several of these A-type HATs are important for transcriptional regulation. Gcn5, a transcriptional co-activator was the first type A HAT identified. Several additional yeast HATs were discovered and many of them are involved in the regulation of gene expression. For example, the Sas2 and Sas3 HATs are important for transcriptional silencing, whereas the Elp3 protein is

involved in transcript elongation. Many of the HATs found in yeast, including Gcn5, are evolutionarily conserved, which indicates the importance of their function in gene regulation. Additional description of Gcn5 activity is described below.

## Gcn5 is a canonical histone acetyltransferase

The link between histone acetylation and transcriptional activity was found when the yeast protein, Gcn5, was shown to function as a nuclear HAT (41). Gcn5 was first identified as a co-activator protein important for the full expression of several genes important for amino-acid biosynthesis (42,43). Subsequent analysis showed that Gcn5 functions as a HAT in vitro and preferentially acetylates specific lysines in the H3 and H4 N-terminal tails (44). Gcn5 HAT function in vivo was later shown to be critical for the proper expression of a subset of yeast genes (30,45,46). Importantly, this data described the first direct relationship between histone acetylation and transcriptional activity of target genes.

As is true of many of transcriptional regulators, Gcn5 functions in the context of several large multi-subunit complexes. Early studies showed that Gcn5 was present in at least two chromatographically distinct complexes, SAGA and ADA (47-49). In addition, Gcn5 has been demonstrated to function in the SALSA/SLIK (50,51) and A2 HAT (52) complexes. The SAGA complex has been the focus of

most research and it contains multiple transcription-related proteins. These components are Gcn5 (41), Ada adapter proteins (53), TBP-related Spt proteins (54), multiple TBP-associated factors (Tafs) (55), an essential activator-interacting protein, Tra1 (56-58), and others. The architecture of the SAGA complex is considered to be modular in nature (59,60). This is due to the fact that it contains subunits that participate in histone acetylation (Gcn5, Ada2, and Ada3), TBP-interaction (Spt3 and Spt8), structural integrity (Ada1, Spt7 and Spt20) and histone deubiquitination (Ubp8 and Sgf11) (61,62). Like SAGA, the ADA complex contains Ada2, Ada3, and Gcn5 (47,63). In addition, ADA also has at least one unique subunit called Ahc1 (63). Unlike SAGA much less is known about the function of the ADA complex.

Gcn5 specifically acetylates a subset of the lysines found in the N-terminal histone tails. In vitro acetylation experiments have shown that purified Gcn5 protein can only acetylate free histones (44). Under these circumstances H3 lysine 14 is the primary target of Gcn5-mediated acetylation (44). When similar experiments were performed using the SAGA or the ADA HAT complex, Gcn5 is able to acetylate nucleosomal histone substrates and again, H3 lysine 14 (H3 K14) is the dominant target (64). In vivo experiments using chromatin immunoprecipitation have shown that Gcn5 is responsible for H3 acetylation at the *HIS3* promoter (30). At the *INO1* gene promoter Gcn5 was found to be responsible for H3 and H2B acetylation (65). Genetic experiments performed by Zhang et al. showed that H3 K14 is a major target of Gcn5 in vivo (66).

Importantly, single mutations of Gcn5-acetylated lysines, including H3 K14, did not cause *gcn5*⁻ phenotypes. Moreover, transcriptional defects caused by a *GCN5* deletion were only partially restored when a combination of H3 and H4 lysines were mutated to glutamine (an acetyl-lysine mimic). These studies provide evidence that the major site of Gcn5 acetylation occurs at H3 K14, but the additional lesser-preferred sites are also critical for Gcn5 function. In another study of histone acetylation function, Ito et al. showed that histone acetylation is important for H2A-H2B dimer destabilization and removal from nucleosomes. Taken together, histone acetylation might function to change chromatin structure by alteration of the composition of the nucleosome.

## The *HIS3* gene as a model for Gcn5 study

The *HIS3* gene is one of the known targets of Gcn5 regulation. *HIS3* encodes imidazoleglycerol-phosphate dehydratase, which catalyzes the sixth step in histidine biosynthesis (67). Transcription of *HIS3* is regulated by the pathway known as general amino acid control (68). The transcriptional activator, Gcn4, regulates most of the genes involved in this pathway. The *HIS3* gene has a low basal level of transcription under normal growth conditions, which does not require the function of Gcn4 (69,70). When a starvation situation occurs for histidine or any of the amino acids, Gcn4 production is stimulated and *HIS3* and the other amino acid biosynthetic genes are up regulated (71). The starvation

condition for histidine can be created by addition of the chemical, 3-amino triazole (3-AT) to synthetic yeast media. 3-AT is a competitive inhibitor of the *HIS3* gene product and cells that are defective in Gcn5 function grow slowly in the presence of 3-AT (30,72).

The HAT activity of Gcn5 is critical for both basal and activated *HIS3* expression and acetylation (30). In addition, during the activated condition, the Gcn4 activator is required to recruit Gcn5 to the *HIS3* promoter (73). Genetic experiments suggest that Gcn5 functions in the context of the SAGA complex at the activated *HIS3* promoter. These studies showed that mutations or deletions in multiple SAGA components reduce the activated *HIS3* expression (74). Although Gcn5 is required for the basal expression and acetylation of *HIS3*, it is unknown how Gcn5 activity is directed to *HIS3* under the basal condition, as the Gcn4 activator is not expressed during this situation. Because of all these features, the *HIS3* gene provides an attractive model for the study of Gcn5 function.

## Research Objective

The main objective of this thesis research is to study how Gcn5-mediated acetylation activates gene transcription and to understand the importance of its participation in multiple HAT complexes. To study the acetylation function, a genetic screen to identify histone H2A and H3 mutations that are Gcn5-independent for *HIS3* expression was performed. Several mutations were found

to increase resistance to the drug 3-AT, an inhibitor of the *HIS3* gene product, in the absence of Gcn5 function. Contrary to expectations, resistance to 3-AT caused by these mutants was not the result of increased *HIS3* expression. To test the function of the Gcn5-containing ADA complex, expression of *HIS3* was determined in an *AHC1* deletion strain. In addition, ChIP was used to test the association of Ahc1 at the *HIS3* promoter. The ChIP assay was also used to determine potential ADA complex regulation of H3 acetylation levels at the *HIS3* promoter. The results indicated that the ADA complex has no role in basal *HIS3* expression, but it might regulate the activated expression through a mechanism other than histone acetylation.

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# Chapter 2

# Screening for histone H2A and H3 mutations that are Gcn5-independent for *HIS3* expression.

# Introduction

The DNA of eukaryotes is organized in the highly dynamic nucleoprotein structure known as chromatin. Compaction and organization of DNA by chromatin, although critical, creates a barrier to several processes that require access to the DNA template. Chromatin is composed mainly of nucleosomes, a complex of the histone octamer wrapped by 147 base pairs of DNA (1). Histone octamers are composed of one H3-H4 tetramer and two histone H2A-H2B dimers. There are two distinct regions of each histone protein, the histone fold domain and the amino- and carboxy-terminal tails. Each histone fold is composed of three $\alpha$-helices, $\alpha$1, $\alpha$2, and $\alpha$3, which are separated by the looped segments called L1 and L2. Maintenance of nucleosome structure is achieved by the protein-protein and protein-DNA interactions created by the histone fold domains. The N-termini of all four core-histones and the C-termini of histones H2A and H2B exist largely on the exterior of the nucleosomes (1) and are subject to many post-translational modifications (2).

Eukaryotes have developed elaborate mechanisms to overcome the repressive environment created by chromatin structure during transcriptional activation. These activities can be broadly categorized into four classes; those that utilize ATP-hydrolysis to change the structure of chromatin; a second group that function through covalent modification of the histone tails; a third group that

functions by removal of histones or exchange of common histones with variant subtypes; and the fourth group involves methylation of the DNA. The first three classes of chromatin modulation are most relevant to this study and are described in more detail below.

SWI/SNF is a prototypical complex that belongs to the first class, which uses the energy of ATP-hydrolysis to modify chromatin structure. Genes encoding SWI/SNF proteins were originally identified in genetic screens for mutations that cause defective mating type switching or sucrose fermentation (3,4). Subsequently, some of these genes were shown to be part of a multi-subunit complex that is required for efficient transcription of several yeast genes, e.g. *SUC2* and *HO* (4,5). Early studies of SWI/SNF implicated its function in changing the accessibility of chromatin (6,7). Additional studies also showed that mutations in genes encoding histones H3 and H4 could partially suppress defects in gene expression caused by loss of SWI/SNF function (8). These data provided extra evidence that SWI/SNF mediates transcriptional activation by the modulation of chromatin structure. Biochemical and biophysical studies of histone mutations showed that some of these SWI/SNF-independent (sin) alleles weaken histone-DNA interactions (9) and resulted in defects in the folding of nucleosome arrays (10). Recent studies have shown that SWI/SNF achieves remodeling by altering the path of the DNA on the nucleosome (11).

The second class of chromatin modifiers can covalently attach acetyl, methyl, phosphate, or ubiquitin groups to the histone tails. Of these, acetylation is one of the best-studied modifications and it has long been associated with transcriptionally active regions of chromatin (12). Gcn5 was the first nuclear histone acetyltransferase identified (13). It was initially discovered as a transcriptional co-activator for several yeast amino acid biosynthetic genes (14). This provided the first direct link between histone acetylation and gene activation. Moreover, Gcn5 as a transcriptional co-activator requires this HAT activity, as mutations that abolish this enzymatic activity eliminate its ability to activate transcription of target genes (15). Histone acetylation by Gcn5 has been positively correlated with transcriptional activation of several genes, but how acetylation leads to changes in gene activation remain elusive.

The third class of chromatin modifications employs removal of nucleosomes or changing of the nucleosome composition. The yeast complex FACT (facilitates chromatin transcription) is important for transcriptional elongation and it can interact with H2A/H2B dimers (16). In an in vitro study, Belotserkovskaya et al. showed that the FACT complex aids transcription through nucleosomes by removal of an H2A/H2B dimer (17). Other complexes function by incorporation of variant histone subtypes into nucleosomes. The SWR1 complex is an example and is known to incorporate the histone variant H2AZ into nucleosomes (18-20). Histone H2AZ is thought to poise genes for activation and also block the spread of silent chromatin. In addition, nucleosome destabilization and/or loss

seem to depend on ATP-dependent chromatin remodeling and histone acetylation (21). For instance, Ito et al. have shown in an in vitro system that chromatin remodeling followed by histone acetylation is required for H2A/H2B dimer displacement to the histone chaperone NAP-1 (22). Hence, removing nucleosomes or changing their composition is intimately linked to ATP-dependent remodeling and histone acetylation.

Clues about protein function can be found by identification of genetic suppressors and genetic interactions. For example, a genetic screen conducted by Sternberg et al. (23) showed that mutations in several genes could partially relieve the transcriptional defects caused by disruption of SWI/SNF function. One of these SWI/SNF-independent (sin) alleles was later found to be a mutation in a gene encoding for histone H3 (8). Several additional studies on sin alleles in histone H3 and H4 suggested that SWI/SNF functions by altering chromatin structure (10,24); and more specifically by disruption of DNA contacts within the nucleosome (9). In a separate screen, Park et al. recently identified new *lrs* (loss of rDNA silencing) histone mutations in the vicinity of H3 lysine 79 (25). H3 K79 is a site of methylation linked to transcriptionally active chromatin and is important for proper regulation of gene silencing (26). The identification of these mutations resulted in the characterization of a nucleosome surface that is critical for regulation of gene silencing.

The link between acetylation of histone amino-terminal tails and transcription activation has been documented extensively. Histone acetylation disrupts higher-order folding of chromatin (27) and causes increased susceptibility to DNA-modifying enzymes, supporting the notion that a function of histone acetylation is to increase accessibility of the chromatin template (reviewed in (28). Exactly how this occurs in unknown. One hypothesis is that acetylation acts in concert with other modifications of the histone tails to act as a stage for recruitment of specific factors to promoters during gene activation (2). Evidence for this theory came from the discovery of transcription factors containing regions that recognize modified histone tails. The bromodomain is one such motif, which recognizes acetylated lysines (29). Another theory is that acetylation may simply act to neutralize the positive charge of the lysine side chains (30). Elimination of the positive charge associated with amino-terminal lysines could reduce their affinity for linker-DNA or adjacent nucleosomes, thereby creating a less compact promoter region (31),(32). Zhang et al. introduced a series of lysine mutations to the H3 and H4 tails. This study showed that mimicking the acetyl-lysine group was not sufficient to restore transcription in the absence of Gcn5 acetylation, which suggests the function of HATs and lysine acetylation may be redundant. Moreover, the work described above by Ito et al. implicates a function of histone acetylation might be to destabilize nucleosomes to allow transcription to occur (22).

This work focuses on elucidating the mechanism(s) by which histone acetylation facilitates transcription. To do this, I used Gcn5 of the budding yeast *Saccharomyces cerevisiae* as the model for these studies. I test the hypothesis that histone mutations that decrease H2A/H2B dimer association with the nucleosome might mimic the chromatin structure induced by Gcn5 acetylation. Toward this end, I have created a yeast strain with a mutant *gcn5* allele from a previously engineered strain that has only one copy of each histone gene (see materials and methods). Random mutations in histone genes, contained on plasmids, were introduced using in vitro PCR mutagenesis. The mutagenized histone gene was used to replace its wild type counterpart in our *gcn5*$^-$ strain. Potential Gcn5-independence (gin) histone alleles of H2A and H3 for expression of the *HIS3* gene (a well-known Gcn5 target gene) were identified based on their increased resistance to the chemical 3-amino-1, 2, 4-triazole (3-AT). Each gin allele candidate was sequenced to identify and analyze the mutations. Contrary to our hypothesis, none of the gin allele candidates showed reproducible and significant restoration of activated *HIS3* expression, as determined by Northern blot hybridization and real-time RT-PCR experiments. Single mutations in the genes for histones H2A and H3 were created based a subset of gin allele candidates originally thought to be Gcn5-independent for *HIS3* expression. None of these mutations restored *HIS3* activated expression in the absence of Gcn5-HAT function. I also investigated the possibility that the SWI/SNF-independent histone alleles are also Gcn5-independent. The H3 sin mutations are lethal

when present as the only copy of histone H3. In addition, H3 E105K and T118I cause a dominant synthetic sick phenotype with Gcn5 mutations.

# Materials and methods

**Strains and Media.** YPD, synthetic complete media (SC), synthetic defined media (SD), and 5-FOA media were prepared as described (33). Media containing 3-amino-1,2,4-triazole (3-AT) was prepared by first autoclaving the media and then adding from a 1M stock of filter-sterilized 3-AT (Sigma) to give the final desired concentration. Yeast transformations were performed by the lithium acetate technique (34). All yeast strains used are described in Table 1 and were derived from a histone quadruple-knockout strain, JHY205 (35). Transformation of JHY205 with pQQ18, a low-copy histone plasmid (35), and subsequent selection in SC-Leucine media was used to create DA9. *LEU*$^+$ transformants were streaked on to 5-FOA media to select for *LEU*$^+$ *ura*$^-$ cells (DA9). DA9 was transformed with *Bam*HI cut pJJ217, which contains the entire *HIS3* gene (36), to create the *HIS3*$^+$ strain, DA10. To introduce the *gcn5 F221A* allele, DA10 was transformed with *Eco*RI cut pMK415 (created by Soumya Singh-Rodriguez, unpublished), which contains the *Bam*HI/*Hind*III *gcn5 F221A* fragment in yIPlac211 (37). *URA*$^+$ cells were selected on CAA-uracil media and were subsequently streaked to 5-FOA media to lose one of the two gcn5 copies. The *F221A* bearing strain, DA11, was first identified based on sensitivity to 3-AT, and subsequently confirmed by genomic PCR and sequencing. DA12 was

obtained by replacing the *LEU2*-containing pQQ18 with the *URA3*-containing pJH33 (35). Transformants that spontaneously lost pQQ18 were identified by their *URA*$^+$ *leu*$^-$ phenotype.

