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INVESTIGATING FUNCTIONS OF GCN5 AND SNF1 IN *HIS3* ACTIVATION

By

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ABSTRACT

INVESTIGATING FUNCTIONS OF GCN5 AND SNF1 IN *HIS3* ACTIVATION

By

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Gcn5p serves as the catalytic subunit of the SAGA histone acetyltransferase (HAT) complex and is critical for *HIS3* activation in *Saccharomyces cerevisiae.* To identify molecular functions that act downstream of or in parallel with Gcn5 protein, an EMS and a minitransposon derived mutagensis libraries were screened for suppressors that rescue the transcriptional defects of *HIS3* caused by a catalytically inactive mutant Gcn5p, the E173H mutant. A truncated Reg1 protein, Reg1(1-740), was found to be a dominant suppressor. The function of Reg1(1-740) protein requires an intact *cis*-acting element for the Gcn4 transcriptional activator, and doesn't change the canonical HAT activity of Gcn5p. Reg1 protein functionally and physically interacts with a histone H3 kinase, Snf1p. Indeed, Snf1p plays an important role for normal and Reg1(1-740)-dependent *HIS3* activation. However, substituting the phosphorylation residue in H3, i.e. Ser10, with alanine or glutamate neither attenuates nor augments the suppression phenotypes. These results argue against an essential role of H3 phosphorylation in *HIS3* expression. Both Reg1(1-740) and overexpressed Snf1p rescue the E173H allele of *gcn5* selectively, suggesting physical interactions between Gcn5p and these two proteins. *In vivo* co-purification experiments confirmed this notion. Moreover, Gcn5p is a substrate of Snf1 kinase *in vitro* and the phosphorylation of Gcn5p *in vivo* is dependent on the Snf1p. A quadruple mutation of Gcn5 (T203A/S204A/T211A/Y212A, TSTY/4A) that diminished

the *in vitro* phosphorylation also impairs *HIS3* transcription *in vivo*. Interstingly, this mutant, as well as the *snf1Δ* mutation, is suppressed by deleting *SPT3* or *SPT8*, suggesting that Snf1p and TSTY residues of Gcn5p function antagonized the inhibitory effects of Spt3p and Spt8p. Furthermore, Spt3 protein interacts with Gcn5 protein *in vitro*, which raises the possibility that Spt3p inhibits Gcn5p function by direct interaction and such inhibition is relieved by the Snf1p-mediated phosphorylation of Gcn5p or/and Spt3p. Our study might unravel an uncharacterized regulatory network intrinsic to the SAGA coactivator complex, of which the composition and molecular functions are well conserved in human cells.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER II: HISTONE H3 SER10 PHOSPHORYLATION-INDEPENDENT FUNCTION OF SNF1 AND REG1 PROTEINS RESCUES A *GCN5*⁻ MUTANT IN *HIS3* EXPRESSION

CHAPTER III: SNF1P ACTIVATES *HIS3* TRANSCRIPTION BY ANTAGONIZING THE INHIBITORY EFFECTS OF SPT3P AND SPT8P

CHAPTER I

Literature Review

Literature review

Part I: Post-translational modification of proteins:

The phenotypes and the behavior of an organism are not only decided by the genetic information it contains, but are also largely affected by the environment. The post-translational modification (PTM) of the gene products – proteins, is a strategy of the cell to adapt to the uncertain and frequently changing environment. There is a variety of protein PTMs in nature. Aside from protein degradation and restricted protease digestion, most easily observed modifications are adding a molecule covalently. These molecules can be very small, such as acetylation (lysine), phosphorylation (serine, threonine, tyrosine and histidine), methylation (lysine and arginine), nitrosylation (cysteine) and glycosylation (serine) etc., or relatively large, such as myristoylation (amino terminus), ubiquitylation (lysine) and sumoylation (lysine). These modifications might change the charge or the conformation of the protein, or in other cases, the attached group provides the better surface for the binding partners. In result, the activity or the cellular localization of the protein is altered. Post-translational modifications are involved in almost all cellular processes such as cell sorting, signal transduction, gene transcription, cell cycle regulation and DNA damage repair. The PTMs can be found almost everywhere in the cell, from the outer surface, to the very inside of the nucleus.

Cell surface: Glycosylation and environmental sensing

The first place to encounter extracellular environment change is the cell surface. These environmental factors may include small molecules like hormones and toxins or much larger particles like viruses, bacteria or other cells. Receptors located on the cell surface are sensors for those extracellular factors. However, the receptor per se is not sufficient for recognition. Carbohydrate groups, implanted by multiple glycosylation enzymes, are required for the function of receptors in most cases, and probably are the primary elements that interact with the outside signals (110). We can imagine that in the absence of or incorrect glycosylation will cause problems for cells in their toxin defense, cell-cell communication, and differentiation. Indeed, abnormal glycosylation patterns are known to be markers for, and in some cases the cause of, certain disease states including cancer (30). For exemple, in colonic cancer cells the mucin O-glycan chains, which are responsible for interactions between cancer cells and their microenvironment, are enriched in certain forms instead of a large range of structures (13). Moreover, different cancer cells display unique glycan epitopes, which could serve as targets for cancer diagnosis and treatment (19).

Cytoplasm: phosphorylation and signal transduction

Protein phosphorylation is probably the most common post-translational modification, as the protein kinases are the third largest protein family in the human genome and represent 2% of proteins (79). The roles of protein phosphorylation in signal transduction are evolutionarily conserved among eukaryotes and have been extensively studied over the

years. Adding or removing the phosphate group can serve as an ON/OFF switch in responding to chemical or physical stress. Good examples are the MAP kinase pathways, in which the phosphorylation of the target protein causes increased kinase activity, or elimination of phosphatase activity to deliver the signal to downstream targets (27). Ser/Thr phosphorylation is thought to play intramolecular roles, such as promoting the conformational change or taking part in the catalysis. On the other hand, tyrosine phosphorylation is more likely involved in intermolecular interactions. Accurate phosphorylation regulation of the MAPK cascades is important for cells keep normal functions, including gene expression, cell proliferation, cell survival and death, and cell motility (20). The MAPK pathway is activated in virtually all melanomas (95). The oncogenic activation of tyrosine kinases is a common feature in cancer (93).

Nucleus:

The eukaryotic nucleus is the major place that DNA replication, gene transcription and DNA damage repair happens. More and more proteins involved in those cellular processes have been shown to require post-translational modifications for proper function. Some examples related to gene activation will illustrate the importance of PTMs.

PTMs of activators: p53

The tumor suppressor protein p53 is the target of various post-translational modifications, which modulate p53 functions at different levels. There are at least 17 phosphorylation sites found in human p53 (12). Most of the known phosphorylation sites are located in the

N-terminus of the protein (Ser6, 9, 15, 20, 33, 37 and 46, and Thr18, and 81), within or close to the transactivation domain (12). Multiple kinases show redundancy and each kinase may target several sites. Serine 15 phosphorylation of p53 can be achieved by the function of ATM/ATR (105, 130), DNAPK (6), ERKs (114), and p38 kinase(71). Any of the above reactions will direct the cell to undergo apoptosis. Additionally, p38 kinase is able to mediate the phosphorylation of Ser33, Ser46, and Ser392. This phosphorylation causes either p53 stabilization or increased DNA-binding ability (12).

As a multifunctional protein, phosphorylation of p53, although quite complicated, is insufficient to regulate the complexity of the processes it controls. Some other PTMs, such as acetylation (146), ribosylation (140), ubiquitylation (14) and sumoylation (21) are also important features for accurate behavior of p53. Phosphorylation and acetylation generally result in activation of p53 (12). Sumoylation at lysine 386 was reported to repress gene transcription (83). These modifications are highly interactive. The ubiquitylation, which is the first step for p53 turnover, requires the deacetylation of p53 (59). Also, phosphorylation of several serines by CK1 requires that Ser15 is phosphorylated (106). The dysfunctional p53 proteins derived from incorrect modifications are always associated with different kinds of cancers.

Besides p53, many other transcriptional activators/repressors and coactivator/corepressors are targets of post-translational modifications. In humans, p300-mediated acetylation of c-myc results in turn-over of c-myc (38). Phosphorylation of p300 by Akt kinase will increase its histone acetyltransferase activity and facilitates its

gene activation function (56). In yeast, AMPK/Snf1 will remove the negative effect of Mig1 repressor from gene promoters by phosphorylation under nutrient limiting conditions (96).

Part II: Histone modifications

Eukaryotic DNA is packed into chromatin by wrapping around the histone octamers. Such a highly compact structure forms a barrier for the DNA or chromosome related regulatory processes. Four approaches have been found to temporarily or permanently alter the organization of chromatin structure to overcome such obstacle.

1. Histone variants. Such as H3.3 and H2A.Z, which are incorporated in a DNA synthesis independent manner (2, 47, 61, 144).
2. ATP-dependent chromatin remodeling (121).
3. DNA methylation (111).
4. Covalent histone modifications (see below)

The regulatory machineries ubiquitously utilize post-translational modifications to either alter the charge of the histones or to provide extra binding sites for regulators. It is hard to imagine how diverse and complicated the modification schemes are if a regulator needs to distinguish its target locus from millions of similar nucleosomes in the genome. Histone modification enzymes dynamically mark the nucleosomes with phosphorylation, methylation, acetylation, biotinylation and ubiquitylation etc., at different residues and in different combinations (29, 64, 99, 107). The histone modifications are also cross-regulated to modulate the chromatin functions more precisely. For example, histone H3 S10 phosphorylation facilitates K9/K14 acetylation at the *INO1* promoter (76). The K4 methylation and K9/K14 acetylation of histone H3 correlate very well through different

developmental stages at chicken β-globin locus (75). Rad6-mediated ubiquitination of K123 of histone H2B is a prerequisite for H3 K4 and K79 methylations and is important for telomeric silencing in *S. cerevisiae* (91, 127). A "histone code" hypothesis has been proposed, in which individual modifications or combination of modifications may extend the information potential of the genetic (DNA) code by recruiting different regulatory factors to the specifically modified chromatin loci (125)

To realize a "histone code", two classes of factors are required. The first class includes enzymes that generate or remove post-translational modifications. The second class includes proteins capable of interacting with histones bearing specific modifications.

The extensively studies on histone post-translational modifications vote "YES" to the above question. Here I will summarize the two classes of factors identified using the examples of histone methylation and phosphorylation. Histone acetylation will be discussed in the next part.

Histone methylation

Histone methylation occurs on the side-chain nitrogen atoms of lysine or arginine residues. Neither lysine nor arginine methylation alters the charge status of the residues. Such chemical natures support the assumption that the modification is a "mark" for recruiting regulatory factors but not affects the nucleosomal structure. Lysine can exist in mono-, di- or trimethylated forms, while arginine is able to accept up to two methyl

groups. In fact, different modification stages are reflected in different physiological functions (35, 80).

Histone arginine methylation can be conducted by several protein arginine methyltransferase (PRMTs). Among them, PRMT1 and CARM1 catalyze asymmetric dimethylation of H4 R3 to facilite transcriptional activation (126, 138). PRMT5, a human SWI/SNF chromatin remodeler associated protein, functions as a repressor by symmetrically methylating R8 of histone H3 (94). On the other hand, methyl groups can be removed from arginine residues of histones by PADI4-mediated deimination reaction to create a citrulline residue (28, 139). So far, no methylated arginine specific binding motif has been found. However, PRMT1 mediated H4 R3 methylation is essential and sufficient for histone acetylation at chicken β-globin region (55), which suggests a modification specific recognition mechanism involved.

Lysine methylations of histones are mainly detected in H3 and H4 (107). Several histone methyltransferases (HMTs) with the signature SET domain were identified in past 6 years. The first characterized HMT is Suv39h/Clr4 that conducts H3 K9 methylation (8, 67). Later, more H3 K9 HMTs were found, including G9a, ESET and EZH2 (31, 66, 129, 137, 143). Interestingly, G9a and ESET are di-methylases, whereas Suv39h is a tri-methylase. Consistent with their activity, Suv39h resides in heterochromatin, while G9a is largely localized to euchromatic regions and acts as either a transcriptional corepressor or coactivator (68, 92), implies the number of methyl-groups is important for the information read out. For instance, H3 K9 methylation is always linked to silenced

chromatin. The specific recognition of lysine 9 methylated histone H3 (mainly tri-methylated) by the chromodomain of HP1 protein is a key step in heterochromatin formation (8, 67). Even mono- or di-methylated forms of K9 are usually associated with repressor complexes or inactivated chromatin (84, 98).

Opposite to K9, lysine 4 methylation of H3 usually correlates with activated chromatin, especially the tri-methylated form (41, 108). The H3 K4 HMTs also contain the SET domains. The activity and substrate specificity of K4 HMTs are largely dependent on the protein complexes they are assembled into (107). Set1 of the budding yeast creates permissive chromatin by adding the di-methyl mark (99). MLL1 HMT is associated with coactivator complexes and catalyzes the tri-methylation of K4 to aid gene transcription (32, 141). Chromodomain proteins can also read the methylated K4 code, as Chd1 from yeast and human is able to target K4 methylated H3 (43, 100, 120). WDR5, which contains WD40 repeats, is also shown to bind methylated K4 (141). However, these proteins can not distinguish tri-memethylation from di-methylated forms. Recently, several groups reported that the PHD finger of NURF and ING proteins specifically recognize trimethylated H3 lysine 4 (73, 97, 115, 142).

Methylation of many other lysines in H3 and H4 N-terminal domains or core domains is also important for physiological functions. Set2 mediated H3 K36 methylation is correlated with transcriptional elongation (87, 136). H3 K79 methylation, catalyzed by non-SET HMT Dot1, is a mark to set up chromatin domains (39, 85, 90, 132, 145). Set8

catalyzed methylation of lysine 20 of H4 is involved in chromatin condensation in mitotic regulation (26, 102).

In addition to methylation, lysine residues are the target for several other modifications, like acetylation, sumoylation, ubiquitylation and biotinylation, etc. (64, 103, 107). Indeed, these modifications may compete for the same residues, like lys9 of H3 is found in multiple modification forms (64, 103, 107). It raise the importance of reversal of the methylation. A few enzymes that remove methyl-group have been identified in last two years. LSD1 is an FAD-dependent amine oxidase and capable of using methylated K4 as substrate (116, 117). The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36 (26, 63).

Histone phosphorylation

Phosphorylation is another important feature of histones in chromatin. So far, only serines or threonines in histones are known to be phosphorylated. The phosphorylation sites are found in all of the 4 core histones (S1, T119, and S129 of H2A; S10, S14 and S33 of H2B; T3, S10, T11, S28 and S31 of H3; S1 of H4). Consistent with histone code hypothesis, phosphorylation of different residues results in different cellular regulations. For example, H4 S1 and H3 S10/S28 phosphorylations play important roles in mitosis (9, 22, 54). S14 of H2B is rapidly phosphorylated at sites of DNA double-strand breaks (40). Sterile 20 kinase mediated H2B Ser10 phosphorylation is induced during apoptosis and meiosis in *S. cerevisiae* (3, 4). H3 S10 phosphorylation is correlated with transcriptional

activation (1, 76). Many histone kinases have been identified to write the phosphorylation code. Apart from the Sterile 20 kinase for H2B S10 phosphorylation mentioned above, NHK-1 phosphorylates T119 of H2A (5); Ipl1/Aurora-B kinase is responsible for H3 S10/28 phosphorylation (54); S10 of H3 can be also phosphorylated by Snf1 (76), Rsk2 (109), and MSK1 (69). On the other hand, not many code erasers (phosphatases) have been reported, except the PP1/Glc7 that mediates dephosphorylation of S10 to counteract the Ipl1 kinase (54).

Little is known about the proteins or motifs that specifically interact with phosphorylated histones. *In vitro* studies using phosphorylated peptide indicates the 14-3-3 module is capable of binding phosphorylated histone H3 (78). An independent study using tethered-catalysis yeast two-hybrid system (46) also detected the yeast 14-3-3 proteins Bmh1 and Bmh2 in the screening for phosphorylated H3 associating protein (Guo, et al, unpublished), which support the hypothesis that 14-3-3 proteins are phosphorylation code readers.

Part III: SAGA and histone acetyltransferase

Histone acetylation is one of the best studied post-translational modifications. In the budding yeast *Saccharomyces cerevisiae*, the amino-termini of all 4 core histones possess multiple conserved lysine residues that are acetylated *in vivo*, like K9, K14, K18, and K23 in H3; K5, K8, K12, and K16 in H4; K4 and K7 in H2A; K11 and K16 in H2B (99, 103). The covalent linkage at an acetylgroup with the ε-amino group of lysine residue not only alters the structure of the amino acid, but also neutralizes the positive charge. Based on sequence homology and architecture of the catalytic domain, histone acetyltransferases (HAT) can be divided into five superfamilies, Gcn5-related acetyltransferase (GNAT), MOZ-Ybf2/Sas3-Sas2-Tip60 (MYST) related HATs, p300/CBP HATs, the general transcriptional HATs, and the nuclear hormone-related HATs (17).

Most HATs existed in multisubunit protein complexes and that regulate gene transcription and other cellular processes. The MYST family member Esa1 is the HAT component of yeast NuA4 complex that plays roles in gene silencing, cell cycle progression and DNA repair of the double-strand break (7, 23, 24). The largest TFIID components, the Taf1 protein, mediates histone acetylation in higher eukaryotic organisms to facilite transcriptional initiation (34, 52, 86). In contrast, the Elp3 acetyltransferase of the elongator complex modulates the transcriptional elongation process (50).

The Gcn5 histone acetyltransferase of *Saccharomyces cerevisiae* is the catalytic subunit of multiple coactivator complexes, e.g. ADA, SAGA, and SALSA (44, 122). The ADA complex contains Ada2, Ada3/Ngg1, Gcn5 and Ahc1 (36). This complex might regulate the basal expression of *HIS3* gene (Kuo and Almy, unpublished). The SAGA and SALSA complexes are very similar except the Spt7 subunit is truncated and the Spt8 subunit is missing in SALSA complex.

The yeast SAGA complex is a 1.8MDa protein complex that consists of at least 19 components. Based on the order of discovery, these components are divided into several groups. The first group is Suppressor of Ty (Spt) proteins, includes Spt3, Spt7, Spt8, and Spt20 (44). A subset of the TATA-binding protein (TBP) associating factors (TAFs, Taf5, Taf6, Taf9, Taf10, and Taf12), initially identified as components of the TFIID general transcription factor, composes the second group of proteins in SAGA (44, 45). Transcriptional adapters are another group of proteins, including Ada1/Hfi1, Ada2, Ada3/Ngg1, and Ada4/Gcn5 (11). Among them, Ada2, Ada3 and Gcn5 form a core module for HAT activity (123).

Other than acting as a HAT that acetylates chromatin to stimulate gene transcription, SAGA also possesses other important activities. The Spt3 and Spt8 subunits interact with TBP and either inhibit or activate TBP function in gene transcription (10, 33, 37, 113). Chd1 is a chromodomain-containing protein that recognizes lys4 methylated histone H3, which may aid the nucleosome specific recruitment (100). The Ubp8deubiquitylation activity was also found to copurify with SAGA complex (51, 58, 70). Moreover, the

newly found component of SAGA, Sgf73/Sca7, can be subject to polyglutamine-expansion, which is important for the complex to behave like an acetyltransferase (82). Several subunits play structural roles to maintain the integrity of the complex. Ada1/Hfi1, Spt7 and Spt20 are such backbone proteins (123). The second group of architectural proteins consists of a few essential TAFs. Mutations in *TAF5*, *TAF10*, and *TAF12* also affect SAGA composition and integrity (45). Finally, the largest subunit, Tra1, which is shared between SAGA and NuA4 complexes, is able to interact with the acidic transcription activators. Such activity is thought to be the mechanism for the coactivator recruitment to the gene promoter (7, 15, 42).

About 10% of the *Saccharomyces cerevisiae* genes are controlled by SAGA (57). Most SAGA target genes are related to stress response and are regulated (57). Interestingly, mutations of the Spt3 module and the Gcn5 module affect different classes of genes, implying that the function of SAGA is more than that of a HAT (57). In most cases SAGA plays a positive role in gene transcription. However, SAGA is found to repress the *ARG1* gene when cells were grown in rich media (101).

Part IV: Snf1

Sucrose non-fermenting 1 (Snf1) is the yeast homolog of the AMP-activated protein kinase (AMPK) family that play essential roles in regulation of glucose and lipid metabolism and cellular stress responses (16, 49, 62). The AMPK family is widely conserved through all eukaryotic organisms (49). By sensing the cellular AMP:ATP concentration ratio, AMPK is activated to shut off energy consuming (anabolic) pathways and to stimulate energy generating (catabolic) pathways (16). The mammalian AMPK plays a central role in the regulation of energy balance at the whole body level by responding to hormonal and nutrient signals in the nervous system (62). Mutations in AMPK cause cardiac hypertrophy and arrhythmia (16). The prevalence of obesity and its associated diseases drive the AMPK energy gauge receiving rising attentions. Indeed, AMPK is becoming an important therapeutic target for type 2 diabetes (48).