All gin candidates were isolated as shown in Figure 1. Briefly, randomly mutagenized H2A and H3 genes were co-transformed with the *LEU2*-marked histone plasmid, pQQ18, which was cut with restriction enzymes to remove the corresponding wild type histone gene (see below for details), into DA12. Next, clones that underwent recombination between pQQ18 and the mutagenized PCR fragments were selected for on SC-leucine media. The *LEU*$^+$ clones were replica plated to media containing the chemical 5-FOA, to select against cells that contain the *URA3*-marked histone plasmid, pJH33. Subsequently, 5-FOA-resistant clones were replica plated to synthetic complete media (SC) lacking histidine and containing 20 mM 3-AT. Plates were incubated at 37°C for 4 days to identify 3-AT resistant clones. Clones appearing to grow on 3-AT plates were isolated by picking the corresponding colony from the 5-FOA plates and then were streaked to another 3-AT plate to confirm the resistance phenotype. To ensure 3-AT resistance is caused by the histone plasmid, yeast DNA was isolated (see below) from each 3-AT resistant strain and then transformed into the original strain, DA12. The 3-AT resistance of the resulting strains was verified by spot assay on various concentrations of 3-AT media. Spot assay results were used to describe the level of 3-AT resistance in Table 2.

# Table 1

*S. cerevisiae* Strain List

| Strain | Relevant genotype | Source |
|---|---|---|
| JHY205 | *MATa leu2Δ ura3Δ0 his3Δ200 (hht1-hhf1)Δ::KAN (hht2-hhf2)Δ::NAT (hta1-htb1)Δ::HPH (hta2-htb2)Δ::NAT <pJH33 (HTA1-HTB1 HHT2-HHF2)::URA3>* | 28 |
| DA9 | isogenic to JHY205 except pQQ18 replaces pJH33 | This study |
| DA10 | *MATa leu2Δ ura3Δ0 HIS3 (hht1-hhf1)Δ::KAN (hht2-hhf2)Δ::NAT (hta1-htb1)Δ::HPH (hta2-htb2)Δ::NAT <pQQ18(HTA1-HTB1 HHT2-HHF2)::LEU2>* | This study |
| DA11 | *MATa leu2Δ ura3Δ0 HIS3 (hht1-hhf1)Δ::KAN (hht2-hhf2)Δ::NAT (hta1-htb1)Δ::HPH (hta2-htb2)Δ::NAT <pQQ18 > gcn5 F221A* | This study |
| DA16 | isogenic to DA10 except pJH33 replaces pQQ18 | This study |
| DA12 | Isogenic to DA11 except than pJH33 replaces pQQ18 | This study |
| YL372 | *MATa leu2Δ ura3Δ0 HIS3 (hht1-hhf1)Δ::KAN (hht2-hhf2)Δ::NAT (hta1-htb1)Δ::HPH (hta2-htb2)Δ::NAT <pJH33 > gcn5 E173H* | 32 |
| gin 1-3 | isogenic to DA12 except gin 1-3 replaces pQQ18 | This study |
| gin 1-7 | isogenic to DA12 except gin 1-7 replaces pQQ18 | This study |
| gin 3-17 | isogenic to DA12 except gin 3-17 replaces pQQ18 | This study |
| gin 3-29 | isogenic to DA12 except gin 3-29 replaces pQQ18 | This study |
| gin 3-44 | isogenic to DA12 except gin 3-44 replaces pQQ18 | This study |
| DA22 | *MATa leu2Δ ura3Δ0 HIS3 (hht1-hhf1)Δ::KAN (hht2-hhf2)Δ::NAT (hta1-htb1)Δ::HPH (hta2-htb2)Δ::NAT <pQQ18(HTA1-HTB1 HHT2-HHF2)::LEU2> trp1Δ::hisG-URA3-hisG* | This study |
| DA24 | *MATa leu2Δ ura3Δ0 HIS3 (hht1-hhf1)Δ::KAN (hht2-hhf2)Δ::NAT (hta1-htb1)Δ::HPH (hta2-htb2)Δ::NAT <pQQ18 > trp1Δ::hisG* | This study |
| DA29 | *MATa leu2Δ ura3Δ0 HIS3 (hht1-hhf1)Δ::KAN (hht2-hhf2)Δ::NAT (hta1-htb1)Δ::HPH (hta2-htb2)Δ::NAT <pQQ18 > trp1Δ::hisG swi2Δ::TRP1* | This study |
| DA30 | isogenic to DA12 except *H2A H83R-pmk 439* replaces pQQ18 | This study |

# Table 1 Cont'd

| Strain | Relevant genotype | Source |
|---|---|---|
| DA31 | isogenic to DA12 except *H2A L84M-pmk 439* replaces pQQ18 | This study |
| DA32 | isogenic to DA12 except *H3 A47P-pmk 439* replaces pQQ18 | This study |
| DA33 | isogenic to DA12 except *H3 L48F-pmk 439* replaces pQQ18 | This study |
| DA34 | isogenic to DA12 except *H3 R49T-pmk 439* replaces pQQ18 | This study |
| DA35 | isogenic to DA10 except *H2A I44N-pmk 439* replaces pQQ18 | This study |
| DA36 | isogenic to DA10 except *H2A V50I-pmk 439* replaces pQQ18 | This study |
| DA37 | isogenic to DA10 except *H2A L84M-pmk 439* replaces pQQ18 | This study |
| DA38 | isogenic to DA10 except *H3 L48F-pmk 439* replaces pQQ18 | This study |
| DA39 | isogenic to DA10 except *H3 R49T-pmk 439* replaces pQQ18 | This study |
| DA40 | isogenic to DA10 except *H2A H83R-pmk 439* replaces pQQ18 | This study |
| DA41 | isogenic to DA10 except *H2A L35I H83R-pmk 439* replaces pQQ18 | This study |
| DA42 | isogenic to DA10 except *H2A V50I H83R-pmk 439* replaces pQQ18 | This study |
| DA43 | isogenic to DA10 except *H3 A47P-pmk 439* replaces pQQ18 | This study |
| DA44 | isogenic to DA12 except *H2A I44N-pmk 439* replaces pQQ18 | This study |
| DA45 | isogenic to DA12 except *H2A V50I* replaces pQQ18 | This study |
| DA50 | *MATa leu2Δ ura3Δ0 HIS3 (hht1-hhf1)Δ::KAN (hht2-hhf2)Δ::NAT (hta1-htb1)Δ::HPH (hta2-htb2)Δ::NAT <pQQ18 > gcn5Δ::hisG* | This study |

To create the *swi2Δ::TRP1* strain, DA29, the *TRP1* gene was disrupted in DA10 by transformation with *Eco*RI and *Bgl*II cut pnky1009 (38) to create the *trp1Δ::hisG-URA3-hisG* strain, DA22. DA22 was then subjected to FOA selection to recycle the *URA3* marker, thus creating DA24. DA24 was then transformed with *Sac*I and *Sal*I restriction enzyme digested pDA5 (see Table 2) to create DA29. The *SWI2* deletion was verified by genomic PCR.

**H3 sin allele testing.** For experiments involving the H3 sin alleles, 50 ng of each mutant histone plasmid or wild-type plasmid, pQQ18, were transformed into DA12 (*gcn5 F221A*), DA16 (*GCN5*), YL372 (*gcn5 E173H*) (39), and DA50 (*gcn5Δ*). The transformants were selected on SC-uracil and leucine so that cells maintained the wild-type H3 gene on the URA3-marked plasmid, pJH33 and the sin H3 allele (or wild-type control, pQQ18) on the LEU2-marked plasmid. Transformation efficiency was calculated as the number of transformants per μg of DNA. Subsequently, the transformation efficiency of the sin alleles was normalized to the wild-type plasmid (pQQ18) transformation efficiency in each strain used (Figure 7). In an effort to isolate cells containing only the sin allele plasmid, the above transformants were transferred to 5-FOA plates after 2 days of growth in YPD media.

**Yeast DNA preparation.** Yeast DNA was prepared by spheroplasting cells in early log phase with lytic enzyme (*A. luteus* yeast lytic enzyme, ICN Cat. # 360942). 5 mL of cells were collected and washed with 500 μL 0.9 M filter-

sterilized sorbitol. Spheroplasting of cells was conducted by suspension in 500 µL digestion buffer (1 M sorbitol, 50 mM potassium phosphate, pH 7.2, 14 mM 2-mercaptoethanol, 5 mg/mL lytic enzyme). Spheroplasted cells were disrupted in a 500 µL solution of 0.2% SDS and 50 mM EDTA for 10 minutes at room temperature. Next, 100 µL of ice-cold 3 M potassium acetate and 5 M acetic acid solution was added to the cell solution and placed on ice for 30 minutes to precipitate the cellular contaminants. The DNA was separated from cellular debris by centrifugation at 14,000 rpm for 10 minutes. DNA (supernatant) was removed and precipitated with an equal volume of isopropanol. Finally, the DNA was precipitated by 10 second centrifugation, then was air-dried and dissolved in 100 µL of 1X TE, pH 8.0. This DNA was used for PCR or to transform *E. coli* for plasmid amplification and purification.

**PCR mutagenesis of *hta1* and *hhf2*.** Mutagenic PCR of the *HTA1* (encodes histone H2A) and *HHT2* (encodes histone H3) genes was performed as described (40), except that 0.3 mM $MnCl_2$ was used for *HTA1* and 0.4 mM $MnCl_2$ and 1.0 mM dNTPs were used for *HHT2*. Primers for *HTA1* were mhk 180 and mhk 181 and primers for *HHT2* were mhk 016 and mhk 017 (see Table 3 for sequences). The sizes of the *HTA1* and *HHT2* PCR fragments are 740 and 842 base pairs, respectively.

**Plasmids.** To prepare pQQ18 for co-transformation with mutagenized *HTA1* or *HHT2* PCR products, it was digested with restriction enzymes to remove the wild

type counterpart. For H2A mutagenesis, *Bgl*II and *Nde*I were used to remove the wild type *HTA1* gene and for H3 mutagenesis, *Sal*I and *Bam*HI were used to remove the wild type *HHT2* gene. All specific mutations were introduced by two-step PCR mutagenesis of pQQ18. Briefly, two oligonucleotides (see Table 3) were designed to introduce each specific mutation desired. For *HTA1* (histone H2A) mutagenesis DA01 and DA02 were used with MHK181 and MHK180, respectively, to introduce the H2A H83R mutation; DA03 and DA04 for H2A L84M; DA11 and DA12 for H2A L35I; DA13 and DA14 for H2A V50I; and DA15 and DA16 for H2A I44N. For *HHT2* (histone H3) mutagenesis DA05 and DA06 were used in combination with MHK017 and MHK016, respectively, to create the mutation H3 A47P; DA07 and DA08 for H3 L48F; and DA09 and DA10 for H3 R49T. The 5' and 3' PCR products also contain overlapping sequences surrounding the site of mutagenesis. The primary PCR products were gel-purified and used together as template for the second round of PCR. During this step the two fragments anneal together and allow for reconstitution of the entire *HTA1* or *HHT2* gene with the newly introduced mutation. These secondary PCR fragments were either reintroduced into pQQ18 by standard subcloning techniques or by co-transformation into yeast. All plasmids were sequenced to confirm the presence of only the desired mutation. The H3 sin allele containing plasmids were prepared using similar methods by Xinjing Xu (unpublished work).

# Table 2

Plasmid List

| Plasmid Name | Description | Source |
|---|---|---|
| pJH33 | *pRS316- HTA1-HTB1 HHT2-HHF2* | 28 |
| pQQ18 | pRS315-*HTA1-HTB1 HHT2-HHF2* | 28 |
| pmk415 | *gcn5 F221A* Integration construct | M.-H. Kuo, unpublished |
| pnky1009 | *TRP1* deletion construct | 31 |
| pDA5 | *SWI2* deletion construct | this study |

# Table 3

Histone H2A and H3 Mutagenesis Oligonucleotides

| Name | Sequence |
|---|---|
| MHK180 | 5'-CTACATCATCATTACGGATTTGG-3' |
| MHK181 | 5'-GCACAGAATGTGTTTGCTACTC-3' |
| DA01 | 5'-GACCAGAATTATTCCTAGGAGGTTGCAATTGGCTATC-3' |
| DA02 | 5'-GATAGCCAATTGCAACCTCCTAGGAATAATTCTGGTG-3' |
| DA03 | 5'-ATTATTCCAAGACATATGCAATTGGCTATC-3' |
| DA04 | 5'-TGATAGCCAATTGCATATGTCTTGGAATAAT-3' |
| DA05 | 5'-ATATAAGCCAGGTACCGTTCCATTGAGAGAAATTAGA-3' |
| DA06 | 5'-TCTAATTTCTCTCAATGGAACGGTACCTGGCTTATAT-3' |
| DA07 | 5'-GCCAGGTACTGTTGCATTCAGAGAAATTAGAAGA-3' |
| DA08 | 5'-TCTTCTAATTTCTCTGAATGCAACAGTACCTGGC-3' |
| DA09 | 5'-GTACTGTTGCCTTGACAGAAATTAGAAGATTC-3' |
| DA10 | 5'-GAATCTTCTAATTTCTGTCAAGGCAACAGTAG-3' |
| DA11 | 5'-GTCGGTAGAGTGCACAGATTGATCAGAAGAGGTAACTACGCC-3' |
| DA12 | 5'-GGCGTAGTTACCTCTTCTGATCAATCTGTGCACTCTACCGAC-3' |
| DA13 | 5'-CAAAGAATTGGTTCTGGTGCACCAATCTACTTGACTGCTGTCTTG-3' |
| DA14 | 5'-CAAGACAGCAGTCAAGTAGATTGGTGCACCAGAACCAATTCTTTG-3' |
| DA15 | 5'-GAGGTAACTACGCCCAAAGACAAGGATCCGGTGCTCCAGTCTACTTGAC-3' |
| DA16 | 5'-GTCAAGTAGACTGGAGCACCGGATCCTTGTCTTTGGGCGTAGTTACCTC-3' |
| MHK016 | 5'-TTCTTTTATATAGGACCACTGT-3' |
| MHK017 | 5'-GACCATGATTACGCCAAGCTCG-3' |

**RNA analyses.** Yeast cell cultures were grown in YPD media for several hours to an optical density at 600 nm ($O.D._{600}$) of approximately 0.8. Cultures were centrifuged and cells were washed with an equal volume of sterile water. Next, the washed cells were transferred to YPD for measurement of basal *HIS3* expression or SD plus 10-20 mM 3-AT for induced *HIS3* expression. Prior to harvesting RNA, cells were incubated in YPD or SD + 3-AT for 2-4 hours at 30°C and 37°C, respectively. RNA preparation and Northern blot hybridization techniques were performed as previously described (15). For RNA analyses by real-time RT-PCR, 10-20 μg of total RNA was treated with 10 U of RNase-free DNase I (Roche) in a 100 μL solution containing 50 mM Tris-HCl, pH 7.5 and 5mM $MgCl_2$ for 30 minutes at 37°C. The DNase I-treated RNA (0.5-1.0 μg) was reverse transcribed using 1.0 μL of Improm II reverse transcriptase (Promega) and 0.5 μg oligo dT in a 20 μL reaction. 1.0 μL of each RT reaction was added to 30 μL real-time PCR reactions including 1X SYBR green PCR reagent (Applied Biosystems), 3 mM MgCl2, 0.8 mM dNTPs, 0.15 μM oligonucleotides and 0.75 U ampliTaq Gold (Applied Biosystems). *HIS3* oligonucleotides were: (HIS3 5' RT) 5'-AGCTTTGCAGAGGCTAGCAG-3' and (HIS3 3' RT); 5'-GCGAGGTGGCTTCTCTTATG-3'; *ACT1* oligonucleotides were (ACT1 5' RT); 5'-CTGCCGGTATTGACCAAACT-3' and (ACT1 3' RT); 5'-CGGTGATTTCCTTTTGCATT-3'. *PGK1* oligonucleotides were: (PGK1 5' RT); 5'-TCATTGGTGGTGGTGACACT-3' and (PGK1 3'RT); 5'-GCAACACCTGGCAATTC-3'; *FAA3* oligonucleotides were: (DA28); 5'-GGTGGGTCTGCGATCAGTAT-3' and (DA29); 5'-

GGCTCCAAAACACAAGCATT-3'. Each reaction was performed in duplicate. Gene expression results are presented as the average values from two or more experiments (unless otherwise stated) with standard deviations. Relative expression of *HIS3* was calculated based on the $2^{-\Delta\Delta CT}$ method (41).

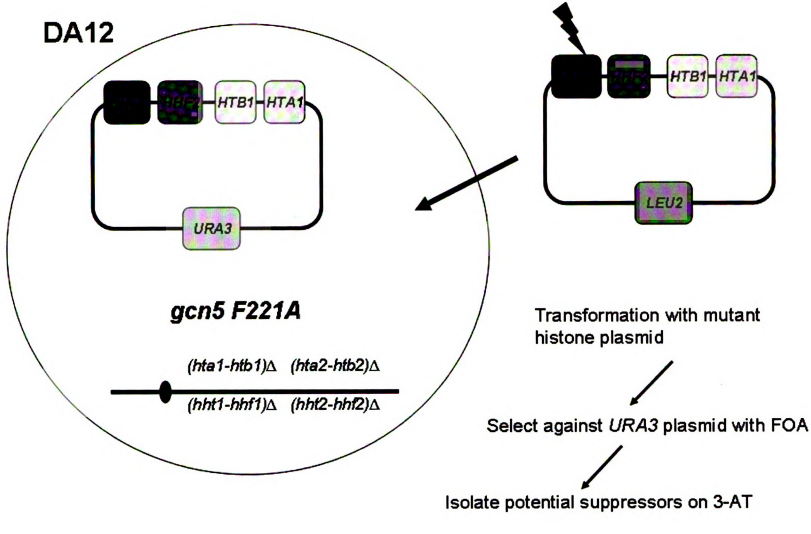
**Doubling time determination.** 25 mL cultures of YPD were inoculated to an $O.D._{600}$ of 0.1 with stationary phase cultures of each strain depicted in Figure 6. Each culture was incubated at 30°C with shaking until stationary phase was reached (up to 30 hr). An $O.D._{600}$ reading was taken every 2 or 2.5 hours of growth. These data were used to calculate the doubling time for each strain during the logarithmic growth phase. Each strain was grown in duplicate and each experiment was performed twice.

# Results

**Screen for Gcn5-independent histone mutations.** To isolate potential histone H2A or H3 mutants that can suppress transcriptional defects caused by the absence of Gcn5 HAT activity, a genetic screen was conducted using the strain DA12 (see Materials and Methods). This strain is deleted for all copies of the four major histone genes and it also contains a catalytically inactive version of Gcn5 (*gcn5 F221A*). For viability, DA12 carries a low-copy plasmid containing each of the four histone genes. DA12 was the parent strain used in the screen;

**Figure 1. Genetic screen for Gcn5-independent histone mutations.** The yeast strain DA12 was created to screen for histone H2A and H3 mutations that are Gcn5-independent for *HIS3* expression. DA12 contains the HAT-defective *gcn5 F221A* allele; deletions of chromosomal *HTA-HTB* and *HHT-HHF* histone genes; and a *URA3* plasmid containing one wild-type copy for each histone gene (pJH33). PCR-mutagenized fragments of the genes encoding histone H2A (*HTA1*) or H3 (*HHF2*) were co-transformed into DA12 with the *LEU2* plasmid (pQQ18), which was previously digested to remove the corresponding wild-type copy of either *HTA1* or *HHF2*. *LEU*$^+$ transformants were replica-plated to 5-FOA media to select against cells with pJH33. These FOA-resistant colonies were then replica-plated to media containing 3-AT as an indirect measurement of the *HIS3* gene. Plasmid DNA from each of the 3-AT resistant mutants was extracted and retransformed into DA12 to ensure that 3-AT resistance was caused by the histone plasmid (see Materials and Methods).

# Figure 1


DA12
HTB1
HTA1
URA3
gcn5 F221A
(hta1-htb1)Δ
(hta2-htb2)Δ
(hht1-hhf1)Δ
(hht2-hhf2)Δ
HTB1
HTA1
LEU2
Transformation with mutant histone plasmid
Select against URA3 plasmid with FOA
Isolate potential suppressors on 3-AT

random PCR mutagenesis and a plasmid-shuffling system was used to identify H2A or H3 mutations that can bypass the Gcn5 requirement for activated expression of *HIS3* (Figure 1). Loss of Gcn5 HAT activity causes reduced *HIS3* expression and increased sensitivity to the chemical 3-amino-1,2,4-triazole (3-AT) (15), a competitive inhibitor of the *HIS3* gene product (42). Therefore, mutations in the histones H2A or H3 that caused elevated resistance to 3-AT are likely to be the result of increased *HIS3* expression.

I isolated 41 3-AT resistant clones from approximately 7,600 transformants during the screen for H2A mutants. In addition, I have found 49 resistant clones from roughly 7,500 histone H3 transformants. Total yeast DNA was extracted from each of these potential suppressors and transformed into bacteria to propagate the histone plasmid (see Materials and Methods). Plasmid DNA was then retransformed into DA12 to ensure the 3-AT resistance phenotype was caused by the histone plasmid and not by spurious mutations. After this additional verification step, 19 histone H2A and 11 histone H3 mutants were found to be responsible for heightened resistance to 3-AT (Table 4). The relative level of 3-AT resistance for each H2A and H3 allele was determined by plating a dilution series of cells from each strain to plates containing various concentrations of 3-AT (Figure 2). Growth was then scored relative to the wild-type Gcn5 strain, DA10, and the *gcn5 F221A* strain, DA12. All of the H3 mutations resulted in a 3-AT resistance that was slightly greater than the *gcn5*

# Table 4

| H2A gin candidate | 3-AT Resistance | Caffeine Resistance | SD colony size |
|---|---|---|---|
| gin 1-1 | +++ | - | +/- |
| gin 1-3 | +++ | +/- | +/- |
| gin 1-4 | ++ | +/- | +/- |
| gin 1-7 | + | - | +/- |
| gin 1-9 | + | +/- | - |
| gin 1-14 | + | +/- | - |
| gin 1-15 | + | +/- | - |
| gin 1-16 | + | +/- | - |
| gin 1-17 | + | - | +/- |
| gin 1-19 | + | - | - |
| gin 1-21 | ++ | - | +/- |
| gin 1-22 | ++ | ++ | - |
| gin 1-27 | ++ | - | +/- |
| gin 1-28 | ++ | undet. | undet. |
| gin 1-29 | ++ | - | +/- |
| gin 1-34 | ++ | undet. | undet. |
| gin 1-39 | ++ | - | +/- |
| gin 1-40 | ++ | +/- | +/- |

| H3 gin candidate | 3-AT Resistance | Caffeine Resistance | SD colony size |
|---|---|---|---|
| gin 3-5 | + | - | - |
| gin 3-6 | + | +/- | - |
| gin 3-9 | + | - | +/- |
| gin 3-15 | + | - | - |
| gin 3-17 | + | - | - |
| gin 3-19 | + | - | +/- |
| gin 3-23 | + | - | +/- |
| gin 3-26 | + | - | - |
| gin 3-29 | + | - | - |
| gin 3-31 | + | - | +/- |
| gin 3-44 | + | - | +/- |

**Symbol Key:** -, denotes less growth than DA12; +/-, equal to DA12; +, more growth than DA12; ++, equal to DA10; +++, more growth than DA10; undet. denotes not tested.

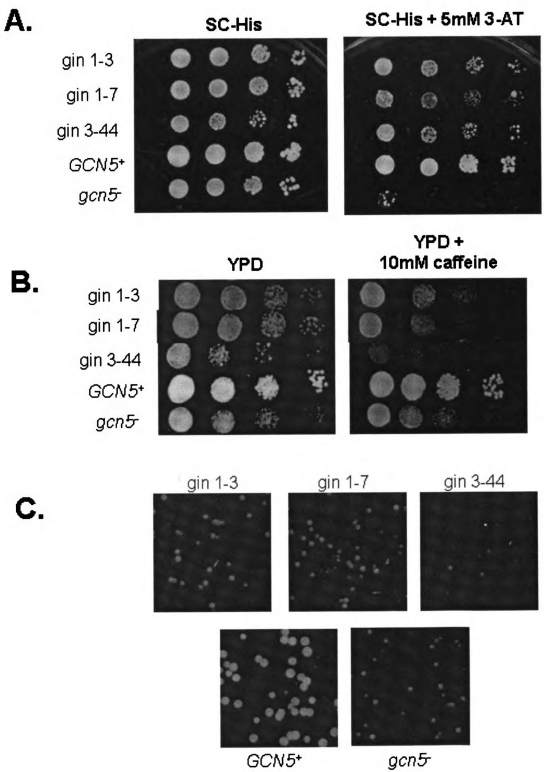
strain, DA12. The H2A mutants showed varying levels of resistance to 3-AT media and most were equal to or greater in resistance than DA10. Altogether, this screen produced 30 potential gin allele candidates based on increased 3-AT resistance in the absence of Gcn5 HAT activity.

**Additional phenotypes of the gin allele candidates.** The gin allele candidates listed in Table 4 might specifically suppress 3-AT sensitivity, and therefore *HIS3* expression defects of the *gcn5 F221A* allele. To quickly determine if the gin allele mutants generally suppress other Gcn5-related phenotypes not related to *HIS3* expression, I tested growth on caffeine containing media of the histone mutation strains. Loss of Gcn5 function causes, by an unknown mechanism, increased sensitivity to media containing caffeine. Caffeine is a nucleotide analog that is known to disrupt several cellular processes (43). Each gin candidate was checked for suppression of sensitivity to caffeine caused by the inactive *gcn5 F221A* allele (Table 4). Only gin 1-22 caused caffeine resistance similar to wild-type cells. The remaining alleles were equal in growth to *gcn5*$^-$ cells or more severely sensitive. These results suggest that the mutant alleles might be specific suppressors of 3-AT sensitivity caused by loss of Gcn5 HAT function.

It is possible that 3-AT resistance of the gin candidates is caused by mechanisms other that restoration of *HIS3* expression. Due to the large number of histone mutation alleles, I wished to narrow the field for gene expression analysis to

**Figure 2. The growth phenotypes of selected gin allele candidates.** (A) 3-AT resistance test. Each strain was grown in rich media until early log phase. Cell density was then determined and serial dilutions were spotted to each plate. Plates were photographed after incubation for 4 days at 30°C or 37°C, for SC-His and SC-His + 3-AT plates, respectively. All strains were tested two or more times. (B) Caffeine resistance test. Cells were spotted to YPD (rich) media or YPD + caffeine plates as described in A. Photographs were taken after 2 or 3 days of incubation at 30°C for YPD and caffeine-containing plates, respectively. (C) Minimal media growth test. All strains were grown as described above and subsequently plated to YPD (not shown) and SD (minimal) plates. Both plates were incubated at 30°C. Photos were taken after 2 days for YPD (not shown) and 3 days for SD.

# Figure 2


A.
SC-His
SC-His + 5mM 3-AT
gin 1-3
gin 1-7
gin 3-44
GCN5+
gcn5-
B.
YPD
YPD +
10mM caffeine
gin 1-3
gin 1-7
gin 3-44
GCN5+
gcn5-
C.
gin 1-3
gin 1-7
gin 3-44
GCN5+
gcn5-

those alleles that were most likely to be functioning by restoring expression to a Gcn5-dependent gene or genes. Gcn5 is important for the expression of several amino acid biosynthetic genes (14); therefore the absence of Gcn5 function causes slow growth on minimal media lacking amino acids (Figure 2). I tested the possibility that the gin candidates might restore expression to several Gcn5-dependent amino acid biosynthetic genes by monitoring the colony size on minimal media (SD) of each histone mutant strain. Contrary to expectations, none of the gin allele candidates show better growth on minimal media than the parent *gcn5*⁻ strain (Table 4). All of the histone mutations resulted in either no effect, or they show reduced growth compared to *gcn5*⁻ cells. These data show that the histone mutations tested do not broadly suppress defects caused by the lack of Gcn5-HAT activity. Instead the gin candidates might specifically restore *HIS3* expression. Another possibility is that the mutations cause increased 3-AT resistance by a mechanism that is unrelated to *HIS3* transcription.

**Histone H2A and H3 sequencing results.** Sequencing each histone mutation was used to determine the mutations contained within each candidate gin allele. In addition, knowing the mutant loci allows for speculation as to which aspects of histone or nucleosome function might be altered to bypass Gcn5 HAT function. If the function of histone acetylation in gene activation is to weaken H2A/H2B dimer contacts within the nucleosome, the majority of histone H2A mutations should be in residues important for this function. The results in Table 5 show that all of the

# Table 5

Histone H2A gin Candidate Mutations

| H2A gin candidate | N-terminus | alpha-1 | L1 | alpha-2 | L2 | alpha-3 | C-terminus |
|---|---|---|---|---|---|---|---|
| gin 1-1 | | | N39I, I44F | | | | P118S, K126E, E130V |
| gin 1-3 | | | I44N | | | L84M | K96M, V108A, L131F |
| gin 1-4 | | R36G | | | | | Q105L, N111Y, K123X |
| gin 1-7 | | L35I | | V50I | | H83R | |
| gin 1-9 | | | | | I79T | L86S | |
| gin 1-14 | | | | I63S | | | N90Y, L109S, N111I |
| gin 1-15 | | R36G | | | | | Q105L, N111Y, K123I |
| gin 1-16 | T25I | R33G | | | K75V | | |
| gin 1-17 | | | I44T | | T77P | R89K | L131I |
| gin 1-19 | G5S, A14T | | R43S | | | | V101P, K123N |
| gin 1-21 | | | | | | I88N | K126R, E130V |
| gin 1-22 | | | R43S | | | | |
| gin 1-27 | K21M | | | | | I88N | N110S, K120R, K126X |
| gin 1-28 | | R36G | | | | | Q105L, N111Y, K123X |
| gin 1-29 | | | | T53S, L59S | | | P118L |
| gin 1-34 | | R36G | | | | | Q105L, N111Y, K123X |
| gin 1-39 | S15A | | | | K75E | Q85H, R89S | L98S, T102I |
| gin 1-40 | K7N | R36G | | | | | Q105L, N111Y, K123X |

X: denotes stop codon

H2A alleles contain multiple mutations, except the allele, gin 1-22. These data make it difficult to ascertain the residue or residues that are responsible for 3-AT resistance. Comparison of the H2A sequencing results also revealed that many residues were mutated in more than one allele and that there are three clusters of mutational "hot spots" (Table 5). The α-1/L1 region is the first cluster of mutations, which is dominated by alterations to Arg 36, Arg 43, and Ile 44. Within the nucleosome, this region is critical for histone-histone and histone-DNA interactions (1). The second cluster contains seven mutations located in the 9 residue α-3 helix, a region important for H2A-H2B dimer interactions (1). Lastly, the largest cluster of mutations is found in the C-terminus of histone H2A. The H2A C-terminus is thought to be important for creation of an interface between the H2A-H2B dimers and the H3-H4 tetramer and potentially for contacting neighboring nucleosomes (1). These results suggest that the gin allele candidates might disrupt histone H2A contacts that are critical for the H2A/H2B dimer stability within the nucleosome.