The functional AMPK complex exists in a heterotrimer that. The α subunit is the catalytic component of the complex. There is only one α subunit in *Saccharomyces cerevisiae*, which is encoded by *SNF1* gene (49). Snf4 is the regulatory γ subunit and is essential for activating the kinase activity (18, 72). Moreover, the complex requires one of the three β subunits, Gal83, Sip1 or Sip2 to tether Snf1 and Snf4 together (60). The three β subunits show great functional redundancy as the *snf1*$^-$ phenotypes can be only achieved with all three subunits deleted (112). Recent studies suggest that the selection of β subunit incorporation may affect the cellular localization of the Snf1 complex (135). Upon glucose limitation, Gal83 directs the Snf1 protein into the nucleus, and Snf1-Sip1

complex relocates around the vacuole, while the Sip2 associated kinase remains cytoplasmtic (135). Other than that, Gal83 has the ability to interact with the Sip4 transcriptional activator (133). Sip2 has been implicated to play a role in aging (74). Interestingly, the recent crystal structure of the Snf1 kinase domain indicates that Snf1 is able to form a homodimer (89, 104).

Snf1 is a 633 amino acid protein with a 330 residue kinase domain at the N-terminus. The C-terminal region contains a regulatory domain that interacts with other subunits of the complex. An auto-inhibition domain is located at residues 367-500. Once cells encounter glucose depletion or other stresses, the T210 residue in the activation loop of Snf1 is phosphorylated by one of the three upstream kinases, Sak1, Elm1 or Tos3, and subsequently a conformational change occurs to activate the kinase activity (53, 81, 88, 128). This activation process is inhibited by the Reg1/Glc7 protein phosphatase, as the T210 is constantly phosphorylated in the *reg1Δ* strain (81). The activated Snf1 will phosphorylate downstream targets with the consensus sequence (*Hyd*-X-Arg-X-X-Ser-X-X-X-*Hyd*), in which the arginine at the P-3 position is crucial (49).

Activation of the Snf1 kinase is a key step in regulating genes related to utilization of alternative carbon sources. However, only a few examples have been established to illustrate what the downstream events are upon the phosphorylation by Snf1. In one case, the phosphorylation of Mig1 disrupts the Mig1-Ssn6 repressor at the promoter and releases the repressor from DNA (131). In other cases, transcription might be stimulated by Snf1-mediated phosphorylation of activator, such as Cat8 and Sip4 (134). Snf1 is also

a histone modification enzyme that regulates transcription at the chromatin level (76). The Snf1 mediated phosphorylation of H3 serine 10 facilities the association of Gcn5 histone acetyltransferase. Such H3 S10 phosphorylation, together with Gcn5 mediated K14 acetylation are important for yeast *INO1* expression (25, 76, 77). Genetic and biochemical studies indicated Snf1 is able to active transcription by other mechanisms, such as interacting with mediator complex or TATA-binding protein directly (65, 118, 119).

Part V: Research interest and significance

The yeast Gcn5 and its histone acetyltransferase activity in gene transcription have been extensively studied. However, the events following Gcn5-mediated histone acetylation are elusive. Moreover, little is know about how histone acetylation facilities transcriptional initiation. Furthermore, Gcn5 is likely to do more things than a HAT, since the catalytic inactive mutant and deletion strains show different expression profile in genomic studies (57, 71). To address the above questions, we sought to isolate extragenic suppressors that allow yeast cells to activate transcription in the absence of HAT activity of Gcn5. Characterizing these suppressors, which represent the factors functionally downstream or in parallel with Gcn5, will help us find out the molecular mechanism of how Gcn5 and its mediated histone acetylation modulates the transcription.

Other than investigating the role of Gcn5 in regulating transcription, we are also curious about how Gcn5 is regulated. So far, the only known mechanism of regulating Gcn5 activity is that the recruitment of Gcn5 containing complex to target promoters by acidic activators. The HAT activity of mammalian histone acetyltransferase p300 is alleviated by Akt-mediated phosphorylation (56), which made us wondering whether Gcn5 is regulated in a similar way. Recent studies showed that Gcn5 is sumoylated in vivo, which raise the likelihood of cellular controlling of Gcn5 activity by post-translational modification, although the biological significance of Gcn5 sumoylation is not clear (124).

In our primary studies, we found that a suppressor, Reg1(1-740), may regulate Gcn5 by modulating Snf1-mediated phosphorylation of Gcn5. Moreover, our studies indicate that Snf1 counteracts the function of Spt3, which is another SAGA component. Such observations suggest that Gcn5 is likely regulated in vivo via intra-complex mechanisms that mediated by Spt3, and by post-translational modifications, such as Snf1-mediated phosphorylation. As a conserved acetyltransferase across species, such regulations of Gcn5 may also exist in other higher eukaryotes. Similarly, given the importance of histone acetyltransferase in the regulation of a wide variety of genes in eukaryotes, the detailed genetics and biochemical studies of the prototype HAT Gcn5 will advance our understanding of the mechanisms of histone acetylation in gene regulation.

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Chapter II

Histone H3 Ser10 Phosphorylation-Independent Function of Snf1 and Reg1 Proteins Rescues a *gcn5*⁻ Mutant in *HIS3* Expression

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Histone H3 Ser10 Phosphorylation-Independent Function of Snf1 and Reg1 Proteins Rescues a *gcn5*⁻ Mutant in *HIS3* Expression

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ABSTRACT:

Gcn5 protein is a prototypical histone acetyltransferase that controls transcription of multiple yeast genes. To identify molecular functions that act downstream of or in parallel with Gcn5 protein, we screened for suppressors that rescue the transcriptional defects of *HIS3* caused by a catalytically inactive mutant Gcn5, the E173H mutant. One bypass of Gcn5 requirement gene (*BGR*) suppressor was mapped to the *REG1* locus that encodes a semidominant mutant truncated after amino acid 740. Reg1(1-740) protein does not rescue the complete knockout of *GCN5*, nor does it suppress other *gcn5*$^-$ defects, including the inability to utilize nonglucose carbon sources. Reg1(1-740) enhances *HIS3* transcription while *HIS3* promoter remains hypoacetylated, indicating that a noncatalytic function of Gcn5 is targeted by this suppressor protein. Reg1 protein is a major regulator of Snf1 kinase that phosphorylates Ser10 of histone H3. However, whereas Snf1 protein is important for *HIS3* expression, replacing Ser10 of H3 with alanine or glutamate neither attenuates nor augments the *BGR* phenotypes. Overproduction of Snf1 protein also preferentially rescues the E173H allele. Biochemically, both Snf1 and Reg1(1-740) proteins copurify with Gcn5 protein. Snf1 can phosphorylated recombinant Gcn5 in vitro. Together, these data suggest that Reg1 and Snf1 proteins function in an H3 phosphorylation-independent pathway that also involves a noncatalytic role played by Gcn5 protein.

INTRODUCTION

Histone acetylation is a well-studied modification of chromatin (67) and has been linked to transcriptional regulation, recombination, DNA replication, and damage repair (13). GNAT (Gcn5 protein-related *N*-acetyltransferases) and MYST (MOZ-Ybf2/Sas3-Sas2-Tip60) families of histone acetyltransferases (HATs) generate both targeted and global acetylation of the chromatin (78). Other HATs, such as TAF1 (formerly $TAF_{II}250$) and nuclear hormone receptor coactivators, though not belonging to either family, have also been shown to play critical chromatin-related functions via their HAT activities (78).

The *Saccharomyces cerevisiae* Gcn5 protein is the catalytic subunit of several chromatographically distinct HAT complexes, including SAGA, ADA (32), SALSA, and SLIK (70, 71, 85). SAGA is recruited to the promoter by certain transcriptional activators and causes promoter-specific nucleosomal hyperacetylation leading to transcriptional activation (4, 5, 48, 51, 72). The SAGA complex also performs HAT-independent functions, such as TATA binding protein (TBP) recruitment and histone deubiquitinylation (8, 9, 19, 24, 38, 44, 55, 75, 86). SAGA and SALSA/SLIK complexes share TBP-associated factors with TFIID (33). Low-resolution electron microscopic studies showed that the architectures of SAGA and TFIID complexes are highly similar (3, 11, 91, 103). TFIID is critical for mostly housekeeping gene expression, and the SAGA-dominated genes (~10% of the nuclear genes) are largely stress-induced and are under the coordinated control of multiple chromatin and transcriptional regulators (43).

Although the promoter-specific histone acetylation function of Gcn5 has been firmly established (48, 51), which molecular activities are modulated by histone acetylation remains an open question. The best-known molecular event triggered directly by histone acetylation is the recruitment of bromodomain-containing proteins (20, 45, 53, 62). Besides this, however, little is known as to what other functions may be triggered or antagonized by histone acetylation. Identification of mutations that suppress defects associated with histone hypoacetylation may reveal factors downstream of histone acetylation. Thus far, the only reported screen for suppressors rescuing *gcn5* null phenotypes was a multicopy suppressor hunt identifying *ARG3* (69), which is likely involved in controlling the global chromatin structure via regulating the balance of nuclear polyamine. On the other hand, Gcn5 protein is important for only a portion of yeast genes (40, 43). Suppressors that display gene specificity, instead of global effects on chromatin structure, may shed light on the molecular basis for Gcn5-mediated transcriptional activation. In our first attempt to identify the bypass of Gcn5 requirement gene (*BGR*) suppressors, we isolated one such mutation mapped to the *REG1* gene.

REG1 (also called *HEX2* and *SRN1*) was identified in several genetic screens of glucose repression and RNA processing (63, 65, 66, 96). Reg1 protein associates physically and functionally with an essential and multifunctional protein phosphatase 1, Glc7 (23, 61, 94), whose substrate specificity is apparently determined by association with different partners, including Reg1 protein. Mutations of *REG1* cause ectopic expression of several genes under repressing conditions (21, 27, 41, 64, 97, 102). Point mutations targeted at the Glc7 interaction domain of Reg1 protein derepress *ADH2* and *SUC2* (23). A similar transcriptional repression defect caused by a *glc7* mutation (T152K) can be suppressed by

overexpressing Reg1 protein (94). These transcriptional derepression phenotypes are likely due to the inability of Glc7 to dephosphorylate the appropriate target protein(s) and consequently the ectopic increase of protein phosphorylation. Indeed, deletion of the Snf1 protein kinase suppresses the derepression defects resulting from *reg1* or *glc7* mutations (23, 28, 42), indicating an antagonistic relationship between the Snf1 kinase and the Reg1-Glc7 phosphatase complex. Consistent with this notion, Reg1 protein interacts directly with Snf1 protein in both yeast two-hybrid assays and affinity purification (61, 79). Furthermore, a hyperactive Snf1 protein caused by *reg1*Δ rescues the Spt^- phenotypes of *spt21*Δ cells (39). Curiously, the interaction between Reg1 protein and Snf1 protein, at least within the yeast two-hybrid context, is enhanced in glucose starvation conditions (61), raising the possibility that Reg1 protein may have a positive role in Snf1 protein action under certain conditions.

Snf1 protein acts as a cellular fuel gauge controlling responses to nutritional crises (37). The animal homologues of Snf1 protein are activated by AMP and are referred to as AMP-activated protein kinases. In plants, Snf1 protein-related kinases (SnRKs) fall into three large families, SnRK1, SnRK2, and SnRK3 (36). Snf1 protein, AMP-activated protein kinases, and SnRKs are the catalytic α subunits of a trimeric complex composed of a scaffold β protein and a regulatory γ subunit. In addition to bridging the α and γ subunits, the β protein contributes to substrate selection as well. The γ subunit of the yeast Snf1 complex is encoded by *SNF4* (14). At least three yeast genes encode the β subunits (26, 104). Snf1 protein plays critical roles in controlling transcription of carbohydrate transporter and metabolism genes (80). Overexpression of Snf1 protein also causes early aging, increased rRNA recombination, and loss of rRNA locus silencing (56),

a set of functions reportedly linked to histone H3 hyperphosphorylation. Indeed, several proteins can be phosphorylated by Snf1 protein in response to glucose starvation, including Reg1 protein (79), Mig1 (92), and histone H3 (60). The histone H3 phosphorylation activity of Snf1 protein has been linked directly to transcriptional activation and TBP recruitment (58, 59). Ser10 phosphorylation facilitates acetylation by increasing the affinity between Gcn5 protein and H3 (15, 18, 60). Both modifications are important for the expression of the *INO1* gene in yeast (59, 60). In addition, genetic interactions between Snf1 protein and Srb/mediator proteins (49, 84) and TBP (83) were reported. Whether these general transcriptional factors can be phosphorylated by Snf1 protein is unclear.

In this work, evidence that a gain-of-function *BGR* allele for Reg1 protein likely adopts a novel function in facilitating transcription of *HIS3* is presented. This function appears to require a functional Snf1 kinase. However, H3 phosphorylation does not play a critical role for the suppression, nor is it important for normal *HIS3* activation. A unique allele specificity for a particular mutant Gcn5 protein is shared by the Reg1 suppressor and overproduction of Snf1 protein. Indeed, both Snf1 and Reg1 suppressor can be copurified with Gcn5 from yeast, linking these three proteins functionally and physically.

MATERIALS AND METHODS

Yeast strains, plasmids, and genetic methods. Yeast strains used in this work are listed in Table 1. All genetic methods were according to reference 81. Yeast transformation was done with the lithium acetate method (29). Plasmids used in this work are listed in Table 2.

To introduce *gcn5* point mutations into the genome, the BamHI-HindIII fragment from wild-type or mutant *GCN5* was inserted into the same sites of YIplac211 (30) to generate pMK284. Constructs pMK284E173H and pMK284F221A were linearized with NgoMIV and transformed into yeast. Integration results in two copies of *GCN5* separated by the YIplac211 sequence containing a *URA3* marker. 5-Fluoroorotic acid (5-FOA) selection and genomic PCR were used to obtain and verify the desired E173H and F221A mutations.

The *HIS3-lacZ* reporter was introduced to yeast by transforming the StuI-linearized pMK334 that generates URA^+ integrants. pMK334 was constructed by inserting the EcoRI-DraI *lacZ* fragment of pLKC482 (90) into the EcoRI-HindIII sites of YIplac211, resulting in pMK333. An EcoRI-BglII fragment containing the *HIS3* promoter was isolated from pMK231 where a BglII site was introduced at the 5′ end of *HIS3* open reading frame (ORF) and inserted into the EcoRI-BamHI sites of pMK333. A unique StuI site within the *URA3* gene was used for integrative transformation. All subsequent integrants were grown in the absence of uracil to maintain the integrated sequence.

To knock out the *SNF1* gene, two disruptors were constructed. *snf1Δ-1::LEU2* was generated by two-step subcloning. First, an ApaLI-HindIII fragment upstream of the *SNF1* ORF was inserted into the XbaI-HindIII sites of pJJ252 (47) to create pMK452. The 3′ flanking region of the *SNF1* gene, an HpaI-SacI fragment obtained by PCR, was inserted into the BamHI-SacI sites of pMK452 to obtain pMK453. In the other disruptor (pYL45, *snf1Δ-2::TRP1*), the PstI-HindIII fragment of *SNF1* was first inserted into pBluescript KS+ (pMK449). The AflII-BglII 200-bp fragment corresponding to amino acids 109 through 176 of *SNF1* in pMK449 was replaced with the EcoRI-BglII fragment of pJJ248 containing the *TRP1* gene (47). To create *snf1* deletion strains, the HindIII-BamHI fragment of pMK453 or the EcoRI-BamHI fragment of pYL45 was obtained by restriction digestion before yeast transformation.

To introduce the *REG1(1-740)* allele, plasmid pYL31 was constructed by replacing the ClaI-BglII fragment of pKD97 (23) with a ClaI-XhoI-digested PCR product that contains the open reading frame of *REG1* up to amino acid residue 740 followed immediately by a stop codon. The ClaI-KpnI fragment of pYL31 was cloned into HindIII-KpnI sites of YIplac211 to obtain pYL35. To replace the entire *REG1* ORF with *REG1(1-740)*, pYL35 was linearized by SnaBI and integrated into the *REG1* locus by homologous recombination. The correct transformants were subjected to 5-FOA selection. Genomic PCR confirmed the correct genotype.

The *reg1Δ* strains were generated by introducing a PCR fragment containing the KanMX6 cassette flanked by *REG1* sequences outside the ORF (10, 99). G418-resistant transformants were examined by genomic PCR to confirm the *reg1Δ* genotype.

To create and test histone H3 mutations, strain JHY205 (2) was first made *HIS3*$^+$ by replacing the *his3Δ1* allele with the BamHI fragment of pJJ217 (47) that contains the entire *HIS3* gene, resulting in yDA10. Each histone H3 mutation was generated by the Quikchange method (Stratagene), using pJH33 as the template. All mutations were confirmed by sequencing.

The 2μ *SNF1* construct pYL41 was created by cloning the BamHI-PstI fragment containing the entire transcription unit of *SNF1* into EcoRI-PstI sites of YEplac112 (30). Deletion of the general control-responsive element (GCRE) was as described previously (51).

pMK547Gcn5 with an N-terminal hemagglutinin (HA) tag was created by cotransforming XbaI-linearized pMK547, derived from pAB8 with the Gal4 DNA binding domain deleted (34), and a PCR-amplified *GCN5* open reading frame. The Gcn5-TAP fusion construct (pYL54) was generated by a strategy essentially equivalent to QuikChange mutagenesis protocol (Roche) except that the mutagenic primers were PCR-amplified TAP sequence (74) flanked with sequences around the stop codon of *GCN5*. pMK144 (52) was the template for mutagenesis and insertion of the TAP sequence. pYL67, a plasmid derived from pBS1479 (74) by replacing the TAP sequence with eight Myc repeats, was severed as PCR template to amplify the *Myc::TRP1* cassette with flanking sequence correlated with residue 740 or the stop codon of *REG1*. Gel-purified PCR products were transformed into yeast cells to generate Reg1-Myc fusions.

HAT and kinase assays. Gcn5 protein amino acids 19 to 348 lacking the bromodomain were cloned into pET21a and expressed as a His-tagged protein (pMK515).

The desired point mutations were generated by the Quikchange method (Stratagene) and verified by sequencing. The recombinant protein was induced in the BL21 strain by adding 1 mM (final concentration) IPTG (isopropyl-β-D-thiogalactopyranoside) when cell culture reached an optical density at 600 nm (OD_{600}) of 0.5/ml. Cell cultures were grown at 37°C for 3 h. Extraction and protein affinity purification were done according to reference 50.

Kinase assays were done with the above Gcn5 protein incubated with glutathione *S*-transferase (GST)-Snf1 (wild-type or K84R) expressed and purified from yeast according to reference 35. The GST-SNF1 constructs were kindly provided by D. Thiele (Duke University).

Suppressor screening. The yeast genomic DNA library (#21) containing the *mTn-lacZ/LEU2* intervening sequence was provided by M. Snyder (Yale University) (77). The DNA was prepared by cesium chloride gradient and digested by NotI before transforming into yMK995. Ten micrograms of the library DNA was digested and isolated by phenol-chloroform extraction and ethanol precipitation. Approximately 26,000 *LEU*$^+$ transformants were replica plated to synthetic complete (SC)-His medium containing 20 mM 3-amino-1,2,4-triazole (3-AT) and incubated at 37°C for 3 to 5 days. 3-AT-resistant colonies were further transferred to nitrocellulose membranes, and the *lacZ* level was tested according to reference 1. Colonies that showed blue color on the *lacZ* filter assays were grown in SC-Leu medium overnight and transferred to yeast extract-peptone-dextrose (YPD) (representing the repressed condition) or synthetic minimal medium (SD) containing 20 mM 3-AT for 4 h. Yeast cells (20 ml; OD_{600} of 0.1/ml) were then harvested

by centrifugation (10,000 × *g* for 5 min at 4°C), washed, and suspended in extraction buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$, 10 mM Tris HCl, pH 7.4, 5 mM EDTA, Complete protease inhibitor cocktail [Roche]). Whole-cell extracts were prepared by vigorous agitation with glass beads using a bead beater (Biospec Products). β-Galactosidase activity was quantified according to reference 1. One clone, renamed yMK1055 henceforth, repetitively showed elevated *lacZ* expression in response to amino acid starvation and was further studied. yMK1055 was backcrossed to yMK1075 before 3-AT tests. To verify that a single *mTn* insertion event was responsible for the *BGR* phenotypes, yMK1055 was crossed to yMK1085. The diploid strain was subjected to sporulation and tetrad dissection; all trp^- segregants were tested for cosegregation of 3-AT resistance and leucine prototrophy. Recommended procedures were employed to map the integration site of the *mTn-lacZ* fragment (http://ygac.med.yale.edu/mtn/insertion_libraries.stm). Namely, yeast genomic DNA was isolated, digested by EcoRI, and subjected to intramolecular ligation prior to bacterial transformation. Plasmid DNA was isolated from *Escherichia coli* cells and sequenced across the junction between *REG1* and *mTn-lacZ/LEU2* using a primer specific to *lacZ*.

Northern analyses. Yeast cells were grown in appropriate selection media until the OD_{600} reached 0.5. Cells were then pelleted by centrifugation (5,000 × *g*, 5 min, 4°C) and transferred to either YPD (for basal expression) or SD supplemented with required nutrients and 40 mM 3-AT (for induced expression). Cell suspensions were further incubated at 37°C for 2 to 3 h before harvesting for RNA preparation. Although these relatively harsh conditions for induction were not essential, such treatment generally

generated more consistent results in *HIS3* activation in our strain background. Procedures for RNA preparation and Northern blot hybridization were described previously (52).

Interaction between Gcn5 and Snf1. To test the interaction between Gcn5 and Snf1 proteins, a GST-Snf1 expression construct (35) or just GST (pYL44) was transformed to the strain carrying pMK547Gcn5. Purification of GST-Snf1 was as described previously (35). Glutathione Sepharose 4B (30 μl; Amersham) was added to whole-cell extracts purified from 1.5×10^9 cells and incubated at 4°C for 3 h under constant rocking. Beads were pelleted and washed twice with HEMGT buffer (25 mM HEPES, pH 7.9, 12.5 mM $MgCl_2$, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1× Complete protease inhibitor cocktail [Roche]) followed by two more washes with HEMGT buffer containing 300 or 500 mM NaCl. The bound fractions were eluted by sodium dodecyl sulfate (SDS) loading buffer and resolved by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The copurified HA-Gcn5 protein was detected by anti-HA antibodies (12CA5; Roche).