Each histone H3 allele was also sequenced to identify the mutations responsible for increased 3-AT resistance phenotype. Unlike the H2A alleles, most H3 alleles contained one or two mutations (Table 6). Additionally, these mutations predominantly occurred in the N-terminus. This domain of H3 is known to be the target of several post-translational modifications that are critical for gene expression and other cellular functions. Interestingly, several lysines and

# Table 6

H3 gin Candidate Mutations

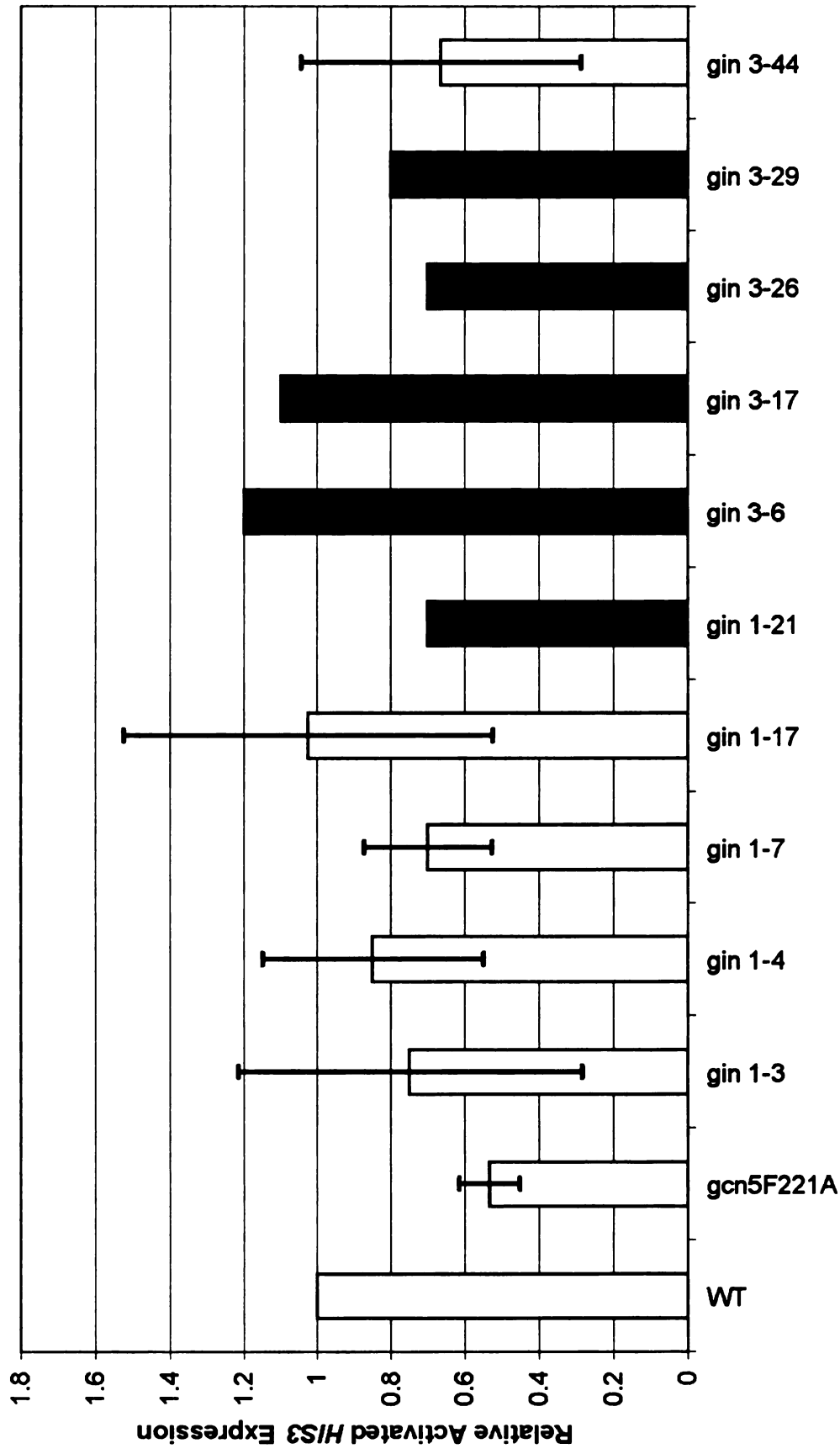
| H3 gin candidate | N-terminus | alpha-1 | L1 | alpha-2 | L2 | alpha-3 | C-terminus |
|---|---|---|---|---|---|---|---|
| gin 3-5 | K14I | R69G | | | | | |
| gin 3-6 | K18N | | | | I119V | | |
| gin 3-9 | K4N, K9E | | | | | | |
| gin 3-15 | Q5L, H39R, R40G | | | V101A | | | |
| gin 3-17 | S10P, G13C, R49T, F54Y | | | | | | |
| gin 3-19 | R40G | | | | | | |
| gin 3-23 | R40G | | | | | | |
| gin 3-26 | R26K, R49T | | | | | | |
| gin 3-29 | A15D, A47P | | | | | | |
| gin 3-31 | G13S, R40I | | | | | | |
| gin 3-44 | L48F | | | | | | |

residues adjacent to the lysines are mutated in the 11 potential H3 gin alleles. Moreover, most of these lysines are mutated to uncharged or negatively charged residues, indicating that the ability of the H3 tail to interact with DNA or neighboring nucleosomes might be disturbed by these mutations. Also, arginine 40 was altered in four different H3 alleles, twice as the only mutation. This residue is known to make histone-DNA contacts as the H3 tail exits the core regions of the nucleosome. A mini-cluster of mutations was also found in the α-N helix of H3, specifically residues 47-49. Intriguingly, this region of histone H3 seems to be important for contacts between H3 and the H2A C-terminus (1), which also contains several mutations as described above. Lastly, the gin 3-6 allele contains the alteration I119V. This mutation is the right next to Thr118, a residue that when mutated, causes the SWI/SNF-independent (sin) phenotype (8). Taken together, most H3 mutations affect the H3 N-terminus and disruption of tail interactions or weakening of the interaction with H2A are possible reasons for the suppression of the Gcn5 defect in 3-AT resistance.

***HIS3* expression of the gin allele candidates.** Cellular resistance to 3-AT can arise from mechanisms other than increased *HIS3* expression. Therefore, real Gcn5-independent alleles will increase *HIS3* activated expression in the absence of Gcn5 HAT activity. To identify the gin alleles, I used Northern blot hybridization and real time RT-PCR to quantitate the activated *HIS3* expression in each of the 3-AT resistant gin candidate strains. Figure 3 represents the *HIS3* expression of a subset of gin allele candidates. Only a subset of the many

**Figure 3. *HIS3* expression of the gin candidate histone alleles.** Relative *HIS3* expression is shown as a ratio of the normalized amount of activated *HIS3* mRNA found in the wild-type strain, DA10. *HIS3* expression was normalized to 18S rRNA for Northern blot experiments. For real-time RT-PCR quantitation, *HIS3* expression was normalized to *ACT1* and *PGK1* gene expression. White bars represent strains tested by multiple experiments; black bars depict the alleles that were only tested by one experiment. Those alleles that never showed increased *HIS3* expression, relative to the parent strain DA12 are not represented. Error bars for *gcn5F221A*, gin 1-3 and gin 1-7 indicate standard deviation. Error bars for gin 1-4 and gin 1-17 indicate the range of *HIS3* expression, since these samples were only measured twice.

Figure 3


gin 3-44
gin 3-29
gin 3-26
gin 3-17
gin 3-6
gin 1-21
gin 1-17
gin 1-7
gin 1-4
gin 1-3
gcn5F221A
WT
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative Activated HIS3 Expression

potential gin alleles showed elevated *HIS3* expression in one or more experiments. Contrary to our hypothesis, none of the alleles showed a statistically significant increase in *HIS3* expression as compared to the original *gcn5 F221A* parent strain. The H3 mutants, gin 3-6 and gin 3-17 showed full restoration of *HIS3* expression, but these samples were not repeated and cannot be described definitively as Gcn5-independent alleles.

Initially, the H2A mutants gin 1-3 and gin 1-7 as well as the H3 mutant gin 3-44 showed increased *HIS3* expression in separate experiments. Therefore, these alleles were thought to be real gin alleles and further mutations were recreated based on these alleles (see below). These alleles failed to show the original increase in *HIS3* expression in later experiments. These data suggest that most or all of the alleles identified in this study are not Gcn5-independent for HIS3 expression. Moreover, these mutations cause increased 3-AT resistance by an unknown mechanism. The H2A mutations described below were selected based on those found within gin 1-3 and gin 1-7. The histone H2A mutations are: I44N, V50I, H83R, and L84M. The H3 mutations studied further were based on gin 3-44 which contains only an L48F substitution. Additionally, the H3 mutations A47P and R49T were also selected for further study based on proximity to the L48F mutation in 3-44 and also based on their presence in gin 3-17, gin 3-26 and gin 3-29.

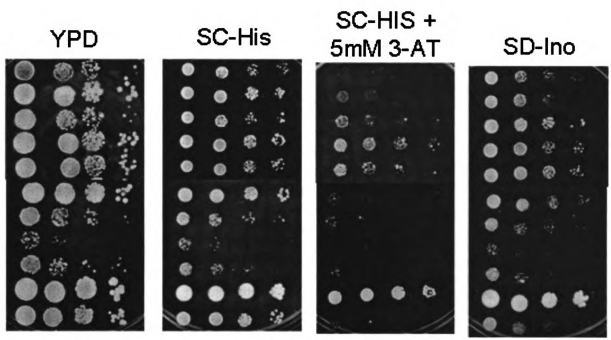
**Recreation of individual histone mutations.** The single mutations of histones H2A and H3 described above were introduced specifically by PCR mutagenesis into the wild-type histone plasmid, pQQ18 (see Materials and Methods). Each mutation was verified by sequencing and subsequently introduced into the *gcn5 F221A* strain, DA12. As before, plasmid shuffling with 5-FOA was performed to isolate cells, which contain only the specifically mutated histone plasmid. These strains were first characterized by growth tests on different media.

Although the 3-AT resistance phenotype was not correlated to *HIS3* expression in all of the histone mutation strains listed in Table 4, it is a quick test that may show which individual mutations are bypassing the Gcn5-requirement for *HIS3* gene expression. As depicted in Figure 4, not all of the newly created histone mutation strains cause increased 3-AT resistance. Only the three histone H3 mutations (A47P, L48F and R49T) give rise to 3-AT resistance. As another growth test for a possible link transcription related Gcn5-indepedence, I have checked the growth of these strains on minimal media that lacks inositol. Gcn5 is required for *INO1* expression (44), whose gene product is important for inositol biosynthesis. Figure 4 shows that the *gcn5 F221A* mutation causes reduced growth on media without inositol. The H2A I44N mutation and the three H3 mutations cause increased growth in the absence of Gcn5-HAT activity. Taken together, all H3 mutations suppressed each *gcn5*$^-$ phenotype tested, while the H2A I44N suppressed only the *Ino*$^-$ phenotype. This suggests that the three H3

**Figure 4. Growth phenotypes of strains containing recreated histone point mutations.** Each strain was grown in rich media until early log phase. Cell density was then determined and serial dilutions were spotted to each plate. All growth tests were performed two or more times. Photos were taken after 2 days for YPD (30°C) and 4 days for SC-His and SC-His + 3-AT (37°C) and SD – inositol (30°C).

# Figure 4

| Histone Allele | *GCN5* |
|---|---|
| H2A H83R | *F221A* |
| H2A L84M | *F221A* |
| H3 A47P | *F221A* |
| H3 L48F | *F221A* |
| H3 R49T | *F221A* |
| H2A I44N | *F221A* |
| H2A V50I | *F221A* |
| H2A L35I H83R | *F221A* |
| H2A V50I H83R | *F221A* |
| WT | WT |
| WT | *F221A* |



alleles are most likely to suppress transcriptional defects caused by the *gcn5 F221A* mutation.

Because the histone proteins are highly conserved and present throughout the genome, introduction of mutations to H2A and H3 are most likely to cause growth defects. To test this possibility, the growth rates of the recreated histone mutants were tested in the presence of wild type *GCN5* and *gcn5 F221A.* Figure 5 shows the results of the growth rate determinations. The *gcn5 F221A* (DA11) mutation causes a moderate decrease in the cell growth rate relative to wild type cells (DA10). Surprisingly, H2A mutations, I44N, V50I and L84M did not alter the growth rate of either the wild-type *GCN5* or the *gcn5 F221A* strain. H2A H83R seems to cause a dramatic growth defect. This defect in growth is not affected by the *gcn5 F221A* mutation. Moreover, this defect of the H83R mutation is not seen in the gin 1-7 allele, of which it is derived. This argues that the two other mutations present in gin 1-7 (L35I and V50I) might intragenically suppress the H83R defect, since combining H83R with either L35I or V50I (Figure 5) is insufficient to alleviate this defect. All three H3 mutations were responsible for decreased growth rate of cells and once again this effect was independent of *GCN5* status. The H3 mutation, A47P, caused the most severe defect and this is most likely attributable to the introduction of proline to the αN helix, which could disrupt histone-DNA and histone-histone contacts in this region of the nucleosome. In conclusion, H2A H83R and the three H3 mutations affect the growth rate, which might indicate the importance of these residues in chromatin

**Figure 5. Doubling time analysis of histone mutations in *GCN5*$^{+}$ and *gcn5*$^{-}$ strains.** The average doubling time of each strain listed is depicted in minutes. Gray bars denote strains with the *gcn5 F221A* allele and the white bars depict the strains with wild-type *GCN5*. Each strain was tested in duplicate in two independent experiments. Error bars represent the range of values obtained in from two independent experiments.

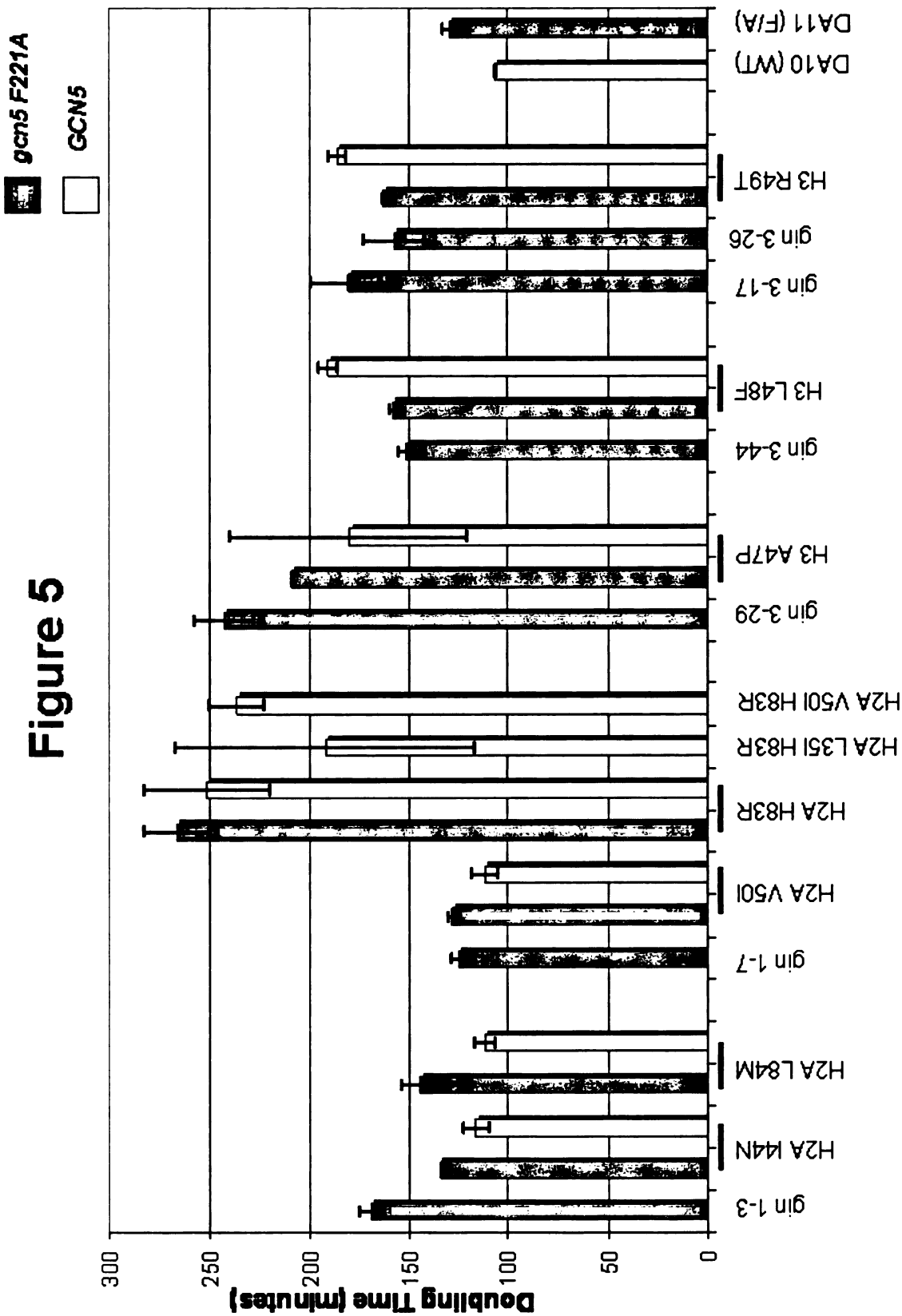


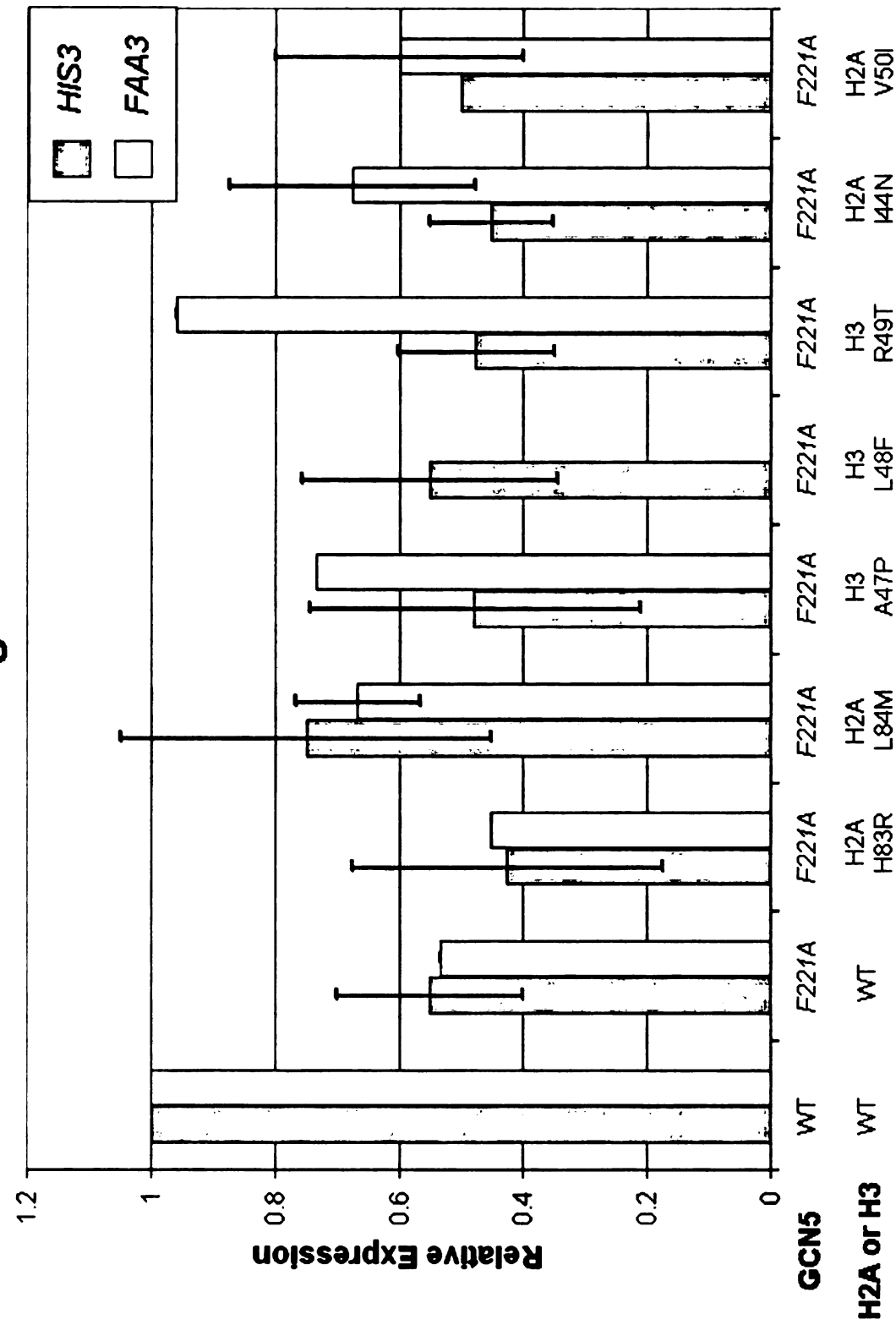
Figure 5
gcn5 F221A
GCN5
Doubling Time (minutes)
0
50
100
150
200
250
300
gln 1-3
H2A I4N
H2A L84M
gln 1-7
H2A V50I
H2A H83R
H2A L35I H83R
H2A V50I H83R
gln 3-29
H3 A47P
gln 3-44
H3 L48F
gln 3-17
gln 3-26
H3 R49T
DA10 (WT)
DA11 (F/A)

maintaining normal chromatin function. In addition, these data allow more precise growth of cultures of the above strains for subsequent RNA analyses.

***HIS3* expression in the histone single mutation strains.** The next question addressed was whether any of the individual mutations described above are responsible for Gcn5-independent expression of *HIS3*. Based on the growth tests described above, I hypothesized that the three H3 mutations are the most likely Gcn5-independent alleles. To test this hypothesis, real time RT-PCR experiments were performed to assess the *HIS3* mRNA levels of each of recreated histone alleles in the *gcn5 F221A* background. Figure 6 shows the results of these experiments. As with the original, multiple mutation alleles, none of these recreated mutants restore *HIS3* expression in the absence of Gcn5 HAT activity. Instead, the H3 alleles cause 3-AT resistance by a mechanism that is independent of *HIS3* expression. I also tested the expression of three other Gcn5-dependent genes, *FAA3*, *FUR4*, and *PHO84*. These genes were selected because they were shown to have 3-fold or greater decreases in expression upon deletion of *GCN5* (45). In addition, I proposed that suppression of transcription defects these other Gcn5-dependent genes would support conclusions based on HIS3 expression for any gin allele found. *FAA3*, *FUR4*, and *PHO84* depend on Gcn5 for full expression in rich media, thus simplifying the experiments. I found that *gcn5 F221A* caused a two-fold decrease in *FAA3* expression (Figure 6). This defect is not as severe as that originally reported (45), most likely because those experiments used a complete *GCN5* knockout.

**Figure 6. *HIS3* and *FAA3* expression of the single mutation histone alleles.** Real time RT-was performed to determine *HIS3* and *FAA3* expression in the single histone mutation strains. Gray bars depict the activated *HIS3* expression and white bars represent the *FAA3* expression of the listed strains. Expression of each gene was normalized to level in the wild-type stain, DA10. Error bars represent the standard deviation or range of expression values based on the averages from two or more independent experiments. The *HIS3* data for the V50I strain (DA45) and the *FAA3* expression of the H2A H83R (DA30) and H3 A47P (DA32) strains have no error bars, as this data represents relative expression of a single experiment.

# Figure 6


HIS3
FAA3
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
GCN5
H2A or H3
WT
WT
F221A
WT
F221A
H2A H83R
F221A
H2A L84M
F221A
H3 A47P
F221A
H3 L48F
F221A
H3 R49T
F221A
H2A I44N
F221A
H2A V50I

In contrast to *HIS3* expression, the defective *FAA3* expression is partially restored by H2A L84M, H3 A47P and H3 R49T. *FUR4* expression was not affected by the *gcn5 F221A* allele (not shown) and was therefore not included in analysis. Finally, I have found that *PHO84* expression was reduced five-fold by in the *gcn5* mutation, but none of the histone alleles showed any suppression of this effect (data not shown). In summary, none of the single mutations cause Gcn5-independence for *HIS3* expression. These data also suggest that Gcn5 functions differently at its target genes, since a subset of the histone mutations partially suppressed defects in *FAA3* expression, but not at *HIS3* or *PHO84*.

**Comparison of gin and other related histone mutant phenotypes.**

Previously, several histone mutations have been isolated in screens for their involvement in multiple aspects of gene regulation. A few sin alleles of histones H3 and H4 are known to suppress the defects in gene expression caused by loss of SWI/SNF activity (8). In addition, the *lrs* mutations of histones H3 and H4 are known to cause defective gene silencing (25). Because these transcriptional regulatory mechanisms function at the level of chromatin, Gcn5-independent histone mutations could possibly also affect these other mechanisms of gene transcription.

To test the above hypothesis, I initially attempted to test three histone H3 SWI/SNF-independent (sin) alleles (H3 E105K, R116H, and T118I) for their potential ability to also function as Gcn5-independent alleles. Each of the H3

mutations was transformed into the *GCN5* and *gcn5 F221A* strains (see Materials and Methods). Plasmid shuffling, as before produced no FOA resistant colonies (*ura*$^-$), indicating when these sin alleles were present, the cells required the wild type *URA3* histone plasmid to maintain viability. In addition, I have found that the H3 E105K allele caused reduced transformation efficiency with either catalytically inactive *gcn5* (*gcn5 E173H or gcn5 F221A*) or the *GCN5* deletion (Figure 7). H3 T118I also resulted in reduced transformation efficiency in the *gcn5 F221A*, which suggests the three Gcn5 mutations are not equivalent. The R116H mutation had no effect on any of the strains tested. In addition, H3 E105K and H3 T118I cause a dominant synthetic sick phenotype in the presence of any of the Gcn5 mutations based on small colony size after transformation (not shown). The H3 sin alleles caused unexpectedly severe growth defects and therefore determination of these mutations to function as gin alleles was not tested.

I also attempted to test whether the histone mutations created in this study were SWI/SNF independent. To this end, I created a *SWI2* deletion strain (see materials and methods). Swi2 is the catalytic subunit of the SWI/SNF complex and is required for its activity as a transcriptional co-activator (46). The *swi2Δ* deletion strain DA29, has the pQQ18 (*LEU2*) histone plasmid, but it must be replaced by the pJH33 (*URA3*) histone plasmid first. This is due to the fact that all of the histone mutations are based on pQQ18. Several attempts to create a

*URA*$^+$ *leu*$^-$, *SWI2* deletion strain were unsuccessful and therefore no testing of the sin phenotype could be performed.

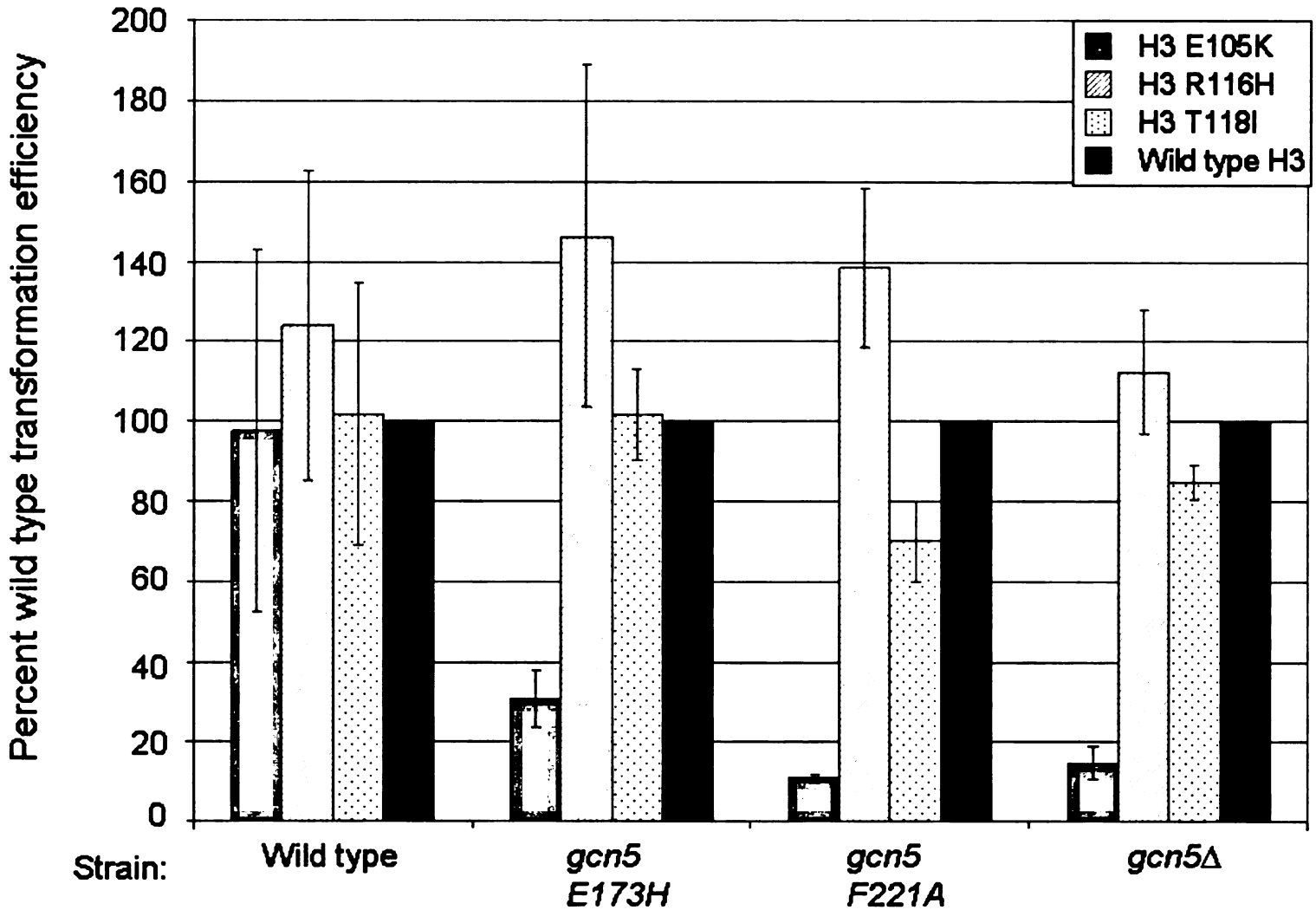
Lastly, I wished to test any possible effects on gene silencing brought about by histone mutations discovered by this study. Gene silencing in yeast occurs at three locations; telomeres, ribosomal RNA gene repeats, and the mating type loci (47). To this end, I obtained constructs for integration of reporter genes for each of the silent loci. As before, great difficulty was experienced during strain construction. None of the reporter constructs were confirmed to be integrated at the proper loci by PCR genotyping.