For Gcn5-Reg1(1-740) copurification, whole-cell extracts from cells carrying pYL54 and C-terminally Myc-tagged Reg1 or Reg1(1-740) protein were prepared with the bead-beating method in FA lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1× Complete protease inhibitor cocktail [Roche]). Lysates from 3×10^9 cells were incubated with 30 μl of immunoglobulin G Sepharose 6 (Amersham) for 2 h at 4°C. After three washes with FA lysis buffer, the beads were boiled in SDS loading buffer and resolved by 8% SDS-PAGE. Western blots were conducted with an anti-c-Myc antibody (Roche).

RESULTS

Screening and identification of a BGR suppressor. To screen for extragenic suppressors that rescue transcriptional defects caused by loss-of-function mutations of Gcn5 protein, we introduced a single mutation to the catalytic domain of Gcn5 protein. This mutation, E173H, is a Glu-to-His mutation at residue 173. Since Gcn5 protein participates in more than one complex, the use of this mutant likely maintains the integral architecture of these complexes (103). Previously, an E173Q mutation was shown by others to drastically reduce the in vitro and in vivo activity of Gcn5 protein (54, 73, 93). However, this E173Q mutant in our hands maintained significant activities in *HIS3* expression after it was integrated back to the native *GCN5* locus (data not shown). We thus designed a Glu-to-His mutation. With the slight positive charge of histidine under physiological pH, a more drastic reduction of the catalytic power of Gcn5 protein was expected (54, 89). The HAT activity of a bacterially expressed E173H mutant was tested using chicken histones as the substrates. As predicted, this mutation significantly reduced the in vitro HAT activity of Gcn5 protein (Fig. 1A). To test in vivo functions, the E173H allele was integrated to the native *GCN5* locus to replace the wild-type allele. In parallel, another well-characterized F221A allele (52) was integrated in the same manner. Both alleles were controlled by the native *GCN5 cis* elements. Yeast strains bearing the wild-type, complete knockout, F221A, or E173H allele of *GCN5* were then tested for responses to amino acid starvation. Each strain was patched to YPD medium and then replica plated to synthetic complete medium lacking histidine and supplemented with various concentrations of 3-AT, a competitive inhibitor of the His3 protein. Very minor growth defects were seen in *gcn5*$^-$ strains when assayed at 30°C in medium

supplemented with 3-AT (Fig. 1B). However, when these cells were incubated at 37°C, 3-AT induced obvious growth defects of all three *gcn5*$^-$ strains. None of these cells were temperature sensitive (compare growth on YPD and SC-His without 3-AT). The clear growth defects of *gcn5*$^-$ cells provide a platform for suppressor screening.

We further modified the E173H mutant strain by introducing a *HIS3-lacZ* reporter to the *ura3-52* locus. Insertion of *HIS3-lacZ* did not change the cellular sensitivity to 3-AT (Fig. 1B, bottom two patches). This *lacZ* reporter, under the control of the *HIS3* promoter, was also activated by amino acid starvation (Fig. 1C) and hence offered a convenient means to verify the 3-AT-resistant suppressor phenotypes.

To identify suppressors, we used a minitransposon (*mTn*)-based mutagenesis approach (77). In this method, the *mTn-lacZ/LEU2* sequence was integrated into a yeast genomic DNA library via transposition. Yeast DNA fragments along with the interrupting sequence were excised from the plasmid pool and transformed into yeast. Each *mTn* sequence integrated to the chromatin via homologous recombination between the flanking yeast sequence and the corresponding genomic locus. *LEU*$^+$ transformants were replica plated to 20 mM 3-AT medium and grown at 37°C. All 3-AT-resistant clones were then screened for increased expression of β-galactosidase induced by amino acid starvation. From approximately 26,000 *LEU*$^+$ transformants, we identified 1 such colony (Fig. 1C). Northern data clearly showed that the *HIS3* expression was upregulated in this suppressor strain compared with the parental *gcn5* E173H cells (Fig. 1D). Similar complementation in transcription was seen in *HIS1*, *HIS6* (not shown), and *HIS4* (Fig. 2C)

as well. Genetic assays showed that a single *mTn* insertion event was responsible for the suppression phenotypes (data not shown and see Materials and Methods).

To map the mutation, we rescued and cloned the *mTn* insertion along with the flanking yeast sequences (see Materials and Methods). DNA sequencing across the junction revealed that the mutagenic fragment had inserted to the coding region of *REG1* (Fig. 2A), resulting in in-frame fusion of *lacZ* to residue 740 of Reg1 protein. While the expression of the Reg1-*mTn-lacZ* fusion protein may have contributed to some β-galactosidase activity shown in Fig. 1C, the *HIS3* transcript quantification results (Fig. 1D) unequivocally demonstrated the rescue of *gcn5*$^-$ defects. Nonetheless, since the in-frame fusion of *lacZ* added a large mass to the truncated Reg1 protein, we were curious whether the β-galactosidase fusion was necessary for the suppression. By integrative transformation, we replaced the chromosomal copy of the native *REG1* with one that is truncated after residue 740 (without the *lacZ* fusion) and tested whether this "clean" *REG1(1-740)* allele was able to suppress the *gcn5* E173H mutation. Figure 2B shows that with a truncated Reg1(1-740) protein, the *gcn5* E173H cells also exhibited significant resistance to 3-AT (Fig. 2B, row 5) and restoration of *HIS3* expression (Fig. 2B, lane 7), although the original *mTn-lacZ* insertion consistently showed better growth than Reg1(1-740). The in-frame fusion of β-galactosidase enhanced but was not essential for suppression efficacy.

We further deleted the *REG1* gene and found that the suppression phenotypes were lost (Fig. 2B, left panel, row 4, and right panel, lane 4). Interestingly, in the presence of a functional Gcn5 protein, deleting the *REG1* gene does not seem to affect *HIS3* expression

(Fig. 1D, lanes 7 and 8). Together, these results showed that a Reg1(1-740) truncated protein is essential and sufficient for suppressing the *gcn5* E173H mutation in *HIS3* transcription.

Gcn5, in the context of SAGA complex, is recruited to the *HIS3* promoter by the transcriptional activator Gcn4 that binds the cognate *cis* element, GCRE (51). To test whether the Reg1(1-740) suppressor exerted its function via Gcn4-GCRE or a novel Gcn4-independent mechanism, we replaced the GCRE 5′ to the *HIS3* gene with an irrelevant sequence (51) and determined whether the suppression was affected. Comparison of mRNA transcribed from the GCRE-less *HIS3* with the wild-type *HIS4* control clearly showed that the GCRE was essential for Reg1(1-740)-mediated suppression (Fig. 2C), indicating that Reg1(1-740) protein modulates an activity downstream of the normal Gcn4 functions.

One possible mechanism for the observed suppression is restoration of the HAT activity of the Gcn5 E173H mutant protein. To see if this was the case, we conducted chromatin immunoprecipitation using an antibody against histone H3 acetylated at Lys9 and/or 14 (52). Figure 2D, lane 1, shows the expected hyperacetylation at the +1 nucleosome of *HIS3* in the presence of a wild-type Gcn5 protein (51). The TATA element and the transcription initiation sites are within the +1 nucleosome. Promoter hyperacetylation was lost in the E173H background (Fig. 2D, lane 5). When *REG1* was replaced with the *REG1(1-740)* suppressor allele, H3 remained hypoacetylated at the *HIS3* promoter (Fig. 2D, lane 7), suggesting that the canonical nucleosomal H3 acetylation by the Gcn5 acetyltransferase was not affected in the *REG1(1-740)* background.

Reg1(1-740) preferentially rescues the E173H allele of *gcn5* in a semidominant manner. To characterize the Reg1(1-740) suppressor further, we first tested whether this allele was dominant or recessive. Figure 3A shows that the *REG1*$^{+}$/*REG1(1-740)* heterozygote retained a growth advantage over the parental *gcn5* E173H *REG1*$^{-}$ homozygous strain at both 30 and 37°C. The strength of the suppression appeared to be somewhat weaker than the haploid strain, suggesting that the Reg1(1-740) protein was a gain-of-function, semidominant suppressor.

Besides E173H, the F221A mutation also causes quantitative defects of Gcn5 functions in vitro and in vivo (Fig. 1B) (52). This mutation selectively impairs the ability of Gcn5 protein to bind acetyl coenzyme A (acetyl-CoA) (57, 76, 88), which is a prerequisite step for histone tail binding (88). It is thus likely that histone tails within the vicinity of the SAGA complex remain free from binding by the F221A mutant protein. In contrast, based on the studies of the E173Q mutant (89) and our yeast two-hybrid tests comparing different disabled *gcn5* mutants (M.-H. Kuo, unpublished data), the E173H allele most likely prolongs its association with both substrates because the catalytic process stalls after the ternary complex is formed. Because of the possible differential effects on histone tail accessibility, we tested whether E173H, F221A, and a knockout allele of *GCN5* responded differently to Reg1(1-740) suppressor.

To test the allele specificity, the *gcn5*Δ and *gcn5* F221A strains were engineered so that *REG1* was replaced with the *REG1(1-740)* allele, and the resultant strains were tested on 3-AT plates (Fig. 3B). Neither the F221A nor the complete knockout allele of *gcn5* was rescued by Reg1(1-740) (Fig. 3B, rows 6 and 8). The strong preference for the E173H

allele suggests that Reg1 protein may interact directly with Gcn5 protein or may control Gcn5 at a step(s) subsequent to the formation of the Gcn5-acetyl-CoA-histone ternary complex.

We further tested whether Reg1(1-740) protein rescued other *gcn5*$^-$ phenotypes. Figure 3C shows that all three *gcn5*$^-$ mutants exhibited severe growth retardation in yeast extract-peptone-glycerol and yeast extract-peptone-ethanol media, as previously reported (58). Moreover, *gcn5*$^-$ cells were also sensitive to 100 mM hydroxyurea, an inhibitor of DNA replication. However, neither the *mTn* allele nor the clean truncation of Reg1(1-740) protein was able to suppress any of these defects. The failure to suppress other phenotypes, such as caffeine sensitivity, and the inability to use galactose or sucrose were also observed (data not shown). In contrast, the sporulation defects (12) of a *gcn5* E173H homozygous strain were partially rescued (not shown). We conclude that the Reg1(1-740) protein suppresses only a subset of Gcn5 target genes.

Snf1 protein plays a critical role for *HIS3* expression. Reg1 protein is best known to inhibit the kinase activity of Snf1 protein and consequently prevents the expression of many genes when glucose is abundant (see the introduction), a function termed glucose repression. Snf1 protein derepresses the expression of these genes via several mechanisms, including histone H3 phosphorylation (59, 60). Phosphorylated H3 was shown to bind Gcn5 protein at a higher affinity (15, 17). It thus seems plausible that the hypoacetylation phenotype caused by *gcn5* mutations may be compensated for by hyperphosphorylation of H3, which either helps anchor Gcn5 protein to the *HIS3* promoter or by itself provides an environment suitable for stronger *HIS3* expression.

To examine the link between Gcn5, Snf1, and H3 phosphorylation, we first tested whether Snf1 protein was involved in *HIS3* expression. To this end, we created two deletion alleles of *SNF1*. The *snf1Δ-1::LEU2* mutant had the entire ORF replaced with a *LEU2* marker. However, this marker was incompatible with the *mTn-lacZ-LEU2* insertion mutant; we thus created another allele, *snf1Δ-2::TRP1*, that was truncated after amino acid 108. Figure 4 shows that both *snf1* mutations caused obvious growth defects on 3-AT plates (Fig. 4A, rows 2 to 4) as well as impaired *HIS3* expression (Fig. 4B, lane 3, and data not shown), demonstrating that the Snf1 protein was also a critical transcriptional regulator for *HIS3*. We next tested whether the Snf1 protein was important for the suppression. Deleting *SNF1* from the original *gcn5* E173H *REG1::mTn-lacZ* suppressor strain significantly attenuated the suppression phenotypes (Fig. 4A, rows 6 and 7, and B, lane 5). Thus, Snf1 protein is critical for normal and Reg1(1-740)-mediated *HIS3* activation.

To see how Gcn5 and Snf1 proteins may genetically interact to activate *HIS3*, we examined whether overexpressing one of these two enzymes can rescue the *HIS3* expression defects caused by a mutation of the other. While a 2μm multicopy *GCN5* construct was unable to rescue the 3-AT hypersensitivity of the *snf1Δ-2::TRP1* strain (Fig. 4C, left panel), overproduction of Snf1 protein effectively rescued the E173H allele of *gcn5* (Fig. 4C, right panel, compare rows 3 and 4). On the other hand, overproduction of a catalytically inactive mutant of Snf1, K83R, failed to rescue the *gcn5*$^-$ phenotypes (data not shown), suggesting that the kinase activity was essential for the suppression. Intriguingly, neither deletion nor the F221A allele of *gcn5* responded to the multicopy *SNF1* plasmid. Thus, the Snf1 multicopy suppressor displays an allele specificity similar

to that of Reg1(1-740). Furthermore, in the presence of a functional *GCN5*, overproduction of Snf1 protein yielded higher resistance to 3-AT (Fig. 4C, right panel, row 2), very similar to the *GCN5*$^-$ *REG1(1-740)* strain (Fig. 3B, row 2).

Taking together the above results, as well as the reports that Reg1 and Snf1 interact genetically and physically for transcription of several inducible genes (see the introduction), it seems likely that Snf1 may be part of the mechanism by which Reg1(1-740) protein suppresses the E173H mutant allele.

H3 Ser10 phosphorylation is not responsible for *bgr* suppression. To test whether H3 Ser10 phosphorylation contributes to the *BGR* phenotypes, we used a yeast strain in which both copies of each of the four core histone genes had been deleted (2). Viability of the cells was supported by a low-copy-number plasmid bearing wild-type histone genes and a *URA3* marker. The desired histone mutations can be introduced into an otherwise identical construct containing a *LEU2* nutrient marker. After transforming the latter plasmid that delivered the specific histone mutation(s), the wild-type histone genes were shuffled out by 5-FOA selection, leaving the mutant allele as the sole copy for histone expression. Additionally, *GCN5* and *REG1* were replaced with the E173H and *REG1(1-740)* alleles, respectively. 3-AT resistance was then compared among different *LEU*$^-$ *Ura*$^-$ strains as shown in Fig. 5. In this genetic background, Reg1(1-740) also effectively rescued the E173H mutant. However, the S10A mutation did not impose a discernible effect on cellular growth (Fig. 5, compare rows 3 and 4), ruling out a critical role played by phosphorylated Ser10 alone. Within the amino-terminal tail domain of histone H3, Ser28 and Ser31 share sequence similarity with Ser10 (^{7}ARKSTGG and

[25]ARKSAPSTGG). Although Snf1 protein has not been shown to phosphorylate either serine residue, Ser28 can be phosphorylated by the Aurora family kinases for chromatin condensation during mitosis (16, 31, 82). We were curious about the possibility that the Snf1 kinase activity might "spill over" to these two residues in the *REG1(1-740)* strain. Thus, a triple Ser-to-Ala mutant, S10A/S28A/S31A, was introduced to the *gcn5* E173H *REG1(1-740)* background. These cells still exhibited robust growth in the presence of 3-AT (Fig. 5, row 6), further supporting the notion that H3 phosphorylation was unlikely to be the driving force for the observed *BGR* phenotypes. Consistent with this, neither single nor triple Ser-to-Ala mutations exacerbated the 3-AT hypersensitivity caused by the E173H mutation in a *REG1*$^-$ background (Fig. 5, compare rows 2, 8, and 10). We therefore conclude that Ser10 phosphorylation, though important for activation of several other genes, does not contribute appreciably to Gcn5 and Snf1 protein-mediated *HIS3* expression. Thus, Snf1 protein most likely controls *HIS3* expression by a novel, H3 phosphorylation-independent mechanism(s).

While preventing H3 phosphorylation imposes no apparent effect on the Reg1(1-740) protein-generated suppression, we were nonetheless interested in knowing whether a constitutively phosphorylated H3 would be sufficient to bring about a chromatin environment that suppresses the *gcn5* E173H transcriptional defects. Toward this end, Ser10, Ser28, and Ser31 were replaced by aspartate or glutamate that mimicked the negatively charged phosphorylation state. Cellular growth in the presence of 3-AT was then assessed. While a single S10E mutation yielded very few differences in *REG1* or *REG1(1-740)* background (Fig. 5, rows 5 and 9), the triple acidic mutation clearly brought about stronger resistance to 3-AT (Fig. 5, rows 7 and 11). Since this phenotype

was independent of the *REG1* status, we conclude that constitutive negative charges at the amino terminus of H3 represent another bypass of Gcn5 requirement suppressor.

Physical interactions of Gcn5, Snf1, and Reg1(1-740). The above data place both Reg1 and Snf1 proteins to the regulatory circuitry of *HIS3* and likely other amino acid starvation-inducible genes. The ability of Reg1(1-740) protein and overproduced Snf1 kinase to rescue preferentially the E173H mutant suggests an intriguing possibility that Gcn5 protein is a functional target for the Snf1 kinase. To test this hypothesis, we purified a wild-type and a catalytically inactive (K84R) GST-Snf1 protein from yeast (35) and incubated these two preps with recombinant Gcn5 protein expressed in *E. coli*. [γ-^{32}P]ATP was included in the reactions to track the phosphorylation status of Gcn5. Figure 6A shows that Gcn5 protein was indeed phosphorylated in the presence of the wild-type Snf1 protein. The K84R mutation effectively diminished Gcn5 phosphorylation, indicating that Snf1 protein was responsible for Gcn5 protein phosphorylation.

Intrigued by the in vitro phosphorylation results, we further tested whether Gcn5 and Snf1 proteins interacted in vivo. To this end, we epitope tagged Gcn5 with HA at its amino terminus. Two yeast strains expressing GST-Snf1 or GST were transformed with the HA-*GCN5* construct and subjected to one-step purification with a glutathione matrix. After extensive washing, the bound materials were resolved by SDS-PAGE and probed with an anti-HA antibody. Figure 6B shows apparent copurification of the HA-Gcn5 protein with GST-Snf1 but not GST alone. Literally identical results were obtained in reciprocal experiments (i.e., immunoprecipitation with the anti-HA antibody, followed by

Western analyses to quantify Snf1 protein in the precipitate) (not shown), confirming the in vivo association between Gcn5 and Snf1 proteins.

We then asked whether Reg1 protein also associated with Gcn5 protein. Figure 6C shows that a Myc-tagged Reg1(1-740) protein was also present in the crude preparation of an epitope-tagged Gcn5 protein. Intriguingly, the full-length Reg1-Myc protein was not detected under the same condition (Fig. 6C, first two lanes), consistent with the gain-of-function trait of the Reg1(1-740) suppressor protein.

DISCUSSION

A putative noncatalytic function of Gcn5 protein. The histone acetyltransferase activity of Gcn5 protein is critical for the expression of multiple yeast genes. Point mutations that eliminate the HAT activity of Gcn5 protein cause defects in promoter acetylation and in transcriptional activation of such model genes as *HIS3* and *PHO5* (7, 52, 73, 100). While these results provide solid evidence that Gcn5 protein uses its HAT activity to activate transcription, microarray studies also showed that a *gcn5* knockout strain has transcriptional defects in more genes than does a strain expressing a catalytically inactive mutant (43), suggesting that Gcn5 protein may perform noncatalytic roles in gene expression. Indeed, Jacobson and Pillus showed that a catalytically inactive Gcn5 protein counteracts transcriptional silencing at subtelomeric loci (46). Such noncatalytic functions of Gcn5 protein may be unveiled by characterizing point mutations that abrogate the catalytic power of Gcn5 protein but permit other functions to be exerted. This notion seems to be consistent with the data presented in this work. For example, *HIS3* and *HIS4* expression are effectively rescued by the Reg1(1-740) suppressor (Fig. 1D and 2C) in the E173H but not the knockout background. No restoration of histone H3 acetylation was detected, suggesting one possibility that the noncatalytic function of the E173H allele is selectively enhanced by Reg1(1-740) protein. This function is likely synergistic with its catalytic counterpart, as more pronounced resistance to 3-AT is exhibited by *GCN5*$^+$ *REG1(1-740)* and *GCN5*$^+$ multicopy *SNF1* strains (Fig. 3B and 4C).

It is also intriguing that the F221A allele is refractory to Reg1(1-740) and higher doses of Snf1 protein. Several other suppressors that are currently characterized by us do not show such unique allele specificity (Y. Liu, X. Xu, and M.-H. Kuo, unpublished data). Molecularly, E173H and F221A mutations abrogate the HAT activity of Gcn5 via different mechanisms and may have different impacts on histone tails. F221A impairs acetyl-CoA binding (57, 88, 93), whereas E173H blocks the nucleophilic attack on the bound acetyl-CoA (89). Association of acetyl-CoA is prerequisite to histone tail binding (88, 89). After the transfer of the acetyl group to histone within the ternary complex, the acetylated histone dissociates first and then follows the consumed coenzyme A. Thus, blocking the association between Gcn5 and acetyl-CoA by the F221A mutation likely prevents Gcn5 protein from binding to the substrate histone, rendering the latter susceptible to other unregulated or untimely chromatin binding and modulating activities. The E173H mutation, on the other hand, may lock Gcn5, acetyl-CoA, and the histone tail in a ternary complex, thus preventing possible usage or modifications of the histone tail by other activities. In addition, it remains a strong possibility that Gcn5 protein uses nonhistone protein substrates (68). If so, the retention of one of these proteins by the E173H mutant enzyme may exacerbate the histone hypoacetylation defects.