# Discussion

I have identified several potential Gcn5-independent histone H2A and H3 mutant alleles based on their increased resistance to the chemical 3-AT. Sequencing results revealed that most of the alleles have mutations in regions of H2A or H3 that could destabilize association of H2A/H2B dimers with the nucleosome or to alter the function of the H3 tail. Actual gin alleles are classified based on elevation of activated *HIS3* mRNA in the absence of Gcn5-HAT activity. Contrary to expectations none of the 3-AT resistant gin allele candidates cause increased *HIS3* expression. Moreover, single mutations were created based on

**Figure 7. Transformation efficiency of the H3 sin alleles.** The transformation efficiency of each histone allele (wild-type, H3 E105K, H3 R116H, and H3 T118I) was determined from three independent transformations of each allele to the *GCN5*$^{+}$, *gcn5 F221A*, *gcn5 E173H*, and *gcn5Δ* backgrounds which have the *URA3*-marked wild type histone plasmid, pJH33. Transformants were selected on synthetic complete media (SC) lacking uracil and leucine to ensure all of the above strains contained at least one wild type copy of the histone H3 gene. The transformation efficiency of the wild-type histone H3 plasmid (pQQ18) was set to 100 percent for each strain used. Error bars represent the standard deviation from the three independent transformations.

# Figure 7


Percent wild type transformation efficiency
200
180
160
140
120
100
80
60
40
20
0
H3 E105K
H3 R116H
H3 T118I
Wild type H3
Strain:
Wild type
gcn5 E173H
gcn5 F221A
gcn5Δ

three histone alleles (gin 1-3, gin 1-7, and gin 3-44) originally suspected to be Gcn5-independent for *HIS3* expression.

As before, all of the individual mutations tested, did not result in suppression of the Gcn5-related *HIS3* activation defect. Additional characterization of these alleles for other transcription-related phenotypes (SWI/SNF independence (sin) and gene silencing defects) was not determined due to difficulties in strain construction.

The gin allele candidates listed in Table 4 were identified on the basis of resistance to 3-AT. Surprisingly, none of these alleles resulted in a statistically significant increase in *HIS3* expression. This is surprising because 3-AT directly inhibits the *HIS3* gene product (48). Moreover, Gcn5 function is required for full activation of *HIS3* in a HAT-dependent manner (15). Therefore, I hypothesized that some of these mutations should have bypassed the Gcn5 requirement at *HIS3* to cause increased 3-AT resistance. Because histone mutations likely alter the expression of several genes, a number of cellular mechanisms could be affected which result in 3-AT resistance. For example, the expression of a transport protein responsible for cellular import of 3-AT could be reduced, leading to less His3p inhibition and therefore cause better growth on 3-AT media. An additional possibility related to *HIS3* expression is that the overall level of *HIS3* mRNA is not increased by the histone mutations; instead the His3 protein levels are increased. This could result from alteration of a number of processes,

including those that regulate *HIS3* mRNA or protein stability. In particular, an increase in His3 protein without a corresponding increase in total mRNA could result by alteration of transcription start site selection. Transcription of *HIS3* is known to initiate mainly from two sites, designated +1 and +13 (49). Gcn4-independent basal expression of *HIS3* produces an equal amount of +1 and +13 transcripts; upon activation, the overall *HIS3* expression is increased and transcription initiation now occurs predominantly from the +13 position (49). The increased +13/+1 transcript ratio depends on Gcn5 HAT activity since catalytic inactivation of Gcn5 reduces the +13/+1 ratio to 1 (50). Therefore, the +13 transcript, which is the dominant species during the activated condition for *HIS3*, could produce more function His3 protein by alteration of the *HIS3* mRNA properties (e.g. increased stability or translation). S1 mapping or primer extension would need to be performed on the histone mutation strains to verify this hypothesis.

Early *HIS3* expression analyses suggested that gin 1-3, gin 1-7 and gin 3-44 partially restored *HIS3* expression in the absence of Gcn5 HAT activity. Additional experiments based on the above mutations showed that these alleles did not cause a statistically significant change in *HIS3* expression. As this was not known at the time, I created single mutation histone alleles based on the above mutations. Initially, the single mutation histone alleles were characterized based on several growth phenotypes (Figures 4 and 5). It was no surprise, due to the presence of histones throughout the genome, that some of the histone

mutations were responsible for a reduction in growth rate. This data was also extremely useful for coordinating the growth of these strains for subsequent RNA analysis. As with the originally identified H2A and H3 mutations, none of the recreated mutations caused a Gcn5-independent increase in *HIS3* expression (Figure 6). All three of the histone H3 mutants caused increased 3-AT resistance, but did not result in elevated *HIS3* expression. The alleles are therefore not gin for *HIS3*. On the other hand, some of these mutations, (H2A L84M, H3 A47P and R49T) partially restored expression to another Gcn5-dependent gene, *FAA3* (Figure 6). Gcn5 regulates the expression of multiple genes and the Gcn5-requirement for each is known to be variable. For example the yeast gene *PHO8* requires Gcn5 function for remodeling of nucleosomes in its promoter and for full gene activation (51,52). On the other hand, Gcn5 functions differently at the *PHO5* gene. Deletion of *GCN5* causes a slight delay in chromatin remodeling and gene expression of *PHO5 (53,54)*. Taken together, this data suggests that Gcn5-target genes are activated by different mechanisms and therefore any histone mutation might only restore expression of a subset of Gcn5-regulated genes.

Attempts to create strains for testing SWI/SNF-independence and gene silencing effects of these alleles were unsuccessful. Strain creation problems have deterred several aspects involving additional and necessary tests to describe the histone mutations described by this research. In yeast, certain phenotypes and genetic interactions are known to be strain specific. For example, Pollard and

Peterson showed that a *snf2Δ gcn5Δ* combination is lethal (44), but later experiments using a slightly different strain showed that this double mutation is viable, although very sick (55). As such, the problems encountered during strain construction might be specific to the S288C-based strain JHY205, used in this study. To avoid these troubles, the *SWI2* deletion and silencing reporter constructs could be introduced into the strain JHY200. JHY200 is highly similar to JHY205, in that it has each of the core histone genes deleted, but it is based on another common laboratory strain, W303 (35).

Biochemical and biophysical characterization of the gin alleles would provide useful details as to the mechanism of their suppression of *HIS3* expression defects in the absence of Gcn5-mediated acetylation. Similar studies performed with the sin alleles have linked their function to disruption of histone-DNA contacts (9), alteration of nucleosome mobility (24), and changing the folding properties of chromatin (10). Functional tests such as those described in these studies could lead to a better understanding of the causative role of Gcn5 acetylation in transcriptional activation.

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# Chapter 3

# Analysis of the role of Ahc1 in *HIS3* gene expression.

# Introduction

Eukaryotic genomes exist in the form of chromatin. This nucleoprotein structure is mainly composed of histone proteins and DNA. Although chromatin is essential for compaction and organization of the DNA, it restricts the access of many factors that require access to the DNA. Several conserved mechanisms exist to modify the chromatin structure in order to enhance or restrict access to the DNA template during processes such as transcription. Histone acetylation is of one of the best-studied chromatin modifications and it is known to play a critical role in the regulation of gene expression. This acetylation is performed by histone acetyltransferases (HATs), many of which are known to be involved in the regulation of transcription (1).

The yeast protein, Gcn5, is the canonical HAT and its histone acetyltransferase activity is required for the proper expression of several genes. Gcn5 functions in the context of at least three multi-subunit complexes. Of these, the most well known is the 1.8 MDa SAGA complex. In addition to Gcn5, the SAGA complex contains several Spt, Taf, and Ada proteins as well as Tra1 and others (2-4). These SAGA subunits can be functionally categorized by their participation in histone acetylation, TBP-interaction, activator-interaction, complex integrity and other functions (5-13). SAGA has been well-characterized and is known to be

important for the expression of approximately 10% of yeast genes, most of which are stress-induced (14).

Gcn5 is also a member of the recently described SALSA/SLIK complex. This complex is highly homologous in composition to SAGA, but lacks the TBP-interacting component, Spt8 (15,16). In addition, SALSA/SLIK is known to contain a truncated version of the Spt7 protein. The SALSA/SLIK complex is thought to be functionally distinct from SAGA, possibly regulating a unique subset of yeast genes (15,16).

The ADA complex shares the Ada2, Ada3 and Gcn5 components with the SAGA complex (2). It was subsequently found to contain the unique component, Ahc1 (ADA HAT Complex component 1) (17). Deletion of *AHC1* chromatographically disrupts the ADA complex, but does not cause any phenotypes common to those caused by SAGA mutations (17). Currently, the function of the ADA complex is unknown.

The *HIS3* gene, a histidine amino acid biosynthetic gene, is regulated by Gcn5 histone acetyltransferase activity (18). Gcn4, the *HIS3* activator, is required for activated *HIS3* transcription and has been shown to recruit Gcn5 in the SAGA complex to the *HIS3* gene under amino acid starvation conditions (19-21). Disruption of the Gcn4 binding site in the *HIS3* promoter eliminates Gcn5 recruitment and causes reduced histone H3 acetylation and gene expression

only during the activating conditions (21). In contrast, both basal and activated *HIS3* expression and histone acetylation is reduced when *GCN5* is deleted (22). Moreover, deletion of *SPT20*, a protein that is critical for the integrity of SAGA, but not ADA, affects only activated *HIS3* expression (23). This evidence suggests that Gcn5 could function in the ADA complex to control basal *HIS3* expression through promoter histone acetylation. To test this hypothesis, I have tested the *HIS3* expression and promoter H3 acetylation levels in an *AHC1* deletion strain. Surprisingly, gene expression data showed that disruption of the ADA complex by deletion of *AHC1* did not affect the basal *HIS3* expression, but may be important for activated expression. Additionally, loss of Ahc1 did not affect the H3 K9/K14 acetylation status of the *HIS3* promoter under basal or activating conditions. Ahc1 was not detectable at the *HIS3* promoter, based on chromatin immunoprecipitation experiments.

# Materials and Methods

**Strains and Media.** Yeast strains used in this work are listed in Table 7. Standard yeast techniques were performed as described in (24). Yeast transformations were performed by the lithium acetate technique (25). Plasmids used in this work are found in Table 8.

pDA8, an Ahc1 expression construct with a N-terminal, trimeric hemagglutinin (HA) tag was made by cotransformation into yeast of *Xba*I digested pMK547

# Table 7

*S. cerevisiae* Strain List

| Strain | Relevant geneotype | Source |
|---|---|---|
| YMK839 | *MATa trp1 leu2-3,112 ura3-52* | 19 |
| YMK842 | *MATa trp1 leu2-3,112 ura3-52 gcn5Δ::hisG* | 19 |
| YMK869 | *MATa trp1 leu2-3,112 ura3-52 his3 gcre-* | M.-H. Kuo, unpublished |
| YMK967 | *MATa trp1 leu2-3,112 ura3-52 ahc1Δ::URA3* | M.-H. Kuo, unpublished |
| YMK968 | *MATa trp1 leu2-3,112 ura3-52 his3 gcre-ahc1Δ::URA3* | M.-H. Kuo, unpublished |
| YMK1019 | *MATa trp1 leu2-3,112 ura3-52 spt20Δ::URA3* | M.-H. Kuo, unpublished |
| DA111 | *MATa trp1 leu2-3,112 ura3-52* pDA8< *CEN TRP1 HA-AHC1*> | This study |
| DA112 | *MATa trp1 leu2-3,112 ura3-52* pDG64<*CEN TRP1Gal4DBD-H3-IPL1-HA*> | This study |
| DA113 | *MATa trp1 leu2-3,112 ura3-52 ahc1Δ::URA3* pDA8<*CEN TRP1 HA-AHC1*> | This study |
| DA129 | *MATa trp1 leu2-3,112 ura3-52* pMK325<*2μ LEU2 AHC1-HA*> | This study |
| DA137 | *MATa trp1leu2-3,112 ura3-52 AHC1-TAP* | This study |
| DA174 | *MATa trp1 leu2-3,112 ura3-52 ahc1Δ::URA3* | This study |
| DA175 | *MATa trp1 leu2-3,112 ura3-52* pYL54<*URA3 leu2d GCN5-TAP* | This study |

# Table 8

**Plasmid List**

| Plasmid Name | Description | Source |
|---|---|---|
| pMK262 | *AHC1Δ::URA3* construct | Kuo, M.-H. unpublished |
| pMK325 | Ahc1-3xHA overexpression construct | Kuo, M.-H. unpublished |
| pDA8 | 3xHA-Ahc1 expression construct | This study |
| pDG64 | Gal4DBD-H3-Ipl1-3xHA expression construct | 24 |
| pYL54 | Gcn5-TAP expression construct | 28 |

(derived from pAB8, lacking the Gal4 DNA binding domain)(26) and a PCR fragment containing the *AHC*1 open reading frame. pDA8 was transformed into YMK839 to create DA111 for subsequent chromatin IP experiments.

For complementation of the *ahc1Δ* (YMK967) phenotypes, pMK325, a pSOS (Stratagene) based plasmid containing the *AHC1* gene, was transformed into YMK967 to create DA129.

The C-terminal TAP tagged Ahc1 strain, DA137, was created as described by Puig et al. (27). Briefly, the TAP sequence was amplified from pBS1479 (27) with oligonucleotides DA38; 5'-TATTACAATCTAAGGTCGAAATCAAGACTGCGTGGTTCTCATACAGCTGGAGGATCCATG-3' and DA39; 5'-ACAGAATATTATATTACGTAATTTACTTATTTATATGTGTGTATACGACTCACTATAGGG-3' with underlined sequence specific to the TAP sequence and non-underlined sequence specific for the *AHC1* 3' region surrounding the stop codon. The *AHC1* homology of this PCR product was extended by amplification of the primary PCR product (DA38/DA39) with the additional oligonucleotides DA52b; 5'-ACTAATGAACTTGAGGGTCCTGATCCTGCAGCAAAATCATCATCTTATTACAATCTAAGG-3' and DA53; 5'-AATCCACTTTTTCTTCCAAAGCGACAAATTAGGTCAGAAAACCAACAGAATAT

TATATTA-3'. The gel purified PCR product from the secondary PCR was used to transform YMK839 to create DA137.

The *ahc1Δ::URA3* strain DA174 was created exactly as YMK967. Briefly, pMK262 (M.-H. Kuo, unpublished) contains the *ahc1Δ::URA3* fragment in pJJ252 (28) and was digested with *Xba*I and *Sph*I prior to transformation into YMK839. The proper integration of the *AHC1* deletion construct was verified by genomic PCR.

***HIS3* expression analyses.** Yeast cell cultures were grown in YPD media for several hours to an optical density at 600 nm ($O.D._{600}$) of approximately 0.8. Cultures were centrifuged and cells were washed with an equal volume of sterile water. Next, the washed cells were transferred to YPD for measurement of basal *HIS3* expression or SD plus 20 mM 3-AT for induced *HIS3* expression. Prior to harvesting RNA, cells were incubated in YPD or SD + 3-AT for 2-4 hours at 30°C and 37°C, respectively. RNA preparation and Northern blot hybridization techniques were performed as previously described (22). For RNA analyses by real-time RT-PCR, 10-20 μg of total RNA was treated with 10 U of RNase-free DNase I (Roche) in a 100 μL solution containing 50 mM Tris-HCl, pH 7.5 and 5mM $MgCl_2$ for 30 minutes at 37°C. The DNase I-treated RNA (0.5-1.0 μg) was reverse transcribed using 1.0 μL of Improm II reverse transcriptase (Promega) and 0.5 μg oligo dT in a 20 μL reaction. 1.0 μL of each RT reaction was added to

30 μL real-time PCR reactions including 1X SYBR green PCR reagent (Applied Biosystems), 3 mM MgCl2, 0.8 mM dNTPs, 0.15 μM oligonucleotides and 0.75 U ampliTaq Gold (Applied Biosystems). *HIS3* oligonucleotides were: (HIS3 5' RT) 5'-AGCTTTGCAGAGGCTAGCAG-3' and (HIS3 3' RT); 5'-GCGAGGTGGCTTCTCTTATG-3'; *ACT1* oligonucleotides were (ACT1 5' RT); 5'-CTGCCGGTATTGACCAAACT-3' and (ACT1 3' RT); 5'-CGGTGATTTCCTTTTGCATT-3'. *PGK1* oligonucleotides were: (PGK1 5' RT); 5'-TCATTGGTGGTGGTGACACT-3' and (PGK1 3'RT); 5'-GCAACACCTGGCAATTC-3'. Each real time PCR contained technical duplicates and each PCR experiment was performed in duplicate. Gene expression results are presented as the average values from three or more independent experiments (unless otherwise stated) with error expressed as standard deviation (Figure 8A) or range (Figure 13B) when appropriate. Relative expression of *HIS3* was calculated based on the $2^{-\Delta\Delta CT}$ method (29).

**Chromatin IP.** Expression of HA-Ahc1 in DA111 and Ahc1-TAP was verified by Western blotting of whole cell extracts from the above strains. Whole cell extracts were prepared from each strain and separated on 8% SDS-PAGE gels. HA-Ahc1 was detected by anti-HA antibodies (12CA5, Roche). Ahc1-TAP was detected by a 1:3000 dilution of rabbit serum (IgG), which binds the protein A epitope in the TAP tag. For chromatin IP experiments, cells were grown as described for *HIS3* gene expression analysis, except that after the cells were switched to fresh YPD or SD + 3-AT; incubation time was 2 hours. Whole cell

extract preparation, sonication, immunoprecipitation and PCR for H3 acetylation and Ahc1 ChIP experiments were performed essentially as described by Kuo et al. (21). Results were based on ChIPs from two independent whole cell lysate preparations with multiple PCR reactions per experiment. As a negative control for the HA-Ahc1 ChIP experiments, whole cell extracts from DA112 (Table 7) were used. DA112 contains pDG64 (26), a plasmid that expresses a Gal4 DNA binding domain, histone H3 tail and Ipl1 fusion construct with a trimeric HA tag. In the Ahc1-TAP chromatin IP studies, whole cell lysates from DA175 (Table 7) were used as a positive control for the TAP immunoprecipitations. DA175 contains the plasmid pYL54, which expresses a C-terminally TAP-tagged Gcn5 (30) . Some minor alterations were used in the ChIP protocol for the Ahc1-TAP ChIP experiments; extracts from approximately 3 x $10^8$ cells (200-300 $\mu$L reaction volume) were immunoprecipitated with 10 μL of immunoglobulin G (IgG) Sepharose 6 beads (Amersham) and the immunoprecipitated DNA was dissolved in 50 μL 1X TE, pH 8.0.

# Results

***HIS3* expression in an *ahc1Δ* strain.** Previously, the deletion of *GCN5* was shown to affect both basal and activated *HIS3* expression (22). Additionally, disruption of the SAGA complex by deletion of *SPT20* does not affect basal *HIS3* expression (23). Because of this, we hypothesized that the ADA complex is important for basal *HIS3* expression. To test this hypothesis, I determined the

*HIS3* expression levels in the *ahc1Δ* strain, YMK967 (Figure 8), because Ahc1 is critical for ADA complex integrity (17). Contrary to the hypothesis, loss of Ahc1 did not affect the *HIS3* basal mRNA levels versus that of the wild-type strain (Figure 8A). As controls for this experiment, strains with a *GCN5* deletion (*gcn5Δ*) or a deleted Gcn4 binding site (*his3 gcre*$^{-}$) were included. As expected, *GCN5* deletion results in a two-fold decrease in basal *HIS3*, while the *gcre-* strain is largely unaffected. In addition, deleting *AHC1* from the *his3 gcre-* strain has no additional effects on basal *HIS3* expression.

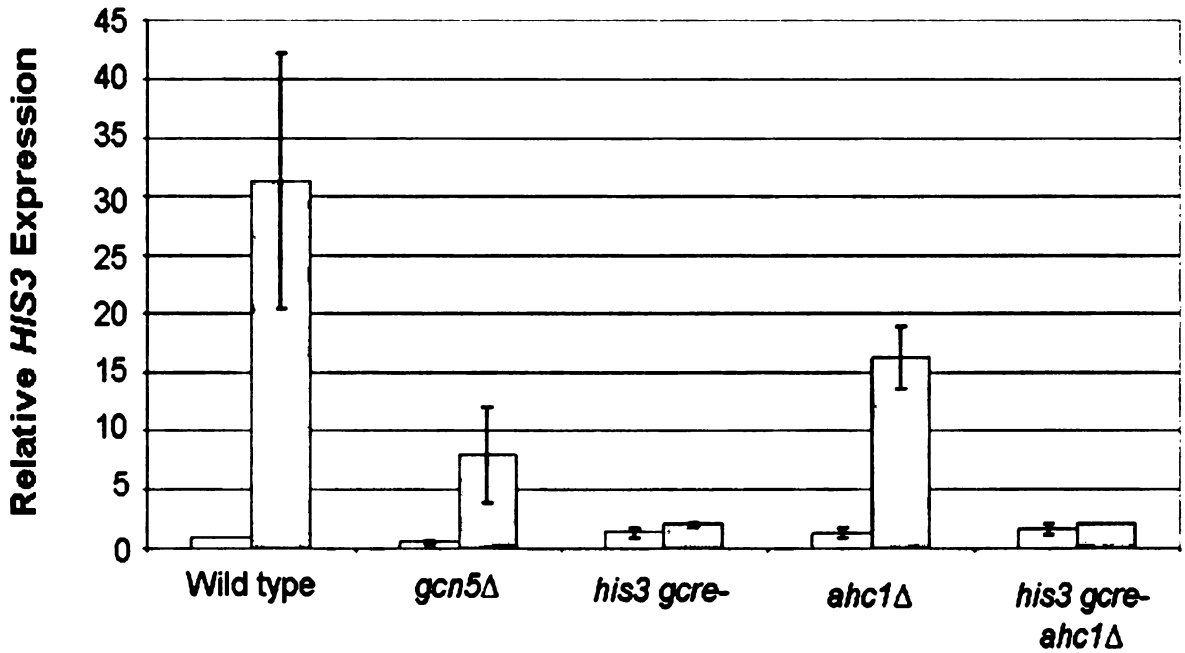
I also determined the effect of *AHC1* deletion on activated *HIS3* expression. Surprisingly, loss of Ahc1 caused defective *HIS3* expression during the activated state and the effect was approximately a two-fold decrease relative to the wild-type strain (Figure 8A). This defect is less severe than the deletion of *GCN5* or of Gcn4 binding site elimination (Figure 8A). In addition, defective *HIS3* expression (activated) was suppressed by heterologous expression of wild-type *AHC1* from a plasmid in the *ahc1Δ* strain (Figure 8B). These results showed that Ahc1 is important for activated *HIS3* expression, but it is not required for the basal expression.

***ahc1Δ* growth phenotypes.** The lack of effect on basal *HIS3* expression and the two-fold decrease in activated *HIS3* mRNA was a surprising result. To determine if the defective activated *HIS3* expression in the *AHC1* deletion strain results in growth defects related to this aberrant expression, I performed a series

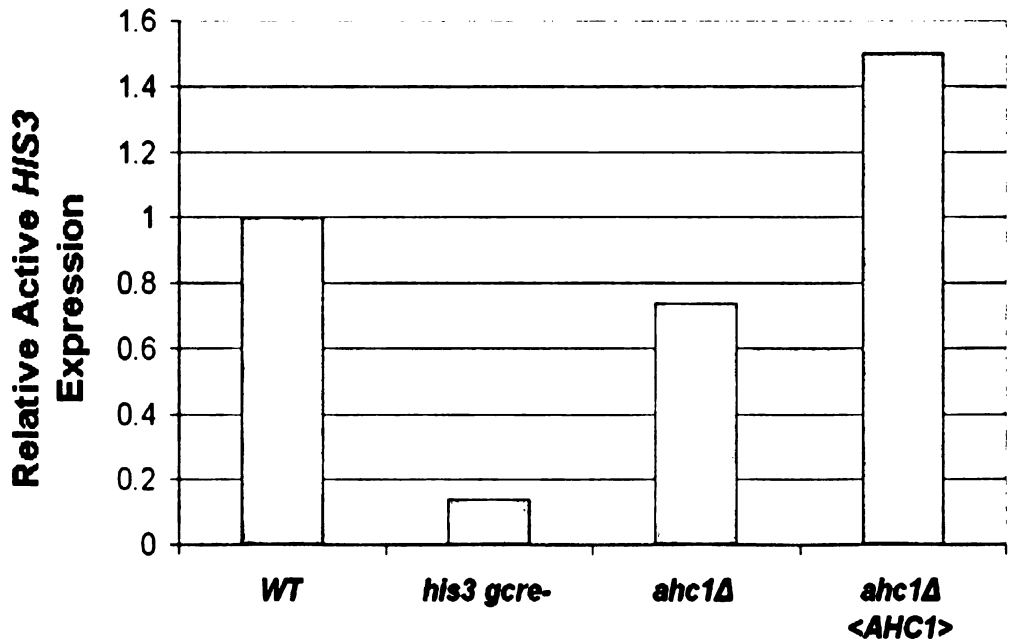
**Figure 8. Loss of Ahc1 reduces activated *HIS3* expression.** Each strain was grown for several hours to early log phase in rich medium prior to shifting to fresh YPD for basal *HIS3* expression and to minimal media containing 3-AT for activated expression. RNA was then harvested and *HIS3* mRNA was determined for each real-time RT-PCR. The relative *HIS3* expression was determined by the $2^{-\Delta\Delta Ct}$ method (29). (A) The level of *HIS3* expression of each strain was normalized to the basal *HIS3* expression of the wild-type strain, YMK839. White and gray bars represent basal and activated *HIS3* expression, respectively. The error bars represent the standard deviation of the averages of multiple PCRs from three independent experiments. (B) Activated *HIS3* expression relative to that of the wild-type strain. This result represents the average of two PCRs from a single experiment.

# Figure 8

A.


Relative HIS3 Expression
45
40
35
30
25
20
15
10
5
0
Wild type
gcn5Δ
his3 gcre-
ahc1Δ
his3 gcre-
ahc1Δ

B.


Relative Active HIS3 Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
his3 gcre-
ahc1Δ
ahc1Δ
<AHC1>

of growth tests. Since *AHC1* deletion caused reduced *HIS3* activated expression, this strain should have increased sensitivity to the chemical 3-AT, an inhibitor of he *HIS3* gene product (31). Strains that are defective in their ability to activate *HIS3* are hypersensitive to 3-AT, relative to wild-type. The results of these growth tests showed that the *ahc1*Δ strain, YMK 967, has surprisingly severe sensitivity to 3-AT (Figure 9A). Curiously, the loss of Ahc1 also causes a strong *his*$^-$ phenotype (Figure 9A). This was unexpected as other mutations, such as deletion of *GCN5*, caused greater reductions in both *HIS3* basal and activated expression, but grew well in the absence of histidine (Figures 8A, B and 9A). Another surprise is that the *his*$^-$ phenotype in the *ahc1*Δ strain is absent when the *AHC1* deletion is combined with the removal of the *HIS3* GCRE (YMK968, Figure 9A). These phenotypes are a result of the loss of Ahc1 function as the defects in the *ahc1*Δ strain are rescued by transformation with, pMK325 a plasmid expressing the wild-type *AHC1* gene (Figure 9B).

**Chromatin immunoprecipitation detection of Ahc1 at *HIS3*.** Taken together, the above results showed a role for Ahc1 and potentially the ADA complex in regulation of *HIS3* expression. Previous chromatin IP experiments pointed to this role as well, but these observations could not be repeated (M.H.K., unpublished result). These experiments showed that Ahc1 is present at the *HIS3* promoter only during basal expression of *HIS3*, or when the Gcn4 activator is absent from the promoter (M.H.K., unpublished results). Because those results could not be repeated, a second strategy was employed.

Fig

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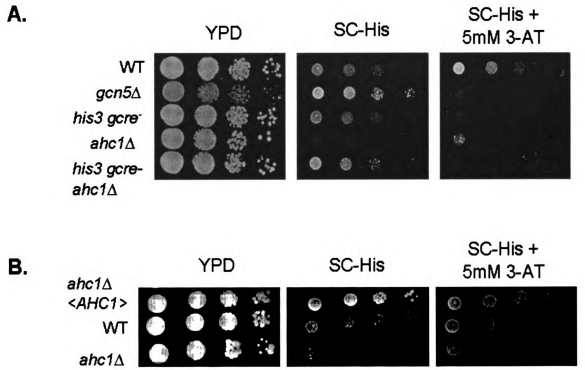
**Figure 9. The *AHC1* deletion causes histidine auxotrophy and 3-AT sensitivity.** (A) Each strain was grown in rich media until early log phase. Cell density was then determined and serial dilutions were spotted onto each plate. Plates were photographed after incubation for 2 days for YPD (30°C) or 4 days at 30°C or 37°C, for SC-His and SC-His + 3-AT plates, respectively. All strains were tested two or more times. (B) Growth tests were performed exactly as in (A). <*AHC1*> represents the wild-type *AHC1* gene in the pMK325 plasmid (see Table 8).

# Figure 9


A.
YPD
SC-His
SC-His +
5mM 3-AT
WT
gcn5Δ
his3 gcre-
ahc1Δ
his3 gcre-
ahc1Δ
B.
YPD
SC-His
SC-His +
5mM 3-AT
ahc1Δ
<AHC1>
WT
ahc1Δ

These experiments used an over-expressed *AHC1* gene which was C-terminally tagged with the Hemagglutinin (HA) epitope (pMK325). Although this construct rescued the defects caused by the loss of Ahc1 (Figures 8A and 9B), the chromatin IP experiments were again unsuccessful (M.H.K, unpublished). Therefore, to show a direct role of Ahc1 in the regulation of *HIS3*, the chromatin IP must be verified reproducibly.