Furthermore, only a subset of defects associated with *gcn5*$^-$ mutants can be rescued by Reg1(1-740) (Fig. 3C). Together, it is highly likely that Gcn5 uses multiple mechanisms to activate transcription in a target gene (or transcriptional activator)-dependent manner.

Reg1(1-740) protein is a gain-of-function suppressor. Reg1 protein is a regulatory subunit for Glc7, an essential and multifunctional type I protein phosphatase (95). Reg1

protein also interacts with several other proteins, including Snf1 (61, 79) and the yeast 14-3-3 homologues, Bmh1 and Bmh2 proteins (22). The binding domains for these proteins are all within the first 500 amino acids that are conserved among Reg1 protein homologues (22, 23, 61). These domain are preserved in our *REG1(1-740)* suppressor allele, suggesting that the prototypical functions of Reg1 protein are not impaired by the C-terminal truncation.

The Reg1(1-740) protein lacks about one third of the total length. The truncation occurs immediately before a stretch of acidic residues (15 of 19 residues are Asp or Glu), and the deleted portion is rich in serine, threonine, and acidic residues (16% Ser, 4.4% Thr, 8.8% Asp, and 7.3% Glu). Little is known about the molecular functions or potential partners of this part of the Reg1 protein. Preliminary sequence search reveals no clear homologues to this region across species (data not shown). Contrary to the gain-of-function *BGR* phenotypes, this C-terminal region is dispensable for glucose repression. For example, Dombek et al. showed that the C-terminal deletion of Reg1 protein (up to residue 693) does not cause appreciable derepression of *ADH2* or *SUC2* (23). Shirra and Arndt reported that a Reg1 protein missing the last 80 amino acids is able to fully complement a recessive *reg1-326* mutant (83). Indeed, we have no evidence of transcriptional derepression of those glucose-repressible genes in the *REG1(1-740)* background (Y. Liu and M.-H. Kuo, unpublished). It is possible that the carboxyl-terminal third of Reg1 protein interacts with a negative regulator(s), or another region of Reg1 protein in *cis*, that restricts specifically the *HIS3* expression-related functions of Reg1 protein. Perhaps this negative regulator selectively controls the residual non-HAT function of the E173H mutant of Gcn5 protein. Upon deleting this Ser-Thr-Asp-Glu-rich

domain, the negative effect of this regulator diminishes, hence unleashing the non-HAT function of Gcn5 protein for *HIS3* activation. This view is consistent with the affinity purification data (Fig. 6) that the Reg1(1-740) but not the full-length Reg1 protein can be copurified with an epitope-tagged Gcn5 protein.

It is important that the suppressing power of Reg1(1-740) protein is abrogated by deleting *SNF1*. While this result alone does not prove that Snf1 protein acts downstream of the Reg1(1-740) suppressor, considering the well-established interaction between Reg1 and Snf1 proteins, we suggest that at least part of the suppressor function of Reg1(1-740) protein is mediated through Snf1 protein. However, we cannot rule out the existence of an intermediary step(s)/factor(s) for the suppression.

One probable factor involved in the *BGR* phenotype is the type 1 protein phosphatase Glc7. Reg1 is one of several regulators of the essential Glc7 enzyme. Unfortunately, our attempts to link Glc7 protein to the *BGR* phenotypes failed to generate conclusive data. Using several known *glc7* point mutations that cause phenotypes in glycogen metabolism and/or glucose repression, we indeed found a few able to confer strong resistance to 3-AT in the absence of a functional Gcn5 protein. However, such elevated 3-AT resistance was not accompanied by increased *HIS3* transcription (Y. Liu and M.-H. Kuo, unpublished). This disparity probably arises from the fact that Glc7 protein controls multiple cytoplasmic and nuclear functions (e.g., see references 87 and 101). Changes in the metabolism and flux of 3-AT may render yeast cells resistant to 3-AT with a low level of *HIS3* transcription. The possible involvement of *GLC7* in *HIS3* regulation awaits further investigation when more mutant *glc7* alleles are available.

Reg1 protein was recently shown to be purified in a complex containing two yeast 14-3-3 homologues, Bmh1 and Bmh2 proteins, and heat shock proteins Ssd1 and Ssd2 (22). Deleting *BMH1* or *BMH2* did not appreciably alter the ability of Reg1(1-740) protein to rescue the *gcn5* E173H mutant (X. Xu and M.-H. Kuo, data not shown), indicating that these two proteins are not part of the suppression mechanism. Alternatively, functional redundancy between Bmh1 and Bmh2 proteins (92% identical) (98) may account for the lack of phenotypes in *bmh1Δ* and *bmh2Δ* strains.

Interestingly, the gain-of-function nature of the Reg1(1-740) suppressor, as well as the phenotypic similarity between Reg1(1-740) and overexpressed Snf1 protein, are at odds with the well-characterized antagonistic relationship with Snf1 protein (see the Introduction). We suggest that the functional relationship between these two proteins may be gene dependent. One precedent for this type of functional variation was reported for Spt3/8 proteins on TBP recruitment. Spt3 protein genetically and physically interacts with TBP (25). While Spt3 protein is required for TBP binding to the TATA elements of *GAL1* and *ADH2* (8, 9, 24, 55), it also plays a negative role in TBP-TATA interaction in other cases (7, 105).

Snf1 protein activates *HIS3* in an H3 phosphorylation-independent mechanism. Snf1 protein is a member of the AMP-activated protein kinase family that serves as a metabolic sensor in eukaryotic cells (37). It thus seems reasonable that Snf1 protein also contributes to the regulation of amino acid biosynthesis genes as shown in this work. Despite the functional interaction between Gcn5 and Snf1 proteins for *INO1* activation (15, 17, 58-60), the H3 phosphorylation function of Snf1 protein is unlikely to be a major

determinant in *HIS3* expression (Fig. 5). However, we cannot rule out the possibility of phosphorylation at other residues or histones by Snf1 protein. In addition, genetic data showed that Srb/mediator complex and TBP are also potential substrates of Snf1 kinase (49, 83).

It is interesting that Snf1 protein can modify a recombinant Gcn5 protein and that these two proteins are copurified from yeast (Fig. 6). We do not yet know the site(s) modified by Snf1 protein in vitro, nor has it been tested whether Gcn5 protein is phosphorylated in vivo. The human Gcn5 protein was shown to be modified and inhibited by the DNA-dependent kinase (6). In our hands, the in vitro-phosphorylated Gcn5 also seems to exhibit a slightly lower activity on histones H3 and H4 (X. Xu and M.-H. Kuo, unpublished). However, it remains an open question as to whether a phosphorylated Gcn5 protein behaves differently within the context of native complexes.

In conclusion, combining the data presented here and those reported by others, we propose a simple model that that Reg1(1-740) protein uses its newly adopted affinity for Gcn5, while maintaining the Snf1 interaction domain (79), to mediate the interaction between Gcn5 and Snf1 proteins. When Snf1 protein is brought to the vicinity of Gcn5, phosphorylation of Gcn5 protein or another factor(s) within or near the SAGA complex may provide the noncatalytic function that rescues the E173H mutation for effective activation of a subset of Gcn5 target genes.

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TABLE 1. Yeast strain list

Strain	Relevant genotype	Source or reference
yMK839	*MAT***a***trp1 leu2-3,112 ura3-52*	52
yMK842	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5*Δ*::hisG*	52
yMK984	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5* F221A	This study
yMK986	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5* E173H	This study
yMK988	*MAT***a***trp1 leu2-3,112 URA3::HIS3-lacZ*	This study
yMK995	*MAT***a***trp1 leu2-3,112 URA3::HIS3-lacZ gcn5* E173H	This study
yMK1055	*MAT***a***trp1 leu2-3,112 URA3::HIS3-lacZ gcn5* E173H *reg1::mTn*	This study
yMK1085	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5* E173H pMK125(*CEN GCN5 TRP1*)	This study
YL232	*MAT***a***trp1 leu2-3,112 ura3-52 snf1*Δ*-1::LEU2*	This study
YL328	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5* E173H *REG1::mTn*	This study
YL338	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5* E173H *reg1*Δ*::KanMX6*	This study
YL351	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5* E173H *REG1(1-740)*	This study
YL352	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5* F221A *REG1(1-740)*	This study
YL375	*MAT***a***trp1 leu2-3,112 ura3-52 REG1(1-740)*	This study
YL376	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5*Δ*::hisG REG1(1-740)*	This study
YL558	*MAT***a***trp1 leu2-3,112 ura3-52 snf1*Δ*-2::TRP1*	This study
YL559	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5* E173H *REG1::mTn snf1*Δ*-2::TRP1*	This study
YL585	*MAT***a***trp1 leu2-3,112 ura3-52 GCN5 REG1(1-740) snf1*Δ*-2::TRP1*	This study
YL603	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5* E173H *REG1(1-740)-myc::TRP1*	This study
YL610	*MAT***a***trp1 leu2-3,112 ura3-52 gcn5* E173H *REG1-myc::TRP1*	This study
JHY205	*MAT***a***his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT* pJH33[*CEN URA3 HTA1-HTB1 HHT2-HHF2*]	2
DA10	*MAT***a***leu2*Δ*0 met15*Δ*0 ura3*Δ*0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT* pQQ18[*CEN*	D. Almy, unpublished

Strain	Relevant genotype	Source or reference
	LEU2 HTA1-HTB1 HHT2-HHF2]	
YL372	*MAT***a***leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5* E173H pQQ18[*CEN LEU2 HTA1-HTB1 HHT2-HHF2*]	This study
YL381	*MAT***a***leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5* E173H *REG1(1-740)* pQQ18[*CEN LEU2 HTA1-HTB1 HHT2-HHF2*]	This study
YL407	*MAT***a***leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5* E173H *REG1(1-740)* pMK439S10A[*CEN LEU2 HTA1-HTB1 hht2-S10A-HHF2*]	This study
YL408	*MAT***a***leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5* E173H *REG1(1-740)* pMK439S10E[*CEN LEU2 HTA1-HTB1 hht2-S10E-HHF2*]	This study
YL409	*MAT***a***leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5* E173H pMK439S10A[*CEN LEU2 HTA1-HTB1 hht2-S10A-HHF2*]	This study
YL410	*MAT***a***leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5* E173H pMK439S10E[*CEN LEU2 HTA1-HTB1 hht2-S10E-HHF2*]	This study
YL457	*MAT***a***leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5* E173H pMK439S10A/S28A/S31A[*CEN LEU2 HTA1-HTB1 hht2-S10A/S28A/S31A-HHF2*]	This study
YL458	*MAT***a***leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5* E173H *REG1(1-740)* pMK439S10A/S28A/S31A[*CEN LEU2 HTA1-HTB1 hht2-S10A/S28A/S31A-HHF2*]	This study
YL459	*MAT***a***leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5* E173H pMK439S10E/S28D/S31D[*CEN LEU2 HTA1-HTB1 hht2-S10E/S28D/S31D-HHF2*]	This study
YL460	*MAT***a***leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5* E173H *REG1(1-740)* pMK439S10E/S28D/S31D[*CEN LEU2 HTA1-HTB1 hht2-S10E/S28D/S31D-HHF2*]	This study

TABLE 2. Plasmid construct list

Plasmid	Description	Source or reference
pMK125	pRS414-*GCN5*	52
pMK284	Integration construct for introducing point mutations to *GCN5*	This study
pMK284E173H	Integration construct for introducing E173H to *GCN5*	This study
pMK284F221A	Integration construct for introducing F221A to *GCN5*	This study
pMK334	Integration construct for introducing *HIS3-lacZ* reporter to *URA3* locus	This study
pMK449	pBluescriptKS-*SNF1*	This study
pMK453	*snf1Δ::LEU2* disruptor	This study
pKD97	pRS316-*HA-reg1Δ6*	23
pYL31	pRS316-*HA-REG1(1-740)*	This study
pYL35	Integration construct for introducing *REG1(1-740)* mutation	This study
pYL41	YEplacI12-*SNF1*	This study
pYL42	pYEX-4T-GST-Snf1	35
pYL44	pYEX-4T-GST	A. Acharya, unpublished
pYL45	*snf1Δ::TRP1* disruptor	This study
pYL54	pYEX-4T-Gcn5-TAP	This study
pYL67	8 × *Myc::TRP1* for tagging proteins with 8 Myc repeats	This study
pQQ18	pRS315-*HTA1-HTB1 HHT2-HHF2*	2
pJH33	pRS316-*HTA1-HTB1 HHT2-HHF2*	2
pMK439S10A	pQQ18 with an H3 S10A mutation	This study
pMK439S10E	pQQ18 with an H3 S10E mutation	This study
pMK439S10A/S28A/S31A	pQQ18 with an H3 S10A/S28A/S31A mutation	This study
pMK439S10E/S28D/S31D	pQQ18 with an H3 S10E/S28D/S31D mutation	This study
pMK515	pET21-6xHis-Gcn5 protein	This study
pMK547Gcn5	3xHA-Gcn5 overexpression	This study

FIG. 1. Identification of a *BGR* suppressor rescuing the *gcn5* E173H mutant. (A) In vitro HAT assays. Chicken core histones were acetylated by recombinant Gcn5 proteins, and the ^{3}H-labeled acetylated histone was detected by fluorography (right panel). CBR, Coomassie blue R250. (B) Temperature-dependent hypersensitivity to 3-AT is generated by three *gcn5*$^{-}$ alleles. The E173H and F221A alleles were introduced to the native *GCN5* locus under the control of its own *cis* elements. Each strain was patched to YPD and grown at 30°C for 2 days. Cells were then replica plated to SC-His medium supplemented with various concentrations of 3-AT and incubated at 30 or 37°C for 3 to 4 days. Relevant genotypes are listed on the right. (C) The *BGR* suppressor rescues both 3-AT hypersensitivity and *HIS3-lacZ* expression. Both plates were from cultures grown at 37°C. The β-galactosidase activities (U/mg of total proteins/min) were measured from cultures grown in YPD or SD (minimal) medium to early log phase. Errors represent variation from two independent cultures of each strain. (D) Northern blot hybridization. Log-phase cells were harvested from rich or minimal medium supplemented with 20 mM 3-AT before RNA preparation and hybridization. 18S rRNA was used as the internal control. The ratio of *HIS3* to 18S rRNA was measured and normalized to the basal expression of a *GCN5*$^{+}$ strain. A, activated; R, repressed. Figures of gel staining, fluorography, and culture plates were scanned with a flatbed scanner and acquired by Photoshop 7.0.

Fig. 1.

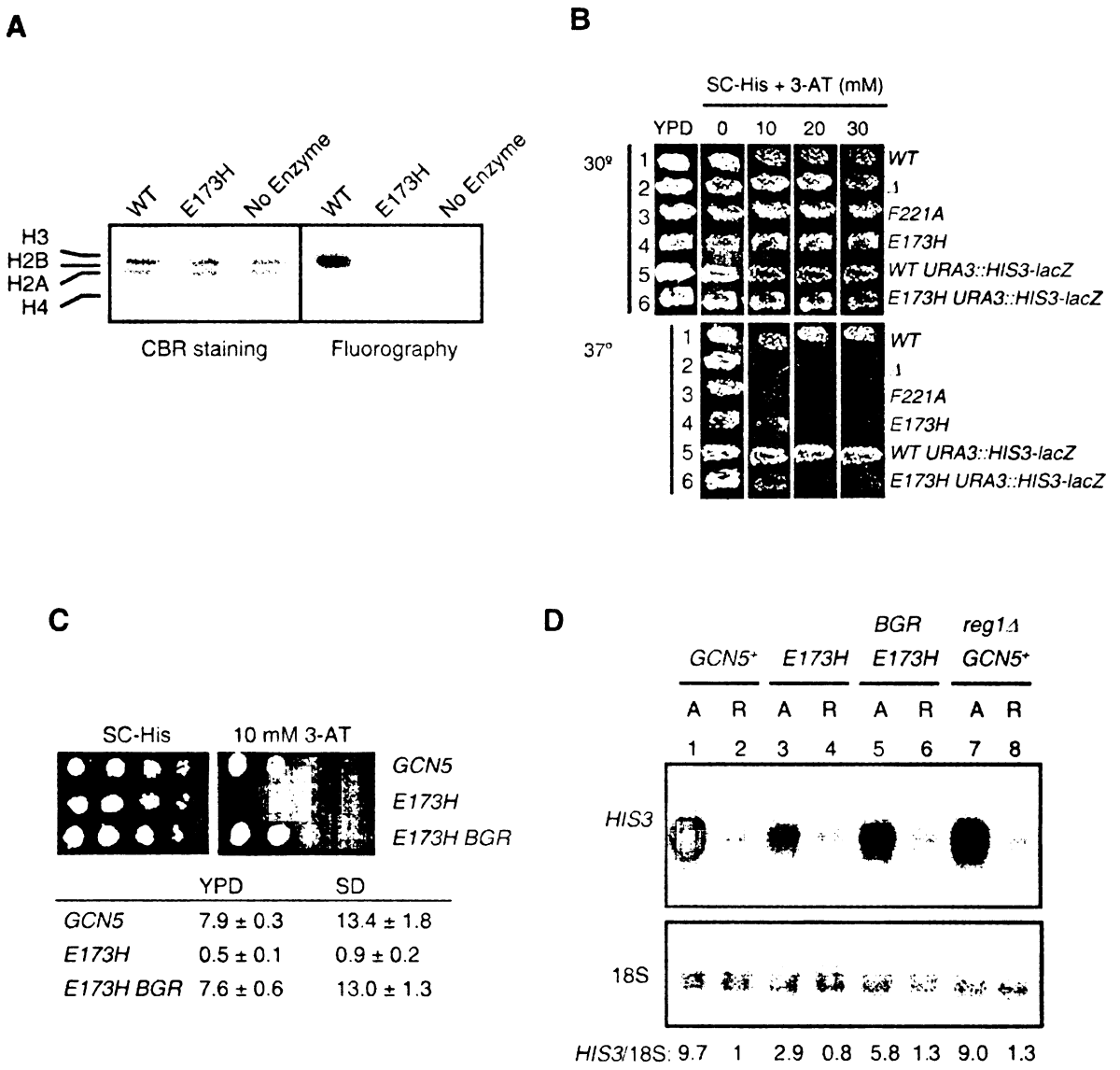

A
WT
E173H
No Enzyme
WT
E173H
No Enzyme
H3
H2B
H2A
H4
CBR staining
Fluorography
B
SC-His + 3-AT (mM)
YPD
0
10
20
30
30°
1 WT
2 Δ
3 F221A
4 E173H
5 WT URA3::HIS3-lacZ
6 E173H URA3::HIS3-lacZ
37°
1 WT
2 Δ
3 F221A
4 E173H
5 WT URA3::HIS3-lacZ
6 E173H URA3::HIS3-lacZ
C
SC-His
10 mM 3-AT
GCN5
E173H
E173H BGR
YPD
SD
GCN5 7.9 ± 0.3 13.4 ± 1.8
E173H 0.5 ± 0.1 0.9 ± 0.2
E173H BGR 7.6 ± 0.6 13.0 ± 1.3
D
GCN5+
E173H
BGR E173H
reg1Δ GCN5+
A R A R A R A R
1 2 3 4 5 6 7 8
HIS3
18S
HIS3/18S: 9.7 1 2.9 0.8 5.8 1.3 9.0 1.3

FIG. 2. Characterization of the Reg1(1-740) *BGR* suppressor. (A) DNA and protein sequences across the *mTn-lacZ* integration site. Insertion of the *mTn-lacZ* fragment at nucleotide 2122 results in an in-frame fusion between the Reg1 protein and the Tn3 long terminal repeat (LTR; lowercase) and the *lacZ* gene. Ser740 of Reg1 protein is marked. The nucleotide and amino acid residue numbers are relative to the start codon of the *REG1* open reading frame. (B) Reg1(1-740) protein is essential and sufficient for the *BGR* phenotypes. The left panel shows 3-AT tests (37°C) of isogenic strains bearing different alleles of *REG1*. The right panel shows Northern hybridization results. All samples were obtained from induced conditions (see legend to Fig. 1D). The *HIS3*/18S rRNA ratio of each sample was calculated and then normalized to that of the *gcn5* E173H strain (lane 2 or 6). (C) Reg1(1-740) protein-mediated suppression requires the Gcn4 activator binding site, GCRE. Shown are reverse transcription-PCR results. *PGK1* is an internal control. (D) Promoter H3 hypoacetylation is not affected by the Reg1(1-740) suppressor. Shown are quantitative PCR results of chromatin immunoprecipitation using an antibody against H3 acetylated at Lys9/14. *ACT1* open reading frame was used as the internal control

Fig. 2.