**Creation and analysis of N-terminally tagged Ahc1.** The location of the HA-tag at the C-terminus of Ahc1 in the pMK325 construct renders it inaccessible to the antibodies used for immunoprecipitation. To test this hypothesis, I constructed the pDA8 plasmid, which expresses an N-terminally, HA-tagged Ahc1 (see Table 2 and Materials and Methods). The expression of Ahc1 from this construct was confirmed by Western blot experiments (not shown). Although the HA-Ahc1 fusion is expressed, I was unable to detect its enrichment at the *HIS3* promoter, during the basal or inducing conditions, relative to *ACT1* (Figure 10B). Because of this result, the HA-Ahc1 expression construct was tested for functionality by determining if it suppressed growth defects of the *ahc1*Δ strain. As depicted in Figure 10C, the pDA8 plasmid did not suppress the *his*$^-$ or 3-AT sensitive phenotypes in YMK967. This data argues that the HA-Ahc1 fusion protein in pDA8 is functionally inactive and could be the reason it was not detectable at *HIS3* by chromatin IP.

Figu

(A)

GC

bot

chr

the

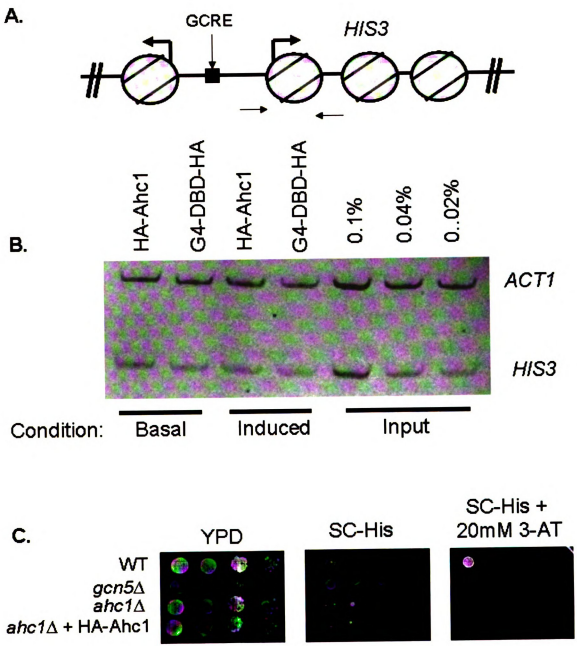
ant

G4

pla

**Figure 10. Chromatin IP and functional testing of the HA-Ahc1 construct.** (A) Cartoon depiction of the chromatin structure of the *HIS3* promoter. The GCRE represents the Gcn4 binding site and the two horizontal arrows on the bottom of the figure represent the oligonucleotides used for *HIS3* detection in chromatin IP experiments. (B) HA-Ahc1 chromatin IP results. The figure shows the semi-quantitative PCR results of chromatin IP experiments using an anti-HA antibody. PCR of the *ACT1* open reading frame was used as the internal control. G4-DBD-HA represents the pDG64 construct (see Table 8) (C) The HA-Ahc1 plasmid, pDA8, does not suppress the growth defects of YMK967.

# Figure 10


A.
GCRE
HIS3
B.
HA-Ahc1
G4-DBD-HA
HA-Ahc1
G4-DBD-HA
0.1%
0.04%
0..02%
ACT1
HIS3
Condition:
Basal
Induced
Input
C.
YPD
SC-His
SC-His +
20mM 3-AT
WT
gcn5Δ
ahc1Δ
ahc1Δ + HA-Ahc1

**Chromatin IP with TAP-tagged Ahc1.** Due to the failure of the above attempts to produce definitive evidence of the presence of Ahc1 at the *HIS3* promoter, a new strategy was developed. I constructed a C-terminally TAP-tagged version of Ahc1 (see Materials and Methods), which is expressed from its endogenous chromosomal locus. The addition of TAP does not appear to disrupt Ahc1 function, as this strain is still *HIS*$^{+}$ and 3-AT resistant, similar to the wild-type strain (Figure 11A). Additionally, Ahc1-TAP expression was verified by IP-Western (not shown). Chromatin IP experiments showed that Ahc1-TAP was not detected at the *HIS3* promoter (see Figure 11B). Because it is unknown if Ahc1 or the ADA complex directly binds to any gene promoters, there is no positive control for PCR for the Ahc1-TAP samples. Therefore, it is possible that Ahc1 does bind directly to the *HIS3* promoter and that this TAP construct is unsuitable for ChIP. Moreover, to ensure *HIS3* DNA immunoprecipitated by the Gcn5-TAP fusion is due to Gcn5 and not non-specific enrichment by over-expression of the TAP tag, the empty TAP vector should be used as a future control.

**Ahc1 and H3 acetylation of the *HIS3* promoter.** Ahc1 is required for activated *HIS3* expression, either directly or indirectly. It is also a critical member of the ADA histone acetyltransferase complex. This evidence suggests that Ahc1 could control expression of the *HIS3* gene through acetylation of its promoter. To test this hypothesis, I determined the H3 acetylation levels of the *HIS3* promoter in the *AHC1* deletion strain by chromatin IP (see Figure 12 and Materials and

**Figure 11. Analysis of TAP-tagged Ahc1.** (A) Growth tests of the yeast strain with an integrated *AHC1-TAP* fusion (DA137). (B) Chromatin immunoprecipitation detection of Ahc1-TAP at the *HIS3* promoter. IgG sepharose beads were used for immunoprecipitation of the TAP-tagged constructs. PCR of the *ACT1* open reading frame was used as the internal control.

# Figure 11

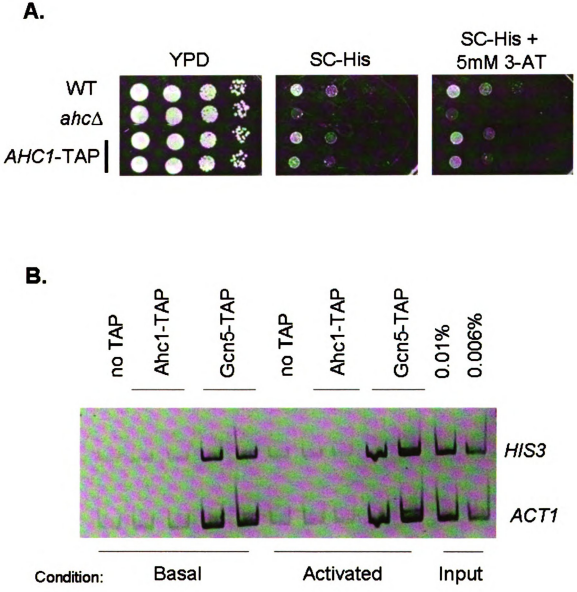
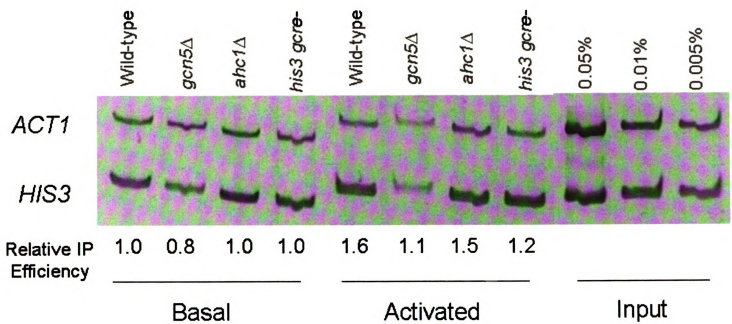

A.
YPD
SC-His
SC-His +
5mM 3-AT
WT
ahcΔ
AHC1-TAP
B.
no TAP
Ahc1-TAP
Gcn5-TAP
no TAP
Ahc1-TAP
Gcn5-TAP
0.01%
0.006%
HIS3
ACT1
Condition:
Basal
Activated
Input

**Figure 12. *AHC1* deletion has no effect on H3 acetylation at the *HIS3* promoter.** Chromatin IP experiments were performed using antibodies against acetylated H3 K9/K14 antibodies. Shown is a representative semi-quantitative PCR result for the *HIS3* promoter relative to the *ACT1* open reading frame. Relative IP efficiency (IP sample divided by input sample) was determined by dividing each sample by the IP efficiency of that in the wild type sample during basal conditions for *HIS3*. Relative IP efficiency was calculated from two independent experiments (see Materials and Methods).

## Figure 12


Wild-type
gcn5Δ
ahc1Δ
his3 gcre-
Wild-type
gcn5Δ
ahc1Δ
his3 gcre-
0.05%
0.01%
0.005%
ACT1
HIS3
Relative IP Efficiency
1.0 0.8 1.0 1.0 1.6 1.1 1.5 1.2
Basal
Activated
Input

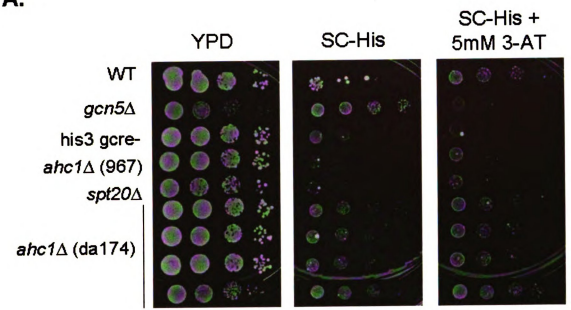
Methods). Contrary to *GCN5* deletion, the deletion of *AHC1* did not affect the H3 acetylation status at the *HIS3* promoter during basal or activating situations. This data suggests that the regulation of *HIS3* expression by Ahc1 is indirect or it functions by a mechanism that is independent of histone H3 acetylation.

**Creation and testing of a new *ahc1Δ* strain.** Although *AHC1* disruption causes reduced activated *HIS3* expression, this defect is not as severe as deletion of *GCN5* (Figure 8A). On the contrary, the growth defects related to *HIS3* expression (*his*$^-$ and strong 3-AT sensitivity) resulting from the absence of Ahc1 are much more severe than those caused by *GCN5* deletion (Figure 9A). In addition, the *his*$^-$ phenotype of YMK967 (*ahc1Δ*) is absent when *AHC1* was deleted in the *his3 gcre-* background (YMK968, Figure 9A). Because of the contradictory growth phenotypes and gene expression, I recreated the *AHC1* deletion strain and named it DA174 (see Materials and Methods). First, the spot assay growth tests were repeated with the newly prepared *ahc1Δ* strains. As shown in Figure 13 panel A, the recreated *AHC1* knockout strain isolates are neither his$^-$ nor 3-AT sensitive. This suggests that YMK967 could bear additional mutations, which alone or in combination with the *AHC1* deletion resulted in the growth defects described. In light of this result, I then tested the *HIS3* expression of the newly created *AHC1* deletion strain. In the first experiment, I found that four DA174 clones showed no change in basal or activated *HIS3* expression. A repeated experiment then showed that as before with YMK967, *HIS3* activated expression was reduced. The average expression from these two experiments is

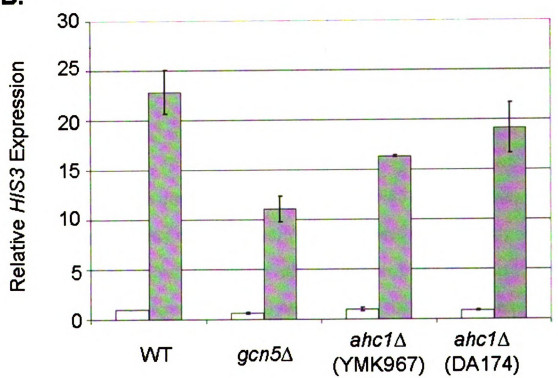
**Figure 13. Characterization of the recreated *AHC1* deletion strain, DA174.** (A) The new *AHC1* knockout strain is not *his*$^-$ or sensitive to 3-AT. The four separate DA174 strains shown were all verified by genomic PCR to have a complete *AHC1* disruption. (B) Real-time RT PCR quantitation of *HIS3* expression. Results depict the averages from two independent experiments with standard deviation. Dark bars are basal *HIS3* expression and light bars are activated *HIS3* expression. All samples were normalized to the basal *HIS3* expression in the wild-type sample.

# Figure 13

**A.**


YPD
SC-His
SC-His +
5mM 3-AT
WT
gcn5Δ
his3 gcre-
ahc1Δ (967)
spt20Δ
ahc1Δ (da174)

**B.**


Relative HIS3 Expression
30
25
20
15
10
5
0
WT
gcn5Δ
ahc1Δ (YMK967)
ahc1Δ (DA174)

shown in Figure 13 panel B. At the time of preparing this thesis, these experiments have not been repeated to accurately determine the role of Ahc1 in *HIS3* activated expression.

# Discussion

Contrary to the initial hypothesis, I found that Ahc1 is dispensable for *HIS3* basal expression, but could be necessary for full expression under activating conditions. To determine if Ahc1 acts at the *HIS3* promoter to directly regulate its expression, I performed multiple chromatin IP experiments. Based on these studies, I was unable to detect Ahc1 at the *HIS3* promoter; suggesting that Ahc1 might control *HIS3* expression indirectly. Additional chromatin IP experiments showed that loss of Ahc1 did not affect the histone H3 acetylation status of the *HIS3* promoter.

The majority of this work describing the role of Ahc1 in *HIS3* expression was conducted using the *AHC1* deletion strain, YMK967. These studies clearly showed the Ahc1 is involved in activated *HIS3* expression, by an unknown mechanism (Figure 8). Recreation of the *AHC1* deletion (DA174) was then carried out to solve intriguing phenotypes found in YMK967. These results showed that DA174 did not share the growth defects of the original *AHC1* deletion strain (Figure 13A). It seems that 3-AT sensitivity and histidine

auxotrophy in YMK967 could be the result of an additional mutation present in this strain. Because the wild type *AHC1* gene suppressed the phenotypes in YMK967 (Figure 9), it is a possibility that the presence of an additional mutation causes cells to require *AHC1* for normal growth and/or normal activated *HIS3* expression.

Because of the differences in growth phenotypes of the two *AHC1* deletion strains, I also tested the *HIS3* expression in DA174 (Figure 13B). Two separate experiments were performed comparing the *HIS3* expression in YMK967 and DA174. The initial result showed that the new strain has no defect in *HIS3* basal or activated expression, while the second experiment showed that DA174 is defective for activated *HIS3* expression. At the time of preparation of this manuscript additional experiments to accurately report the role of Ahc1 in *HIS3* gene expression had not been performed. Therefore, I cannot conclude that Ahc1 alone is responsible for the loss of *HIS3* expression under the activating condition.

Contrary to the discrepancy concerning Ahc1 and activated *HIS3* expression, all of the expression experiments indicated Ahc1 did not have an effect of the basal *HIS3* expression as originally proposed (Figures 8A and 13B). Because of this, it seems Gcn5 functions to control *HIS3* basal expression and acetylation in the context of another HAT complex or through an indirect mechanism. Spt7 and Spt20 are two SAGA components known to be essential for complex integrity

(reference). Moreover, *spt7Δ* and *spt20Δ* strains have normal basal *HIS3* expression. This argues that basal *HIS3* expression is not regulated by Gcn5 in the context of SAGA. On the other hand, deletion of the Spt3 and Spt8 TBP-interacting components of SAGA cause derepression of *HIS3* under non-inducing conditions. This data appear contradictory as to the role of Gcn5 and the SAGA complex in regulation of basal *HIS3* expression. Therefore, additional experiments should be performed to determine how Gcn5 controls the acetylation and expression of the *HIS3* gene during the uninduced state.

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