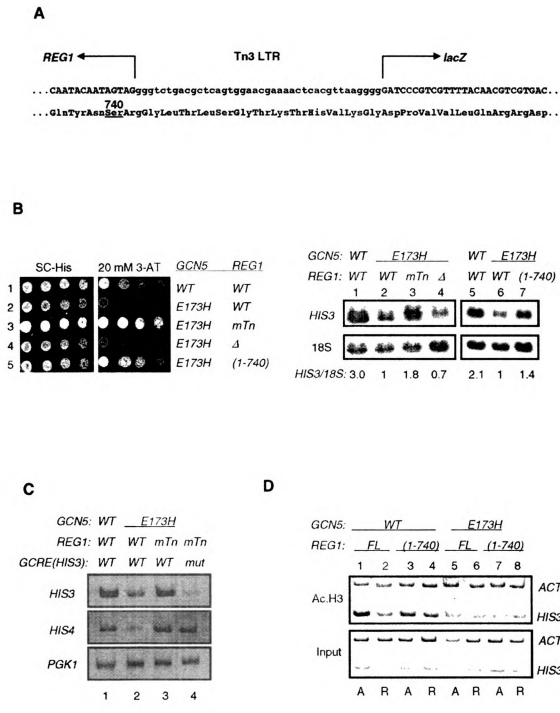
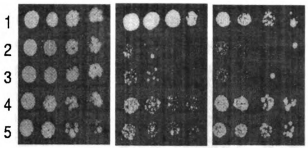

A
REG1
Tn3 LTR
lacZ
...CAATACAATAGTAGgggtctgacgctcagtggaacgaaaactcacgttaaggggGATCCCGTCGTTTTACAACGTCGTGAC...
740
...GlnTyrAsnSerArgGlyLeuThrLeuSerGlyThrLysThrHisValLysGlyAspProValValLeuGlnArgArgAsp...
B
SC-His
20 mM 3-AT
GCN5 REG1
1 WT WT
2 E173H WT
3 E173H mTn
4 E173H Δ
5 E173H (1-740)
GCN5: WT E173H WT E173H
REG1: WT WT mTn Δ WT WT (1-740)
1 2 3 4 5 6 7
HIS3
18S
HIS3/18S: 3.0 1 1.8 0.7 2.1 1 1.4
C
GCN5: WT E173H
REG1: WT WT mTn mTn
GCRE(HIS3): WT WT WT mut
HIS3
HIS4
PGK1
1 2 3 4
D
GCN5: WT E173H
REG1: FL (1-740) FL (1-740)
1 2 3 4 5 6 7 8
Ac.H3
ACT1
HIS3 +1
Input
ACT1
HIS3 +1
A R A R A R A R

FIG. 3. The *BGR* suppressor is semidominant and selectively rescues the E173H defects of the GCN pathway. (A) Semidominant features of the *REG1(1-740)* allele. Diploid strains bearing different combinations of *GCN5* or *REG1* alleles were tested for their resistance to 3-AT at 30 or 37°C. (B) Allele specificity of the suppression. The *REG1(1-740)* allele was integrated to the chromosome to replace the wild-type *REG1* gene in different *gcn5*$^-$ strains. Resultant strains were then spotted to 3-AT medium and grown at 37°C. (C) Growth defects in different conditions caused by *gcn5*$^-$ mutations are not affected by the Reg1(1-740) protein. Indicated strains were grown to log phase in YPD, spotted to yeast extract-peptone-glycerol, yeast extract-peptone-ethanol, or YPD containing 100 mM hydroxyurea (HU), and incubated at 30°C for 3 to 4 days

Fig. 3.

A



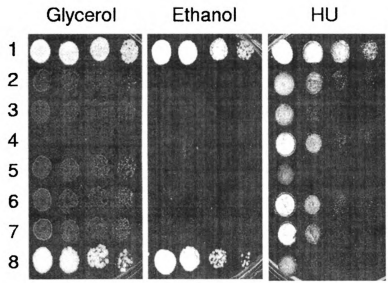
	GCN5 / GCN5	*REG1 / REG1*
1	*WT / E173H*	*WT / WT*
2	*E173H / E173H*	*WT / WT*
3	*E173H / E173H*	*WT / WT*
4	*E173H / E173H*	*WT / mTn*
5	*E173H / E173H*	*WT / mTn*

B

	GCN5	*REG1*
1	*WT*	*WT*
2	*WT*	*(1-740)*
3	*E173H*	*WT*
4	*E173H*	*(1-740)*
5	*Δ*	*WT*
6	*Δ*	*(1-740)*
7	*F221A*	*WT*
8	*F221A*	*(1-740)*

Fig. 3.

C

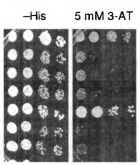


GCN5	*REG1*
WT	*WT*
E173H	*WT*
E173H	*mTn*
E173H	*(1-740)*
Δ	*WT*
F221A	*WT*
F221A	*(1-740)*
WT	*Δ*

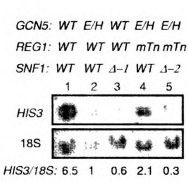
FIG. 4. *SNF1* is important for *HIS3* expression and *BGR* phenotypes. (A) Deleting *SNF1* causes hypersensitivity to 3-AT in *REG1* and *REG1*::*mTn* strains. The *snf1*Δ*-2*::*TRP1* allele contains a *TRP1* insertion at amino acid 109. The *snf1*Δ*-1*::*LEU2* allele lacks the entire ORF of *SNF1*. (B) Northern hybridization of some of the strains shown in panel A. Only induced transcription is shown. The ratios of *HIS3*/18S rRNA were normalized to the *gcn5* E173H sample (lane 2) and are shown at the bottom. (C) Overexpression of Snf1 protein also selectively rescues the E173H allele of *GCN5*. (Left panel) A multicopy *GCN5* construct does not rescue *snf1*Δ*-1*. (Right panel) *gcn5* E173H was selectively rescued by a multicopy *SNF1* construct. Growth at 37°C is shown.

Fig. 4.

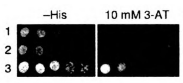
A


–His
5 mM 3-AT
GCN5
REG1
SNF1
1 WT WT WT
2 WT WT Δ–1::LEU2
3 WT WT Δ–2::TRP1
4 WT WT Δ–2::TRP1
5 E173H mTn WT
6 E173H mTn Δ–2::TRP1
7 E173H mTn Δ–2::TRP1

B


GCN5: WT E/H WT E/H E/H
REG1: WT WT WT mTn mTn
SNF1: WT WT Δ–1 WT Δ–2
1 2 3 4 5
HIS3
18S
HIS3/18S: 6.5 1 0.6 2.1 0.3

C


–His
10 mM 3-AT
GCN5 snf1Δ–/+
1 vector
2 2μ GCN5
3 2μ SNf1

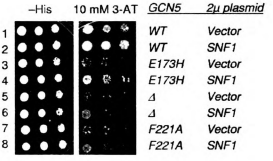
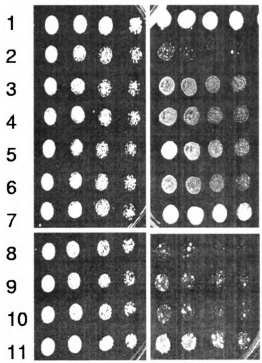

–His
10 mM 3-AT
GCN5
2μ plasmid
1 WT Vector
2 WT SNF1
3 E173H Vector
4 E173H SNF1
5 Δ Vector
6 Δ SNF1
7 F221A Vector
8 F221A SNF1

Fig. 5.

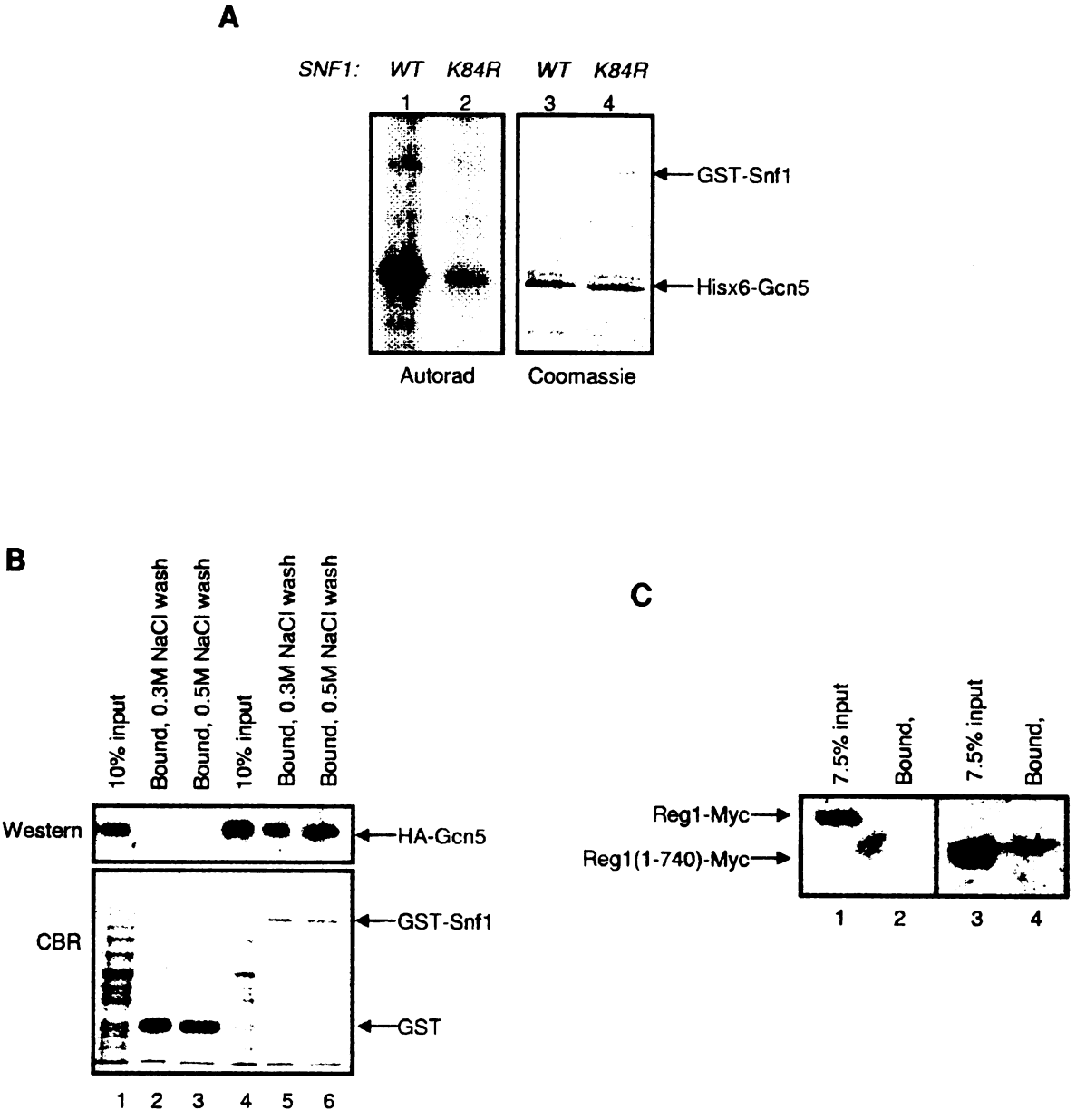


	GCN5	REG1	H3
1	WT	WT	WT
2	E173H	WT	WT
3	E173H	(1-740)	WT
4	E173H	(1-740)	S10A
5	E173H	(1-740)	S10E
6	E173H	(1-740)	S10A/28A/31A
7	E173H	(1-740)	S10E/28D/31D
8	E173H	WT	S10A
9	E173H	WT	S10E
10	E173H	WT	S10A/28A/31A
11	E173H	WT	S10E/28D/31D

FIG. 5. H3 Ser10 phosphorylation is not required for the *BGR* phenotypes. Yeast strains expressing the desired H3 mutants were tested on 3-AT plates at 37°C. Strains derived from this background (2) were more sensitive to 3-AT. The choice Asp or Glu in site-directed mutagenesis was based on whether a restriction site could be generated.

FIG. 6. Biochemical interactions of Gcn5/Snf1 and Gcn5/Reg1(1-740) proteins. (A) Recombinant Gcn5 is phosphorylated by the wild-type but not the K84R Snf1 protein. GST-Snf1 or GST alone was purified from yeast and incubated in the presence of [γ-^{32}P]ATP with recombinant Gcn5 protein. (B) Copurification of Gcn5 and Snf1 proteins from yeast. HA-Gcn5 and GST-Sfn1 or GST alone were expressed in yeast, and the whole-cell extracts were subjected to glutathione affinity purification. The bound materials were washed with 0.3 or 0.5 mM NaCl prior to SDS-PAGE and Western analyses using an anti-HA antibody (top). (C) Copurification of Reg1(1-740) and Gcn5 proteins from yeast. Gcn5 was C-terminally tagged with protein A and coexpressed in yeast strains with Reg1-Myc or Reg1(1-740)-Myc recombinant proteins. Whole-cell lysates were bound to immunoglobulin G beads and analyzed by SDS-PAGE and anti-Myc Western analyses.

Fig. 6.


A
SNF1: WT K84R WT K84R
1 2 3 4
GST-Snf1
Hisx6-Gcn5
Autorad
Coomassie
B
10% input
Bound, 0.3M NaCl wash
Bound, 0.5M NaCl wash
10% input
Bound, 0.3M NaCl wash
Bound, 0.5M NaCl wash
Western
HA-Gcn5
CBR
GST-Snf1
GST
1 2 3 4 5 6
C
7.5% input
Bound,
7.5% input
Bound,
Reg1-Myc
Reg1(1-740)-Myc
1 2 3 4

Supplementary Data:

Snf1 and Reg1(1-740) likely function together for the *bgr* phenotypes

That deleting *GCN5* or *SNF1* abolishes the *bgr* phenotypes demonstrates the importance of both Gcn5 and Snf1 proteins. While the unique allele specificity for the E173H mutation suggests that Reg1(1-740) may act directly on Gcn5, it is unclear whether Snf1 is a target for the Reg1(1-740) suppressor protein. Given that Reg1 and Snf1 can interact physically and functionally (see Introduction), and that the Snf1 interaction domain of Reg1 remains intact in the *bgr* allele (79), it is tempting to speculate that the Reg1(1-740) protein elicits the *bgr* phenotypes via the Snf1 kinase. If so, deleting *SNF1* from a *GCN5 reg1(1-740)* strain will eliminate the suppressing power of the Reg1(1-741) protein. To test the above hypothesis, we introduced the *reg1(1-740)* mutant into a 3-AT sensitive *GCN5 snf1*Δ strain and tested the cellular growth on 3-AT plates. Unlike the strong *bgr* phenotypes for E173H, the Reg1(1-740) protein fails to rescue the 3-AT sensitivity of the *snf1*Δ strain. Although at 10 mM 3-AT, a slightly better growth was observed in the *GCN5 reg1(1- 740) snf1::TRP1* strain, this improvement in 3-AT resistance is minute compared with the *SNF1*+ cells (row 3, Figure S1). Similarly, knocking out *SNF1* from the 3-AT resistant *GCN5 reg1(1- 740)* strain sensitizes drastically the cellular growth on 3-AT plates (data not shown). These results suggest that Snf1 is likely a key co-factor for Reg1(1-740) to carry out the *bgr* functions.

Fig. S1.

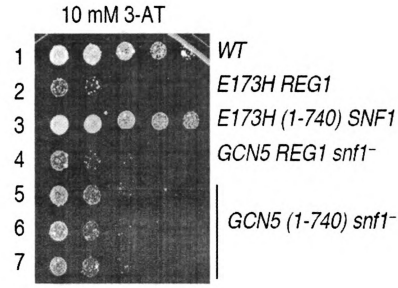


Figure S1. Reg1(1-740) protein does not rescue *snf1*– 3-AT hypersensitivity. The *reg1(1-740)* allele was introduced to the *GCN5 snf1::TRP1*(*snf1*⁻) strain. After confirming the correct genotype by genomic PCR, three integrants (rows 5-7) and other control strains were tested on 3-AT plates at 37°C.

Fig. S2.

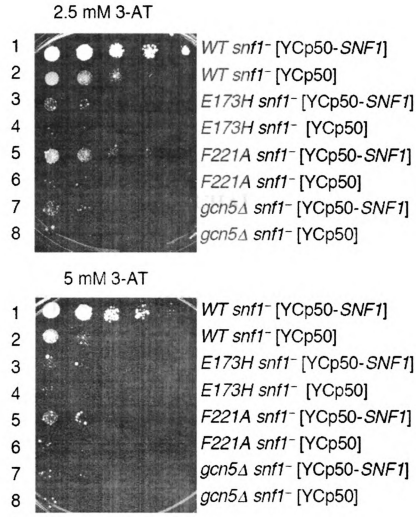


Figure S2. Genetic interaction between Gcn5 and Snf1 for *HIS3* expression. A. Gcn5 and Snf1are likely part of two pathways for *HIS3* expression. *SNF1* was deleted from the indicated *gcn5*⁻ strains (all *snf1*⁻ strains used in this panel were *snf1::TRP1*), and the resultant strains were transformed with YCp50, an *ARS CEN* plasmid, or YCp50-*SNF1* that contained a wildtype *SNF1* gene under the control of the native *SNF1* regulatory elements.

CHAPTER III

Snf1p activates *HIS3* transcription by antagonizing the inhibitory effects of Spt3p and Spt8p

(Manuscript for Mol. Cell. Biol.)

Snf1p activates *HIS3* transcription by antagonizing the inhibitory effects of Spt3p and Spt8p

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ABSTRACT

Transcriptional activation of the *Saccharomyces cerevisiae HIS3* gene requires both Gcn5 histone acetyltransferase (HAT) and Snf1 kinase. Our studies suggest Gcn5 protein is a likely target for Snf1 in *HIS3* regulation because the phosphorylation of Gcn5 *in vivo* is dependent on the Snf1 dosage. Moreover, the Gcn5 residues (T203, S204, T211 and Y212; TSTY) that are important for Snf1 mediated phosphorylation are critical for *HIS3* activation as well. Interestingly, the *HIS3* transcriptional defect caused by substitution of the above essential residues is suppressed by deleting the Spt3 component of the Gcn5 related coactivator complexes, which indicates that Spt3 protein plays negative roles in *HIS3* transcription. The notion that Snf1 and TSTY residues of Gcn5 protein are in the same regulatory pathway is further supported by the observation that deleting *SPT3* also rescues *snf1Δ* in *HIS3* activation. As expected, knocking out Spt8, which is functionally related to Spt3 protein, is also able to rescue the impairment caused by *snf1Δ* and *gcn5 TSTY/4A* (TSTY residues substituted to alanine) mutants. The *in vitro* pull-down assays demonstrate direct interaction between Gcn5 protein and Spt3 protein, suggesting that Spt3 protein inhibits Gcn5 function by physical association. Thus, one possible function of Snf1 is to disrupt the interaction by phosphorylating Gcn5 protein or Spt3 protein or both.

INTRODUCTION

Eukaryotic DNA is packaged into a structure called chromatin. Chromatin modifying enzymes, e.g. ATP-dependent chromatin remodelers and covalent histone modification enzymes, dynamically alter the chromatin structure and/or chromatin surface to change the accessibility of genetic information that is carried by DNA. Histone acetylation is a well studied covalent modification involved in the regulation of many cellular processes (5). In particular, the promoter acetylation of histones is usually coupled with gene activation; and histone deacetylases (HDACs), the enzymes that mediate deacetylation reactions, are considered to be one of the most promising targets of anticancer drugs (31).

In *Saccharomyces cerevisiae*, the Spt-ADA-Gcn5-acetyltransferase (SAGA) complex is one of the histone acetyltransferase complexes that regulates transcription of about 10% of yeast genes (18). The 1.8 MDa SAGA complex is composed of more than 19 subunits and possesses multiple activities related to transcriptional regulation. The SAGA complex has two known histone modification activities. Gcn5, together with the adapter proteins Ada2 and Ngg1/Ada3, mediates histone acetylation (12, 22, 23, 34). In addition, SAGA is able to remove ubiquitin from histone H2B through the activity of Ubp8 and Sgf11 subunits (16, 19, 24). Polyglutamine extension of Sca7/Sgf73 protein is critical for HAT activity of SAGA (30). The Tra1 protein, the largest SAGA component, interacts with acidic activators for promoter recruitment of the complex (3, 4). The chromodomain containing protein Chd1 might be involved in substrate recognition by interacting with

methylated lysine 4 of histone H3 (33). Three backbone proteins, Hfi1/Ada1, Spt7 and Spt20, are essential for SAGA integrity (42). A group of TBP association factors (TAFs) that are shared between TFIID and SAGA (Taf5, Taf6, Taf9, Taf10 and Taf12), are also thought to help maintain the architecture of the complex (13, 50). A module containing Spt3 and Spt8 proteins regulates gene transcription by modulating TBP activity (9, 42).

Snf1 is the yeast ortholog of the conserved AMPK kinase that is important for responses to metabolic stress (15). The kinase exists in several heterotrimers, each consisting of the catalytic α subunit, Snf1, the regulatory γ subunit, Snf4, and one of the three scaffold β subunits, Gal83, Sip1 or Sip2, which tether Snf1 and Snf4 together (20). The three β subunits show functional redundancy as *snf1*$^-$ phenotypes are achieved when all three subunits are deleted (37). Recent studies suggest that the selection of β subunit incorporation may affect the cellular localization of the kinase complex (45). Moreover, Snf1 protein is able to form a homodimer, based on the crystal structure study of the Snf1 kinase domain (32, 35). Snf1 is activated by upstream kinases, Sak1, Elm1 and Tos3, through phosphorylation of threonine 210 in the activation loop (17, 29). On the other hand, the Snf1 activity is repressed by Reg1 protein, which helps recruit the protein phosphatase Glc7 that dephosphorylates and inactivates Snf1 (8, 36). The roles of Snf1 protein in regulating transcription of genes related to adaptation of alternative carbon sources have been well characterized. Snf1 kinase may stimulate transcription by phosphorylating activators Cat8 and Sip4 (44). Snf1 mediated phosphorylation of Mig1 protein disrupts the Mig1-Ssn6 repressor at the promoter, leading to derepression of the downstream genes (43). Genetic and biochemical studies indicated Snf1 interacts with the

mediator complex or TATA-binding protein directly (21, 39, 40). Interestingly, Snf1 also phosphorylates Ser 10 of histone H3 for INO1 activation (27). H3 phosphorylated at Ser 10 exhibits a stronger affinity for Gcn5 protein, linking H3 phosphorylation to acetylation (7, 27, 28).

Our previous studies showed that Snf1 protein positively regulates *HIS3* transcription, as deletion of *SNF1* abolished the *HIS3* activation under amino acid starvation conditions. In addition, we showed that *SNF1* is a multi-copy suppressor of the E173H mutation of *GCN5* (26). Instead of acting as a histone kinase, we found that Snf1 protein more likely regulates the function of the SAGA coactivator complex, since replacing Ser10 of H3 with alanine or glutamate neither attenuates nor augments the suppression phenotypes. Moreover, Snf1 protein interacts with Gcn5 protein *in vivo* and phophorylates Gcn5 protein *in vitro* (26). We suggested that Gcn5 phosphorylation is critical for *HIS3* activation,

We now present evidence for Snf1-dependent Gcn5 phosphorylation in *vivo*. The residues in Gcn5 protein that are important for Snf1-mediated *in vitro* phosphorylation are important for Gcn5 *in vivo* functions as well. Interestingly, transcription defects resulting from the mutations of the putative phosphorylation sites are rescued by deletion of another SAGA component, *SPT3*. Deletion of *SPT3* also suppresses the defect in *HIS3* activation caused by *snf1Δ*. Similar to Gcn5, Spt3 protein copurified with Snf1 *in vivo*. Moreover, *in vitro* pull-down assays revealed that Spt3 protein can bind Gcn5 protein directly. Together, we suggest that Spt3 protein performs a negative role in *HIS3*

activation by interacting directly with Gcn5 protein, and this inhibition is alleviated by Snf1-mediated phosphorylation of Gcn5 protein or Spt3 protein.

MATERIALS AND METHODS

Yeast stains, plasmids and genetic methods.

Yeast strains used in this work are listed in Table 1. All genetic methods were performed according to reference (38). Yeast transformation was done using the lithium acetate method (11). Plasmids used in this work are listed in Table 2.

The *spt3Δ* strains were created by introducing a PCR fragment containing the KanMX6 cassette flanked by *SPT3* sequences outside the open reading frame (46). G418-resistant transformants were examined by genomic PCR to confirm the *spt3Δ* genotype. The *spt8Δ* strains were generated with similar strategy except the *SPT8* open reading frame is replaced by the *TRP1* gene.

Gcn5-myc and Snf1-myc strains were generated by transforming the gel-purified PCR products using pYL67 (26) as template to amplify the *8xmyc::TRP1* cassette with flanking sequences of *GCN5* or *SNF1*. The integration was confirmed by genomic PCR and expression was confirmed by western blot with α-c-myc antibody (9E10, Roche).

pYL89 with an N-terminal hemagglutinin (HA) tag was created by co-transforming XbaI-linearized pMK547 (26), and a PCR-amplified *SPT3* open reading frame. The HA-Spt3-TAP fusion construct (pYL99) was generated by co-transformation of the large

fragment of BamHI-NgoMIV digested pYL54 and a PCR amplified *HA-SPT3* fragment from pYL89 flanked with pYL54 sequences outside the *GCN5* open reading frame. The PCR product containinig *HA-SPT3* was digested with EcoRI-XhoI and inserted into the same sites of pET21a to create pYL90 that expresses HA-Spt3 in bacteria. All of the above constructs were verified by PCR and the expression was tested by Western blot with α-HA antibody (12CA5, Roche) or Rabbit antiserum (for TAP tagged construct). Plasmids with Gcn5 mutations were created by following the QuikChange mutagenesis protocol (Roche).

RNA preparation and RT-PCR.

Yeast cells were grown in appropriate selection media until the OD_{600} reached 0.5. Cells were then pelleted by centrifugation (5,000 × *g*, 5 min, 4°C) and transferred to SD supplemented with required nutrients and 40 mM 3-AT (for induced expression). Cell suspensions were further incubated at 37°C for 2 to 3 h before harvesting for RNA preparation. Procedures for RNA preparation was described previously (23). 10 μg of RNA was treated with 10-unit DNaseI (Roche) in 100μL (50mM Tris-HCl, pH7.5, 5mM $MgCl_2$), and incubated at 37°C for 1 hour. The cDNA was created following the instruction of ImpronII reverse transcriptase (Amersham) kit using of 30 ng of poly(dT) for priming. The resultant cDNAs were diluted 50 fold. PCR reactions contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0) at 25°C, 1% Triton X-100, 2 mM $MgCl_2$, 0.1 mM each dNTP, 0.5 μM each primer, and 1.25 U Taq DNA polymerase (Promega), and

appropriately diluted DNA templates. PCR parameters were (94°C, 4 min; 50°C, 4 min; 72°C, 30 sec) for 2 cycles; (94°C, 45 sec; 50°C, 45 sec; 72°C, 30 sec) for 24 cycles; and 72°C, 3 min. PCR products were resolved in polyacrylamide gels followed by Ethyldium Bromide staining.

***In vitro* kinase assays.**

Kinase assays were performed with recombinant Gcn5 protein incubated with glutathione *S*-transferase (GST)-tagged Snf1 (wild-type or K84R) expressed and purified from yeast according to reference (14). The GST-Snf1 constructs were kindly provided by D. Thiele (Duke University).

Affinity purification and Immunoblot.

Snf1 and Gcn5 interaction was tested as previous described (26) except the Gcn5 was myc-tagged at the c-terminus within the chromosomal copy. To test the interaction between Spt3 and Snf1 proteins, a GST-Snf1 expression construct (14) or just GST (pYL44) was transformed to the strain carrying pYL89 (HA-Spt3). Purification of GST-Snf1 was performed as described previously (26). Glutathione Sepharose 4B (10 µl; Amersham) was added to whole-cell extracts purified from 3×10^9 cells and incubated at 4°C for 1 hour with constant rocking. Beads were pelleted and washed three times with HEMGT buffer (25 mM HEPES, pH 7.9, 12.5 mM $MgCl_2$, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1× Complete

protease inhibitor cocktail (Roche)). The bound fractions were eluted with SDS loading buffer and resolved by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The copurified Gcn5-myc protein or HA-Spt3 protein was detected by α-c-myc (9E10, Roche) or α-HA antibodies (12CA5, Roche).

***In vitro* interaction.**

The recombinant protein was induced in the *E. coli* BL21 (DE3) strain by adding 0.1 mM (final concentration) IPTG when cell culture reached an optical density at 600 nm (OD_{600}) of 0.5/ml. Cell cultures were grown at 16°C overnight. Extraction and protein affinity purification were done according to reference (23) except the *E.coli* cells containing the recombinant HA-Spt3 were first broken with buffer containing 2% SDS, and diluted 20 fold afterwards to solve the insolubility problem. The *in vitro* pull down assays were done by incubating the immobilized protein with bacterial whole cell lysates containing the protein of interest at 4°C for 1 hour. After washing 3 times with HEMGT buffer, the proteins were eluted with SDS loading buffer and subjected for western analyses with α-HA (12CA5, Roche) or α-His-tag (BD Scientific) antibodies.

Phosphoprotein staining.

To detect *in vivo* phosphorylation of proteins, the yeast cells were grown in appropriate media at 30 °C until late log phase. Then the cultures were harvested by centrifugation and washed with PBS buffer. Whole cell lysate (WCL) from 3 x 10^9 cells was prepared

by agitate beating with a mini-bead-beater (BioSpec Inc.) as described (26), except the 800 μL PiPT buffer (50 mM potassium phosphate, pH 7.5; 140 mM potassium chloride; 0.1% TritonX-100; 1 mM DTT; 1 mM EDTA; 1 mM sodium orthovanadate; 10 mM sodium fluoride; 1 tablet/20 mL Protease Inhibitor Cocktail (Roche); 1 mM PMSF) was used. Affinity purification was done by incubating the 500 μL of WCL with 20μL of IgG sepharose 6G (Amersham) at 4°C for 1 hour. The IgG beads were collected with gentle centrifugation and washed twice with PiPT buffer without protease inhibitors, followed by two more washes with PiPT buffer with higher concentration of potassium chloride (500 mM).

The IgG sepharose affinity purified proteins were eluted from the beads with SDS loading buffer and resolved on an SDS-PAGE mini-gel. Then the gel was fixed with 100 mL of 50% methanol/ 10% acetic acid (v/v) for 1 hour to over night. The residual methanol and acetic acid was removed by washing with 50 mL deionized water for 10 minutes with gentle agitation. After repeating the washing for 3 times, the gel was incubated with 50 mL of Pro-Q Diamond Phosphoprotein Gel Staining solution (Molecular Probes) for 1 hour, with gentle agitation in dark. To reduce the background and non-specific staining, the gel was treated with destaining solution [50 mM NaOAc, pH 4.0; 20% acetonitrile (v/v)] for 30 minutes with 3 repeats. Finally, the gel was washed twice with deionized water at room temperature for 5 minutes per wash. The staining was detected by Molecular imagerFX-PRO Plus (BioRad). Commassie staining was performed after the gel was scanned to check the evenly loading.

RESULTS

Overproducing Snf1p causes Gcn5p hyperphosphorylation *in vivo*.

Previously we showed that Snf1p phosphorylates Gcn5p *in vitro* and that Snf1p and Gcn5p co-purified from yeast extracts (26). It is likely that Snf1p phosphorylates Gcn5p in a physiological environment. To test this hypothesis, we partially purified TAP-tagged Gcn5 protein from yeast with IgG affinity matrix. Phospho-staining with Pro-Q Diamond (see Materials and Methods) was used to examine the Gcn5 phosphorylation status (Fig. 1A). Clear staining of Gcn5-TAP was observed (Lane 2, about 70 KDa) and the band shifted upon TEV protease digestion that removed the Protein A moiety of the TAP-tag (Lane 1, about 55 KDa). The phospho-staining is specific, as the fluorescence signal was diminished upon λ protein phosphatase treatment (Lane 3). These results clearly demonstrate that Gcn5p can be phosphorylated *in vivo*.

To test whether Snf1 protein is responsible for Gcn5 phosphorylation *in vivo*, we compared the phospho-staining intensity of Gcn5-TAP proteins isolated from strains containing different doses of Snf1 protein (Fig. 1B). While Gcn5-TAP protein isolated from a *snf1Δ* strain showed positive, albeit weak, phospho-staining, a much stronger signal was seen in the Snf1 overproducing background. We thus conclude that Gcn5p is a substrate for the Snf1 kinase *in vivo*. The weak phospho-staining of Gcn5p in the *snf1Δ* background also suggest a Snf1-independent phosphorylation of Gcn5p *in vivo*.

Potential phosphorylation sites in Gcn5p are essential for *HIS3* induction

To understand the biological function of Gcn5 phosphorylation, we first mapped the phosphorylation sites in Snf1p modified recombinant Gcn5 by mass spectrometry. The preliminary data suggested that T203, S204, T211 and Y212 might be phosphorylated (not shown). Because Snf1p is a Ser/Thr kinase, we tested the *in vitro* phosphorylation efficiency of Gcn5p bearing mutations at T203, S204 and /or T211 (Fig. 2A). While none of the single mutations caused significant reduction in Gcn5 phosphorylation, T203A/S204A and T203A/S204A/T211A mutations drastically diminished the *in vitro* labeling, indicating that all 3 residues are likely phosphorylated by Snf1p. We consistently observed elevated phosphate labeling of T203A and T211A mutants. If Gcn5 phosphorylation is physiologically relevant to *HIS3* activation, we expect that the mutation of phosphorylation site(s) will affect *HIS3* transcription. Indeed, a quadruple mutation (T203A/S204A/T211A/Y212A, TSTY/4A) impaired the cellular ability to survive under 3-AT stress and *HIS3* induction as well (Fig. 2B and 2C). Without visible defects in rich (YPD) and histidine dropout media (SC-His), the TSTY/4A allele caused poor growth upon 3-AT treatment. Consistent with the 3-AT growth defect, the *HIS3* expression was barely detectable by RT-PCR (Fig. 2B). These results indicate the T203/S204/T211/Y212 residues of Gcn5p, which are important for Snf1p-mediated phosphorylation *in vitro*, are essential for Gcn5p function in *HIS3* transcription.

The *gcn5 TSTY/4A* mutant is suppressed by *spt3Δ*

Spt3p is a subunit of the SAGA complex, and it interacts with the TATA-binding protein, TBP (9). Under the non-inducing condition, *HIS3* expression is repressed by Spt3 protein as the deletion of *SPT3* derepresses *HIS3* transcription in synthetic complete media (1). Gcn5p HAT activity is still required to active HIS3 transcription as *gcn5Δ spt3Δ* and *gcn5E173H spt3Δ* strains are sensitive to 3-AT (Fig. 2B). Different from the *gcn5Δ* and *gcn5E173H* mutations, the *gcn5 TSTY/4A* mutation was suppressed by *spt3Δ* (Fig. 2B and 2C). The *gcn5 TSTY/4A spt3Δ* strains showed robust growth under a harsh amino acid starvation condition, as evidenced by resistance to 30 mM 3-AT. *HIS3* mRNA level increased significantly in a double mutation strain as well. The negative effect of Spt3p is further confirmed by complementation of the *gcn5 TSTY/4A spt3Δ* strain with a multicopy plasmid carrying the wild type *SPT3* gene. By overexpression of *SPT3*, the suppression phenotype is lost (Fig. 2D, row 8). Notably, increasing the copy number of *SPT3* caused cellular sensitivity to 3-AT, even in a strain background containing wild type Gcn5p (Fig. 2D, row 4; and Fig. 3C, row 2). This result provides another piece of evidence that Spt3p functions as a repressor.

In summary, the results above confirmed the negative roles of Spt3 protein in *HIS3* expression, which are related to Gcn5 TSTY functions. Such functions related to TSTY residues of Gcn5p might be activated by Snf1 mediated phosphorylation, as these residues are essential for *in vitro* phosphorylation by Snf1. The TSTY residues of Gcn5 might represent an activity different from the canonical HAT function, since the

gcn5E173H and *gcn5Δ* are not rescued by removing *SPT3*. Moreover, the allele specific suppression of *spt3Δ* on *gcn5 TSTY/4A* implies that Spt3 protein might physically interact with Gcn5p.

The defect of *HIS3* expression in *snf1Δ* strains is rescued by deleting *SPT3*

That the mutations of the likely Snf1p targets (i.e. TSTY/4A) can be rescued by *spt3Δ* raises a possibility that *spt3Δ* might also rescue the *snf1Δ* defects. Indeed, in a *snf1Δ spt3Δ* double deletion strain, the *HIS3* mRNA level is much higher than that of the *snf1Δ* single mutation (Fig. 3A). The 3-AT sensitivity test results conserved with the RNA quatitation data (Fig. 3B). Moreover, re-supplying *SPT3* to the *snf1Δ spt3Δ* cells attenuated resistance to 3-AT (Fig. 3C), which means the *spt3Δ* is a genuine suppressor. Importantly, the suppression by *spt3Δ* requires the presence of Gcn5 HAT activity, since the introduction of E173H mutation eliminates the suppression thoroughly (Fig. 3B). Together, these data suggest that Snf1p functions upstream of Gcn5p, and that the HAT activity of Gcn5p might be subjected to regulation by both Snf1p and Spt3p.

The *gcn5TSTY/4A* mutation and *snf1Δ* are suppressed by *spt8Δ*.

Spt8p is functionally similar to Spt3p in the regulation of TBP recruitment to the promoter, as null mutations in *SPT8* are suppressed by some *spt3* mutations (10). Similar to Spt3p, Spt8p inhibits *HIS3* and *TRP3* basal expression (1). However, Spt8p and Spt3p

are not equivalent in all situations. For example, Spt8p is not required for TBP recruitment at *GAL1* promoter (2). Furthermore, Spt8p is absent in the SAGA like coactivator complex SLIK/SALSA (41). Accordingly, we were curious as to whether Spt8p behaved similarly to Spt3p in regulating Gcn5p and Snf1p. To address this question, *SPT8* was deleted from *snf1Δ* and *gcn5TSTY/4A* strains. The resultant strains were then analyzed for their 3-AT sensitivity. Fig. 4 shows that *spt8Δ* is also a suppressor for the *snf1Δ* and *gcn5TSTY/4A* mutations. These results implicate that Spt3p and Spt8p function together to achieve the inhibitory function.

Snf1p interacts with and phosphorylates Spt3p *in vivo*

Previously, we showed that Snf1p and Gcn5p interact *in vivo* (26). The *in vitro* kinase assay suggests that Snf1p is able to bind Gcn5p directly, although this association might be transient. Because both Snf1p and Gcn5p are associated with multi-subunit complexes, we are unsure if the Snf1p complex binds to free Gcn5 protein or in the context of SAGA or SLIK/SALSA. By examining the interaction between Snf1p and other components of the Gcn5p related complex, we believe Snf1p is likely associate with the Gcn5p in complex. In Fig.5A, an HA-epitope-tagged Spt3 protein was co-expressed in yeast with GST-Snf1 or GST. With the partial enrichment of the GST-Snf1 protein by incubating the whole cell lysate with glutathione beads, the Spt3 protein was copurified with GST-Snf1 (Lane 2) but not with GST alone (Lane 4).

The Lys84-to-arginine substitution of Snf1p is a dominant-negative mutation that eliminates the ATP binding capability (6). Presumably the K84R mutant will bind to its protein substrates more stably. Interestingly, we found a stronger interaction between Snf1K84R protein and Spt3p (Fig.5A, lane 3), but a weaker interaction between the K84R allele and Gcn5p (Fig.5B, lane 3). It suggests that Spt3p is another substrate of Snf1p in SAGA complex. Indeed, the partially purified HA-Spt3-TAP fusions were labeled by the Pro-Q Phosphoprotein staining solution (Fig. 5C). The construct bearing HA-Spt3-TAP cassette was transformed into strains in the absence of, with the chromosomal copy of or with overexpression of *SNF1* gene. By comparing the relative phospho-staining of Spt3p isolated from these strains, we observed a Snf1p dosage dependent phosphorylation of Spt3p. These results indicate that Snf1p is able to phosphorylates Spt3p and probably associates Spt3p in a direct manner *in vivo*.

Spt3p interacts with Gcn5p *in vitro*

The allele specific suppression of *gcn5TSSY/4A* by *spt3Δ* raises a possibility of direct interaction between Spt3p and Gcn5p. The Snf1p mediated phosphorylation may disrupt the association between Spt3p and Gcn5p to remove the inhibition from Spt3p.

To test the direct interaction between Gcn5p and Spt3p, we performed *in vitro* pull-down assays, using bacterially expressed Gcn5p and Spt3p. Such preparation decreases the

chance of protein phosphorylation that might affect the interaction between Gcn5p and Spt3p. Spt3p was tagged with 3xHA repeats at the amino terminus, and Gcn5p was tagged with 6xHis-tag. The fusion HA-Spt3 protein was immuno-purified and bound to Protein G agarose beads. Then the immobilized Spt3p was incubated with bacteria lysate with 6xHis-tagged Gcn5p. The bound fractions were analyzed by immunoblotting against His-tag. As shown in Fig.6A, Gcn5p was effectively pulled down by HA-Spt3 fusion (Lane 3). As a control, the protein G beads in absence of Spt3p association did not pulldown Gcn5 protein (Lane 1). The α-His-tag signal is specific because the Spt3-beads can not enrich any signal at Gcn5 position by incubation a lysate without Gcn5-6xHis protein expressed. Consistently, in the reciprocal experiment the HA-Spt3 protein is preferentially pulled down by the Ni^{2+}-Talon beads with the Gcn5-6xHis protein bounded, as shown in Fig.6B. These results demonstrated that Spt3p and Gcn5p are capable of interacting with each other directly.

DISCUSSION

Snf1 protein, as a kinase regulating cellular stress responses, has been shown to play important roles in transcriptional control. It is not surprising that Snf1p is also involved in gene activation upon amino acid starvation. In this study, we showed that Snf1p is able to interact with and phosphorylate the Gcn5p related coactivator complex *in vivo*. The phosphorylation mediated by Snf1p may antagonize the inhibitory function of Spt3p by a mechanism of altering the architecture of the SAGA complex.

Snf1p interacts with and phosphorylates SAGA or SALSA coactivator complexes

Snf1 kinase plays positive roles in transcription. Snf1-mediated phosphorylation of the repressor protein Mig1p leads to the dissociation of the corepressor complex from the promoter (43). Snf1p also phosphorylates activators, such as Cat8 and Sip4 (44). As we reported in this study, the coactivator proteins, another category of protein in transcriptional regulation, can be targeted by Snf1p. At least two components of the SAGA or SALSA/SLIK complexes, Spt3p and Gcn5p are co-purified with Snf1p and are phosphorylated *in vivo* in a Snf1p dosage dependent manner. SAGA and SALSA/SLIK are closely related coactivator complexes (41). The differences between these complexes include a SALSA/SLIK-specific truncated Spt7p subunit, and SAGA specific Spt8p subunit (41). We do not know as yet which complex(es) is targeted by Snf1p. One proposed experiment, the co-purification of full-length and truncated Spt7p with Snf1p

might be helpful for addressing the question. However, our genetics results implicate Snf1p is functionally associated with SAGA complex, since the deletion of SAGA specific component, *SPT8*, suppresses the *snf1Δ* phenotypes.

Spt3p and Gcn5p represent opposite functions in *HIS3* activation.

Spt3p and Gcn5p are two nonessential components of SAGA complex (42). In electron microscopy structure of SAGA, Spt3p and Gcn5p are located in two physically separate modules (50). Genomic studies showed that distinct groups of genes are regulated by Gcn5p and Spt3p (25). The different requirement of Spt3p and Gcn5p was further demonstrated by the observation that Spt3p but not Gcn5p is essential for *GAL1* expression (2) and the requirement is opposite toward *HIS3* activation, e.g. the *SPT3* deletion or point mutation *spt3-401* derepressed the *HIS3* gene at the non-induced condition by interacting TATA-Binding protein (1). Moreover, under certain circumstances, the two proteins have opposite functions. For example, the defect of *HO* expression by *gcn5Δ* is suppressed by *spt3Δ* (51). Our results provide further supporting for the inhibitory role of Spt3p in *HIS3* activation. By overproduction of Spt3p, we observed growth defects in the media containing 3-AT (Fig. 2D; Fig. 3C). On the other hand, deleting *SPT3* has no effect on *HIS3* induction (not shown and Belotserkovskaya et al, 1), which implies the Spt3 protein, although existed in the coactivator complex, possesses a negative effect on *HIS3* expression. Interestingly, a specific *gcn5* mutant allele TSTY/4A, rescues the defect of *spt3Δ* in alternative carbon sources usage (not shown), which indicates that Gcn5p has a possible negative effect on Spt3p, too. Such

inhibition is removed by an uncharacterized mechanism to exhibit the Spt3 function in these gene transcriptions.

Spt3 may inhibit Gcn5 function by direct association

The position of Gcn5p and that of Spt3p are spatially separated upon electron microscopy data suggested architecture of SAGA complex (50). However, our genetics and biochemical data imply that the two proteins interact closely with each other. The *in vitro* pull-down studies clearly showed the capability of direct interaction of these two proteins. Thus, we hypothesize that Spt3p binds to Gcn5p and blocks its activity in *HIS3* induction. Given that the recombinant proteins are free of phosphorylation, we further hypothesize that Snf1p mediated phosphorylation of Gcn5p or Spt3p or both may disrupt the Gcn5p-Spt3p interaction and consequently abolish the inhibition. Since Gcn5p is robustly copurified with Snf1p (26), we suspect that Snf1p is present in the SAGA complex. Snf1 is automatically activated during regular protein preparation procedure (47, 49), thus the Gcn5p or/and Spt3p in SAGA is likely in its phosphorylated form that leads to the disassociation of Spt3p and Gcn5p, which results in the separation of the two modules in the electron microscopy analyses (50). Moreover, the affinity purified SAGA complex in the Wu et al study is likely an active form and Spt3p is in a flexible domain (50), which reinforce the likelihood of Spt3p dynamically regulating Gcn5p function in an intra-complex manner.

Future study

From the results and discussion above, we can clearly see the negative roles of Spt3p in *HIS3* transcription. This inhibitory function is likely downstream of Snf1p as *HIS3* transcription defect of *snf1Δ* is suppressed by *spt3Δ*. Such suppression is gene specific since the transcription of another Snf1p target, *GAL1*, is not rescued. The allele-specific suppression of the *gcn5 TSTY/4A* mutation by *spt3Δ* suggests that the inhibition by Spt3p is mediated through Gcn5p. The interaction between Gcn5p and Spt3p *in vitro* raises the possibility that Gcn5p is inhibited by Spt3p *via* direct association, and that Snf1 kinase action alleviates this association. Several experiments are proposed to test the hypothesis in the near future.

First, we want to test whether phosphorylation of Gcn5p prevents its binding to Spt3p. Towards this goal, we will use purified Snf1p with Glutathione beads to phosphorylate recombinant Gcn5-Hisx6 (either purified or using bacteria lysate) *in vitro* and then test the ability to pull down Gcn5p by immobilized HA-Spt3 protein. We can not phosphorylate the Gcn5-Hisx6 bound to Ni^{2+}- beads because Snf1p contains a 13xHistidine stretch that will associate with any Ni^{2+} matrix as well. Such that we can not distinguish the Spt3p bound to Gcn5p or bound to Snf1p. The phosphorylation status of Gcn5p can be monitored by isotope labeling or Pro-Q phosphoprotein staining. The importance of phosphate group(s) for Gcn5p-Spt3p association could be characterized by phosphatase treatment after the kinase reaction. If the phosphorylation is critical for

blocking the Gcn5p-Spt3p association, the Gcn5p treated with the Snf1 kinase will be sequestrated from the Spt3p and the precipitated matrix, and the interaction will be restored by incubation with phosphatase. Alternatively, we will test the impact of Spt3p phosphorylation on Spt3p-Gcn5p interaction.

Second, we are very curious about the biological function(s) that Spt3p has in *HIS3* regulation. The most obvious hypothesis is that Spt3p blocks the HAT activity of Gcn5p. The reason is that Spt3p directly binds to Gcn5p, which suggests Spt3p may regulate Gcn5p function. In addition, the suppression of *spt3Δ* on *gcn5* mutations in HAT domain (i.e. TSTY/4A, Fig. 2) implies HAT domain of Gcn5p maybe the target of inhibition.

To investigate whether Spt3p association will affect Gcn5 HAT activity, we will perform *in vitro* HAT assays with recombinant Gcn5p in the presence or the absence of Spt3p. The recombinant Gcn5p, either full length or just the catalytic domain will be enriched by Ni^{2+}-talon beads followed by elution with imidazole. The recombinant HA-Spt3 protein can be affinity purified by α-HA antibody. Because the HA-Spt3 protein has poor solubility in *E.coli* cells, a newly created construct (pYL99) that expresses HA-Spt3-TAP proteins in yeast cells will be used as the backup source of Spt3p. In this case, the Spt3p could be obtained by IgG affinity purification and elution with TEV protease digestion. The Spt3 sample will be analyzed by α-Ada2 western before adding to the HAT reaction to ensure the complete removal of other HAT components that might regulate enzymic activity of recombinant Gcn5p.

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Table 1. Yeast strain list.

strains	relevant genotype	Source
yMK839	*MATa trp1 leu2-3,112 ura3-52*	(23)
yMK842	*MATa trp1 leu2-3,112 ura3-52 gcn5Δ::hisG*	(23)
yMK986	*MATa trp1 leu2-3,112 ura3-52 gcn5E173H*	(26)
yYL232	*MATa trp1 leu2-3,112 ura3-52 snf1Δ::LEU2*	(26)
yYL515	*MATa trp1 leu2-3,112 ura3-52 spt3Δ::KanMX6*	This study
yYL516	*MATa trp1 leu2-3,112 ura3-52 gcn5E173H spt3Δ::KanMX6*	This study
yYL590	*MATa trp1 leu2-3,112 ura3-52 GCN5-8xmyc::TRP1*	This study
yYL591	*MATa trp1 leu2-3,112 ura3-52 SNF1-8xmyc::TRP1*	This study
yYL600	*MATa trp1 leu2-3,112 ura3-52 gcn5E173H SNF1-8xmyc::TRP1*	This study
yYL607	*MATa trp1 leu2-3,112 ura3-52 gcn5E173H-8xmyc::TRP1*	This study
yYL622	*MATa trp1 leu2-3,112 ura3-52 SPT7-13xmyc::TRP1*	This study
yYL682	*MATa trp1 leu2-3,112 ura3-52 gcn5Δ spt3Δ::KanMX6*	This study
yYL683	*MATa trp1 leu2-3,112 ura3-52 snf1Δ::LEU2 spt3Δ::KanMX6*	This study
yYL782	*MATa trp1 leu2-3,112 ura3-52 gcn5TSTY/4A-8xmyc::TRP1*	This study
yYL783	*MATa trp1 leu2-3,112 ura3-52 gcn5TSTY/4A-8xmyc::TRP1 spt3Δ::KanMX6*	This study
yYL786	*MATa trp1 leu2-3,112 ura3-52 spt8Δ::TRP1*	This study
yYL787	*MATa trp1 leu2-3,112 ura3-52 spt8Δ::TRP1*	This study
yYL788	*MATa trp1 leu2-3,112 ura3-52 spt8Δ::TRP1*	This study

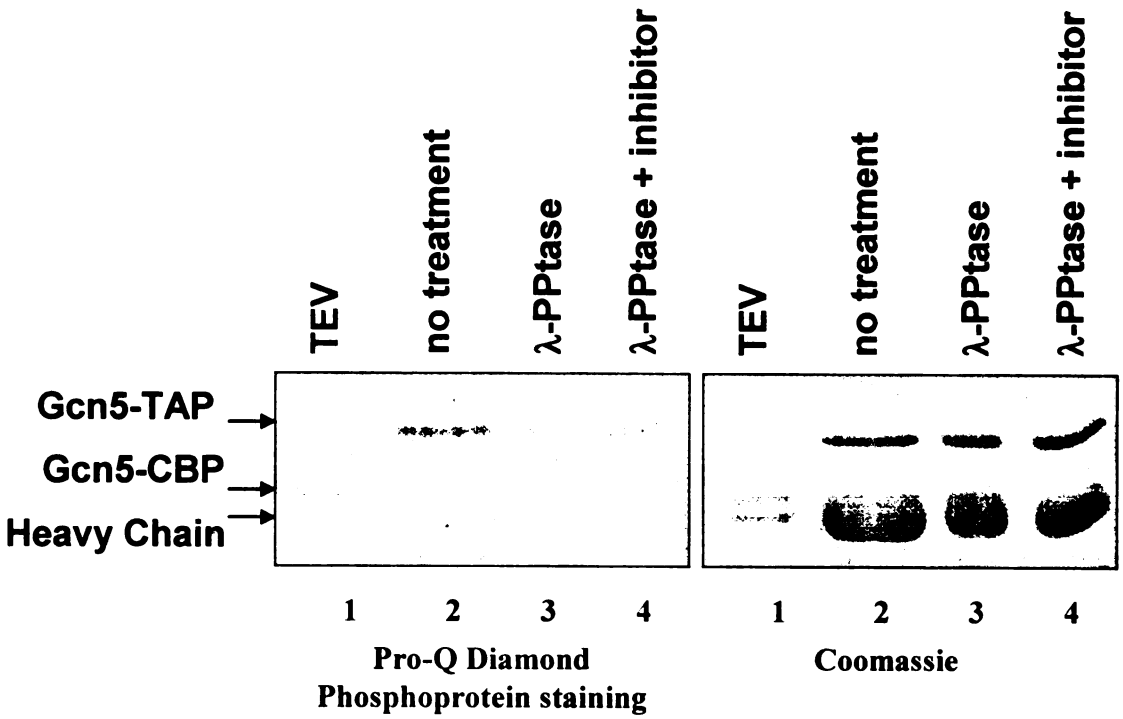
Table 2. Plasmid list.

Plasmid	Description	Source
pMK100	pRSET-Gcn5-6xHis	(23)
pFW32	YEp-*SNF1*	(48)
pYL41	YEplac112-*SNF1*	(26)
pYL42	pYEX-4T-GST-Snf1	(14)
pYL43	pYEX-4T-GST-Snf1K84R	(14)
pYL44	pYEX-4T-GST	(26)
pYL54	pYEX-4T-Gcn5-TAP	(26)
pYL55	pYEX-4T-Gcn5E173H-TAP	This study
pYL67	*8xmyc::TRP1* for tagging proteins with 8 myc repeats	(26)
pYL72	pMK547Gcn5, 3xHA-Gcn5	(26)
pYL89	pMK547Spt3, 3xHA-Spt3	This study
pYL90	pET21a-3xHA-Spt3	This study
pYL93	pYEX-4T-Gcn5TSTY/4A-TAP	
pYL98	pRSET-Gcn5TSTY/4A-6xHis	This study
pMK284TSTY/4A	Integration construct for introducing T203A/S204A/T211A/Y212A to *GCN5*	This study
pMK515	pET21-6xHis-Gcn5 protein	(26)

FIG. 1. Overproducing Snf1 protein causes Gcn5p hyperphosphorylation in vivo. (A) Gcn5 is a phosphoprotein. Gcn5 was fused to the Tandem Affinity Purification (TAP) tag consisting of the calmodulin binding protein (CBP), TEV protease cleavage site, and Protein A. Gcn5-TAP was expressed and purified from yeast by IgG beads. A fraction of the bound materials was treated with the TEV protease that specifically liberates Gcn5-CBP from the IgG beads (Lane 1). Proteins were resolved by SDS-PAGE prior to staining with the Pro-Q Diamond Phosphoprotein Gel Staining solution as described in Materials and Methods. The λ-protein phosphatase with (Lane 3) or without phosphatase inhibitors (Lane 4) was used to treat an aliquot of sample to test the specificity of the assay. The gel was stained with Coomassie Blue R250 (CBR) after scanning with fluorometer to compare the amount of proteins loaded. (B) The level of *in vivo* Gcn5 phosphorylation is correlated with the copy number of *SNF1*. The Gcn5-TAP proteins were purified from strains without (Lane 6) or with overexpressed Snf1 kinase (Lane 7) and subjected to phosphoprotein staining.

Fig. 1

A


TEV
no treatment
λ-PPtase
λ-PPtase + inhibitor
TEV
no treatment
λ-PPtase
λ-PPtase + inhibitor
Gcn5-TAP
Gcn5-CBP
Heavy Chain
1 2 3 4
1 2 3 4
Pro-Q Diamond
Phosphoprotein staining
Coomassie

B

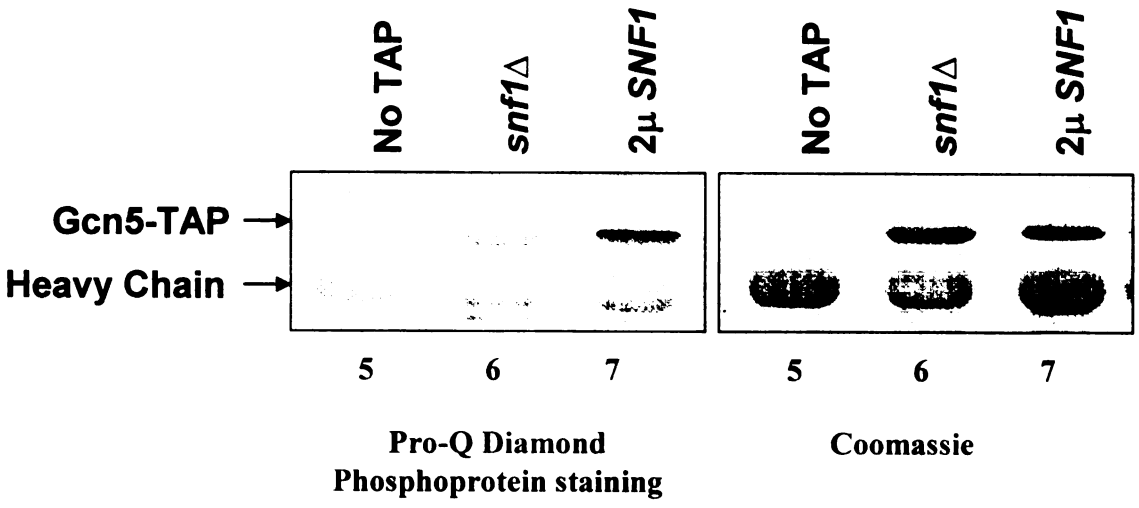

No TAP
snf1Δ
2μ SNF1
No TAP
snf1Δ
2μ SNF1
Gcn5-TAP
Heavy Chain
5 6 7
5 6 7
Pro-Q Diamond
Phosphoprotein staining
Coomassie

FIG. 2. The TSTY/4A mutation of Gcn5 was suppressed by deleting *SPT3*. (A) The T203, S204, and T211 residues are important for Gcn5 phosphorylation *in vitro*. The indicated mutations were introduced into the Gcn5 HAT domain and were expressed in bacteria. Purified recombinant proteins were subjected to *in vitro* kinase assays in the presence of γ-[^{32}P] ATP and GST-Snf1 purified from yeast. The SDS-PAGE gel was stained by CBR prior to autoradiography. (B, C) The substitution of T203, S204, T211, and Y212 residues of Gcn5 with alanines (Gcn5 TSTY/4A) impairs *HIS3* expression. The defect caused by Gcn5 TSTY/4A mutation was rescued by *spt3Δ*. (B) RT-PCR. RNA was isolated from indicated strains that grew under induced condition and subjected to RT-PCR analysis to determine the *HIS3* expression. *PGK1* was used as loading control. (C) 3-AT sensitivity test. Strains indicated were grown in YPD until late log phase. After adjusting to the same cell density, the different cultures were serially diluted and spotted onto plates in the presence or the absence of 3-AT. (D) 3-AT resistant phenotypes of *gcn5TSTY/4A spt3Δ* are suppressed by overexpression of *SPT3*. The Spt3 overproduction construct YEp-SPT3/pFW32 was transformed into *spt3Δ*, *gcn5TSTY/4A*, and double mutation strains. The resultant transformants were subjected for 3-AT sensitivity test as in (C). (E) *gcn5E173H* and *gcn5Δ* are not suppressed by *spt3Δ*. SPT3 was deleted from strains containing *gcn5E173H* mutation or *gcn5Δ* and then examined for 3-AT sensitivity. Pictures were taken after the plates incubated at 30°C or 37°C for 2-5 days.

Fig. 2

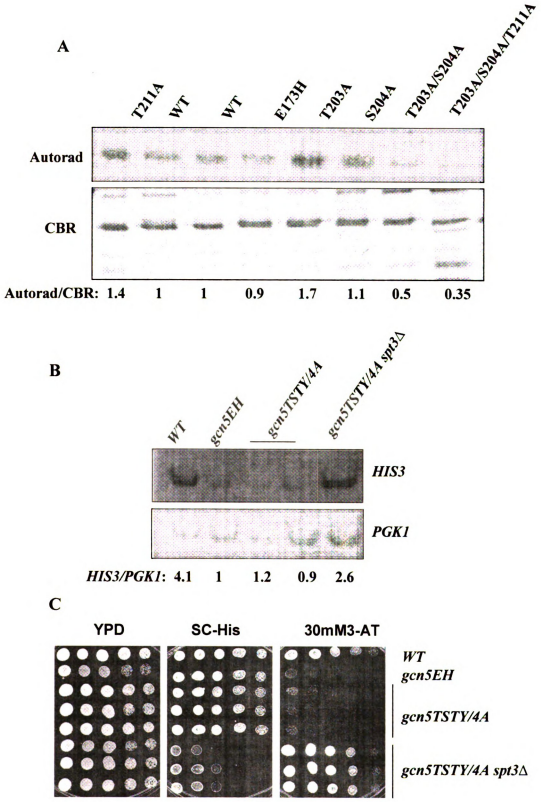
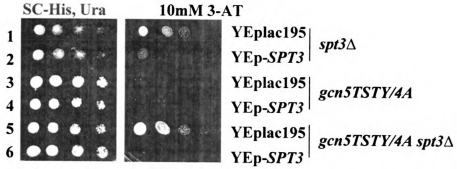

A
T211A
WT
WT
E173H
T203A
S204A
T203A/S204A
T203A/S204A/T211A
Autorad
CBR
Autorad/CBR: 1.4 1 1 0.9 1.7 1.1 0.5 0.35
B
WT
gcn5EH
gcn5TSTY/4A
gcn5TSTY/4A spt3Δ
HIS3
PGK1
HIS3/PGK1: 4.1 1 1.2 0.9 2.6
C
YPD
SC-His
30mM3-AT
WT
gcn5EH
gcn5TSTY/4A
gcn5TSTY/4A spt3Δ

Fig. 2

D


SC-His, Ura
10mM 3-AT
1
2
3
4
5
6
YEplac195
YEp-SPT3
spt3Δ
YEplac195
YEp-SPT3
gcn5TSTY/4A
YEplac195
YEp-SPT3
gcn5TSTY/4A spt3Δ

E


- 3-AT
+ 3-AT
gcn5E173H
gcn5E173H spt3 Δ
gcn5Δ spt3 Δ

FIG. 3. Spt3p antagonizes Snf1p function in *HIS3* activation. (A) RT-PCR. Yeast strains indicated were grown in YPD until late log phase and then were transfered to SD media with 40 mM 3-AT to inducing *HIS3* transcription. RNA isolation and RT-PCR were performed as in Fig. 2B. (B) 3-AT sensitivity test. *SPT3* gene was deleted in *snf1Δ* strains. The *snf1Δ* strain showed the decreased *HIS3* transcription and 3-AT sensitivity as previously reported (26). The deletion of *SPT3* rescues the *snf1Δ* phenotypes as restored *HIS3* mRNA level and wild type-like growth on 3-AT plate were observed in a double deletion strain. The Gcn5 HAT activity is required since the E173H mutation of Gcn5 in *snf1Δ spt3Δ* strain abolished the suppression phenotypes. (C) 3-AT resistant phenotypes of *snf1Δ spt3Δ* were suppressed by overexpression of *SPT3*. The *SPT3* overproduction construct (YEp-*SPT3*) and epitop-tagged Spt3p (HA-Spt3-TAP) were transformed into *snf1Δ spt3Δ* strain to complement the *spt3Δ* phenotypes.

Fig. 3

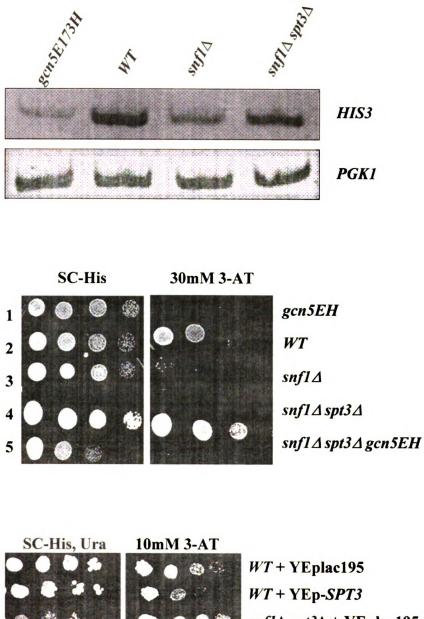

A
gcn5E173H
WT
snf1Δ
snf1Δ spt3Δ
HIS3
PGK1
B
SC-His
30mM 3-AT
1
2
3
4
5
gcn5EH
WT
snf1Δ
snf1Δ spt3Δ
snf1Δ spt3Δ gcn5EH
C
SC-His, Ura
10mM 3-AT
1
2
3
4
5
WT + YEplac195
WT + YEp-SPT3
snf1Δ spt3Δ + YEplac195
snf1Δ spt3Δ + YEp-SPT3
snf1Δ spt3Δ + 2μ HA-SPT3-TAP

Fig. 4

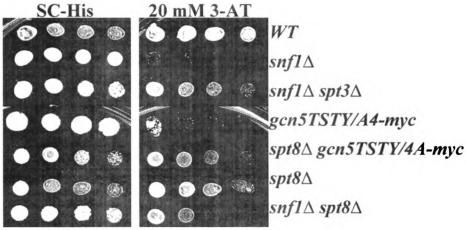
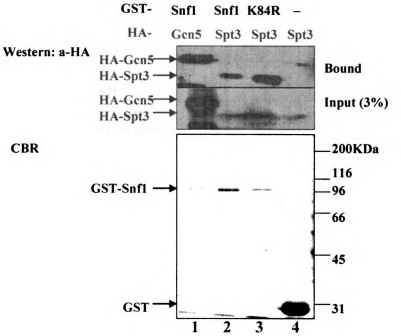


FIG. 4. Both *gcn5TSTY/4A* and *snf1Δ* are suppressed by deleting *SPT8*. *SPT8* was deleted from *gcn5TSTY/4A* and *snf1Δ* strains. 3-AT sensitivity test was performed. While *gcn5TSTY/4A* and *snf1Δ* cells grow poorly in the media in the presence of 3-AT, the further deletion of *SPT8* partially suppressed the growth defect. The *spt8Δ* per se, has no obvious phenotype observed.

FIG. 5. Snf1p interaction with and phosphorylates Spt3p. (A) Snf1p 1interacts with Spt3p. HA-tagged Spt3 protein was co-expressed with GST-Snf1 (WT, Lane 2 or K84R, Lane 3) in yeast cells. Glutathione-purified fractions were detected by anti-HA Westerns to examine the abundance of the co-purified HA-Spt3 protein (top). HA-tagged Gcn5 protein (Lane 1) and GST alone (Lane 4) were processed in parallel as the positive and negative controls. The CBR staining of the duplicate gel is shown at the bottom. (B) K84R mutation of Snf1p attenuates the interaction between Gcn5p and Snf1p. The chromosomal copy of *GCN5* was tagged with 8xmyc tandem repeats. GST-Snf1 was overexpressed in such background. Gcn5 proteins were analyzed by anti-c-myc Western after partially purified by Glutathione Sepharose (top). The blot was stained with Indian Ink after the Western analyses (bottom). (C) Spt3p is phosphorylated *in vivo* in a Snf1p dependent manner. The construct expressing HA-Spt3-TAP fusion was transformed into strain backgrounds without (*snf1Δ*), with single copy (chromosomal copy, *Chr.*) or with multiple copies of *SNF1* gene (O/E). The WCLs from the resultant strains were partially purified by IgG beads and resolved by SDS-PAGE. Then the gel was stained by Pro-Q Diamond Phosphoprotein staining solution as described in Materials and Methods followed by CBR staining. The signals were quantified by Image J and the relative ratio of Pro-Q and CBR signals were listed at the bottom.

Fig. 5

A


GST-
Snf1
Snf1 K84R
–
HA-
Gcn5
Spt3
Spt3
Spt3
Western: a-HA
HA-Gcn5
HA-Spt3
Bound
HA-Gcn5
HA-Spt3
Input (3%)
CBR
200KDa
116
96
66
45
31
GST-Snf1
GST
1
2
3
4

B

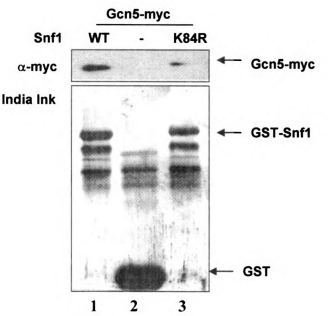

Gcn5-myc
Snf1
WT
-
K84R
α-myc
Gcn5-myc
India Ink
GST-Snf1
GST
1
2
3

Fig. 5.

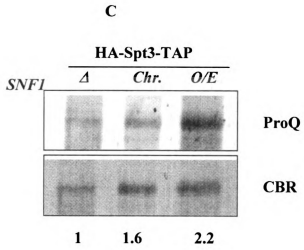
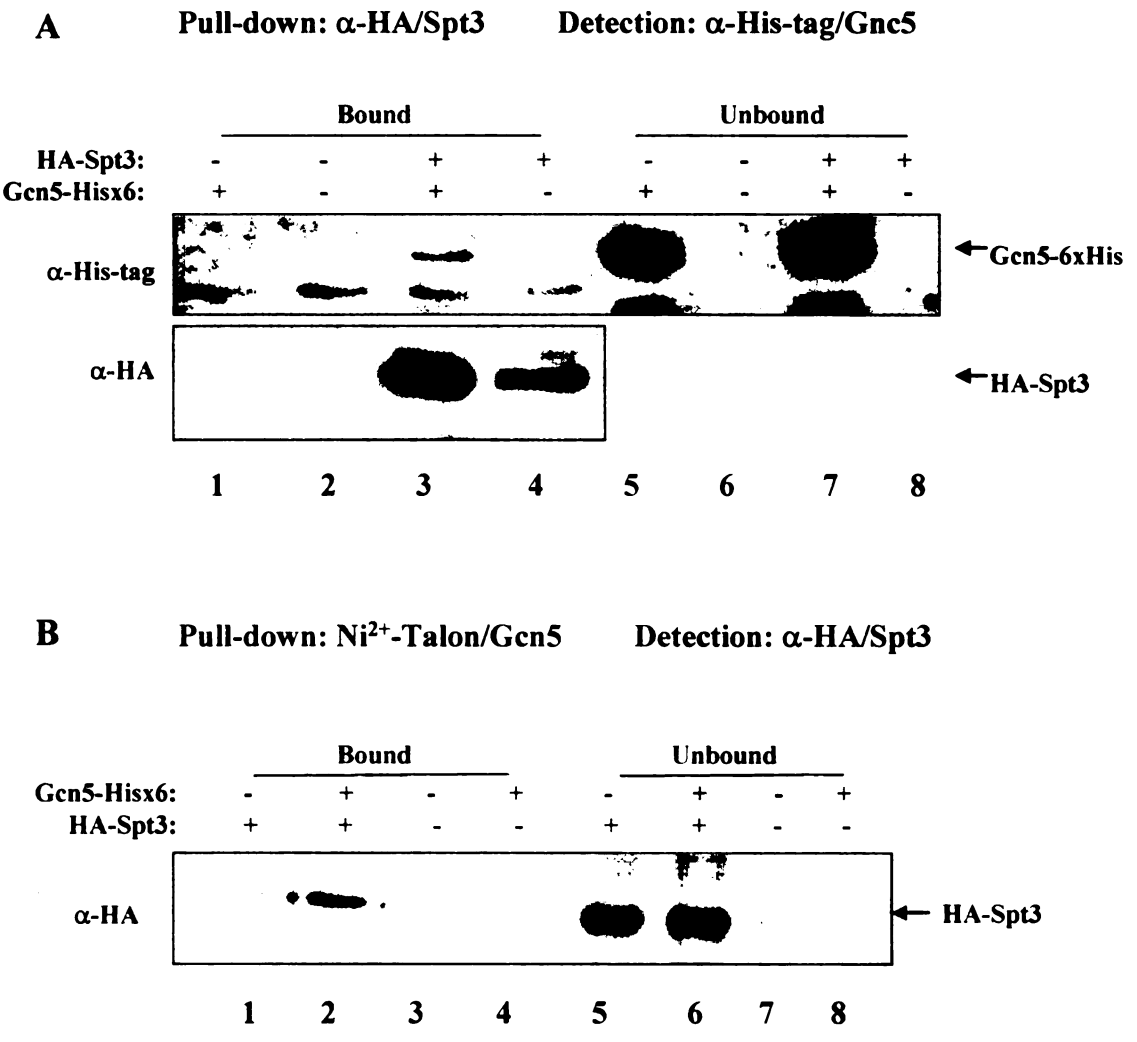


FIG. 6. Direct interaction between Gcn5p and Spt3p. (A) Gcn5p is pulled-down by HA-Spt3 protein *in vitro*. Recombinant HA-Spt3 protein was obtained by expressing pYL90 in BL21 (DE3) cells. The HA-Spt3 fusion was enriched by IP with α-HA antibody (3F10) followed immobilized on Protein G Sepharose 4G (Amersham). Then the beads were incubated with bacteria lysate with His-tagged full length of Gcn5 protein [pMK100 in BL21 (DE3)]. The bound fractions (Lane 1-4) and the unbound fractions (Lane 5-8) were examined by Western blot with α-His-tag antibody (top) followed by a second western blot with α-HA antibody (bottom). (B) Spt3p is pulled-down by Gcn5p *in vitro*. Ni^{2+}-Talon beads bound with His-tagged Gcn5 was incubated with bacteria lysate containing HA-Spt3 protein. The bound fractions (Lane 1-4) and the unbound fractions (Lane 5-8) were examined by Western blot with α-HA antibody.

Fig. 6


A
Pull-down: α-HA/Spt3
Detection: α-His-tag/Gnc5
Bound
Unbound
HA-Spt3: - - + + - - + +
Gcn5-Hisx6: + - + - + - + -
α-His-tag
Gcn5-6xHis
α-HA
HA-Spt3
1 2 3 4 5 6 7 8
B
Pull-down: Ni2+-Talon/Gcn5
Detection: α-HA/Spt3
Bound
Unbound
Gcn5-Hisx6: - + - + - + - +
HA-Spt3: + + - - + + - -
α-HA
HA-Spt3
1 2 3 4 5 6 7 8

APPENDIX

Identification of suppressors that bypass the Gcn5 requirement by screening an EMS mutagenesis library created from a *gcn5*⁻ strain

Identification of suppressors that bypass the Gcn5 requirement by screening an EMS mutagenesis library created from a *gcn5* strain

Introduction

Gcn5-mediated histone H3 acetylation at lysine14 or lysine 18 is a key step in transcriptional activation of such genes as *HIS3*, *INO1* and *HXT4* (6, 9, 14). Similarly, other histone modifications play important roles in different cellular process, such as H3 lysine 9 methylation is a mark of chromatin silencing (11). Serine phosphorylation is critical for cell cycle regulation. Such modifications may alter the charge of the histone tails to modulate the conformation of local chromatin. In other possibilities, these extra chemical groups may provide a different binding surface for other regulators association. The 'histone code' hypothesis was suggested based on the latter assumption, which is different modifications or combination of modifications may extend the information potential of the genetic (DNA) code by recruiting different regulatory factors to the specifically modified chromatin loci (13).

Supporting the "histone code" hypothesis, many histone mark-specific binding proteins and binding motifs were found in the past few years, like bromodomain is a docking site for acetylated histones (2, 5). The chromodomain specifically interacts with lysine 9 methylated H3 (1, 4, 10). The PHD finger domain specifically recognizes methylated H3

lys4 (7, 12, 15). These facts explain the outcome of interaction between modified histones and regulatory factors perfectly. However, these findings cover only a small part of histone modifications. The proteins recognizing the phosphorylated histones and ubiquitylated histones, which are known important regulatory events, are still not unraveled. Moreover, although the "mark reader" was identified, like bromodomain may stick to acetylated H3, the significance of such association and the regulatory mechanism activated are elusive.

To understand the regulatory mechanism associated with histone acetylation during the *HIS3* activation, we initiated a suppressor screening in a strain background with a catalytic inactive mutation of Gcn5 (*gcn5E173H*, reference (8)). The extra mutations were introduced by two separate strategies. One is EMS mutagenesis (summarized in Table 1), in which the alkaline mutagen will cause the transition to introduce the point mutation into the yeast genome randomly. The second approach employed a minitransposon derived library. The minitransposon insertion-derived mutagenesis and screening are discussed in Chapter 2. The screening is based on the cell resistance to a chemical named 3-amino-triazol (3-AT), which is a competitive inhibitor of *HIS3* gene product that requires the cells to express more *HIS3* to achieve the histidine biosynthesis. Thus, when we remove histidine from the media to force the cells to activate histidine production, 3-AT will kill cells that fail to induce *HIS3* gene (like *gcn5*$^-$ cells). Using the random mutagensis approaches outlined above, we are seeking extragenic mutations that survived under 3-AT treatment even in the absence of Gcn5 histone acetyltransferase activity. Given the name Bypass of Gcn5 Requirement (BGR), these mutations may

represent the regulators working downstream or in parallel with Gcn5-mediated histone acetylation. By identifying these factors functionally linked with Gcn5, we expect to draw a much clearer picture of how histone acetylation facilitates *HIS3* activation. In a more broad view, we are trying to find some clues to investigate the principles of how "histone codes" control the cellular progressing.

As with other genetic screening, a lot of false positive candidates may be picked up because of the limitation of experimental set up. The most likely pseudo-suppressors are the factors that may affect the 3-AT transport in or/and out of the cell, e.g. alter the expression or activation of the 3-AT carrier. Other mutations may enhance the production of the substrate of the *HIS3* protein, which makes 3-AT less competitive. To address this concern, β-galactosidase assay against the *HIS3-lacZ* reporter was performed after the 3-AT selection (Fig.1).

Table 1. Summary of Screening:

65% cell death
10^8 colonies screened
144 candidates resistant to 3-AT
34 candidates verified by *HIS3-lacZ* reporter analyses

The *gcn5E173H* mutation was created by Soumya Singh-Rodriguez. The EMS mutagenesis was performed by Dr. Kuo. The initial screening and verification with β-Galactosidase

assays were done by Xin-Jin Xu and Xuqin Wang. I started the characterization of the mutations with the distinguished phenotypes. These assays include Northern analyses, spore analyses and isolation of mutations, dominance test, complementation test, and *gcn5* deletion study. This chapter presents the results for these assays.

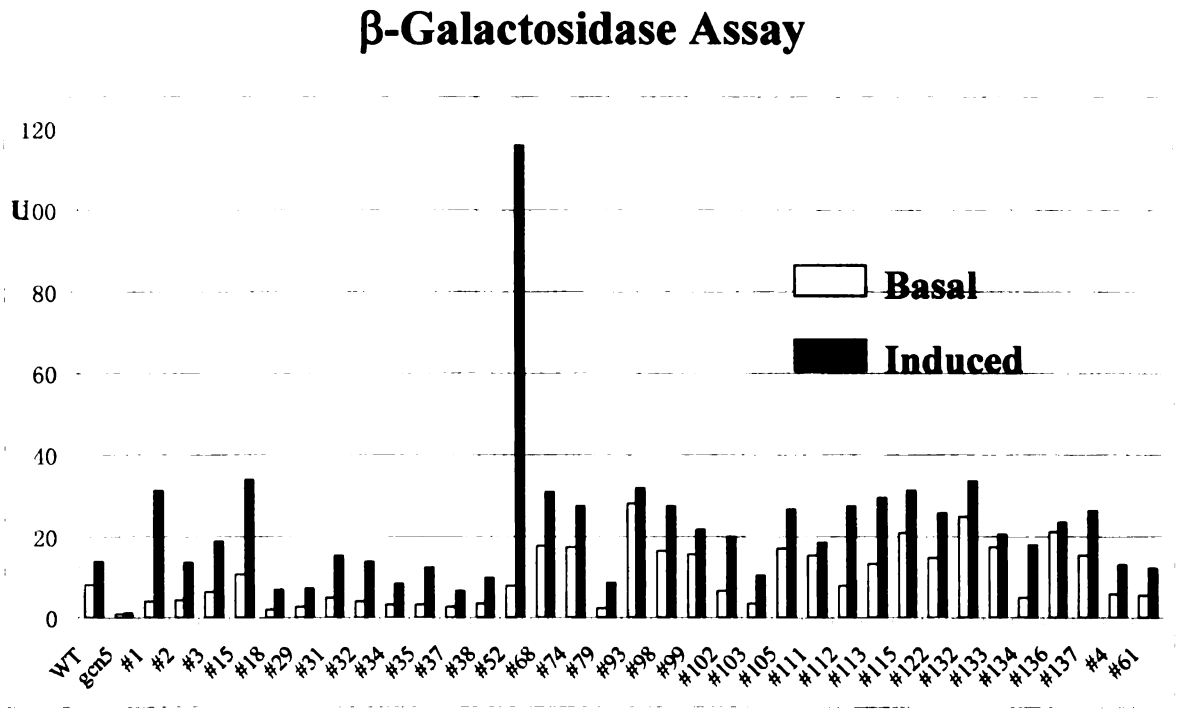


Fig. 1. *HIS3-lacZ* reporter expression in selected BGR mutants. lacZ gene was translational fused to the 3' of *HIS3* promoter and then engineered into *URA3* locus by homologous recombination. The strains were grown in 20 mL Yeast extract-Peptone-Dextrose (YPD) media to log phase and harvested by centrifugation. Then the cell pellet was resuspended and split into 10 mL YPD (for Basal expression) or Synthetic delete (SD, for inducing *HIS3* expression) media and growing for 6 hours. The collected cells were broken by vortex with glass beads.

RESULTS:

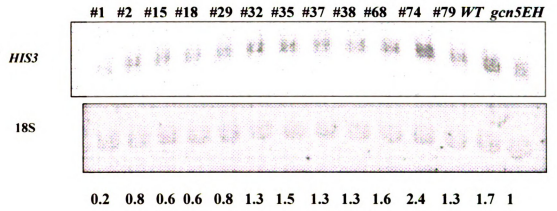
Northern analyses

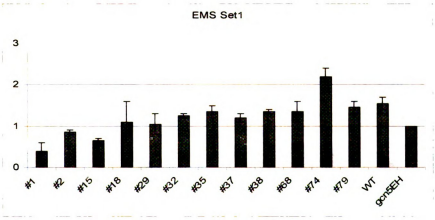
The *HIS3-lacZ* reporter assay provides us a simple and large-scale approach for analyzing *HIS3* transcription. However, the ectopic copy of *HIS3* promoter may not fully represent the endogenous gene transcription level since the local chromatin structure maybe different. Moreover, the *HIS3-lacZ* insertion at *URA3* locus results in the reporter flanked by two repeated sequences. The high frequency of recombination in budding yeast will cause the looping out of the *HIS3-lacZ* fragment. Thus the culture is a mixture of cells with or without reporter, which may under evaluate the *HIS3* induction. In such concerns, we decided to test *HIS3* expression directly by measuring the mRNA level. Fortunately, we had narrowed the number of candidates with strong phenotypes to thirty four, which is the amount feasible for Northern analyses.

Collaborating with Dave Almy in the lab, I grew different mutant strains along with the controls in YPD to log phase and then transferred the cells to SD with the supplement of 20mM 3-AT. After induction at 37°C for 6 hours, cells were harvested for RNA preparation. For each sample, 30μg of total RNA was used for Northern hybridization. The results are listed below.

Fig. 2. Northern analyses of the *hgr* candidates. The selected 34 candidates were divided into 3 sets. For each northern analysis, the strains indicated were grown to log phase and then transfered to SD media with 20mM 3-AT to induce *HIS3* transcription. The RNA isolation and northern hybridization were followed the procedure described before (7). Results from two independent inductions for each set of samples are presented. The results were quantified by PhosphoImager and the summarized results are shown as bar graph at bottom of each figure.

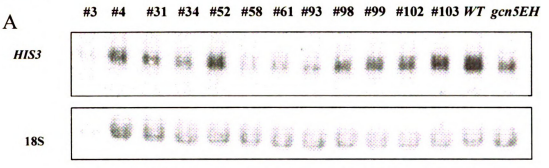
Set1


#1 #2 #15 #18 #29 #32 #35 #37 #38 #68 #74 #79 WT gcn5EH
HIS3
18S
0.2 0.8 0.6 0.6 0.8 1.3 1.5 1.3 1.3 1.6 2.4 1.3 1.7 1
#1 #2 #15 #18 #29 #32 #35 #37 #38 #68 #74 #79 WT gcn5EH
HIS3
18S
0.6 0.9 0.7 1.6 1.3 1.2 1.2 1.1 1.4 1.1 2.0 1.6 1.4 1

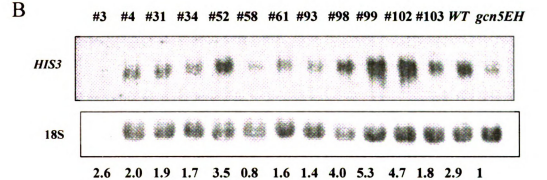

EMS Set1
HIS3/18S
3
2
1
0
#1
#2
#15
#18
#29
#32
#35
#37
#38
#68
#74
#79
WT
gcn5EH

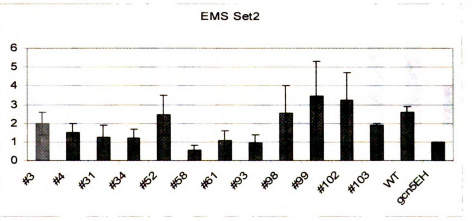
Set2

A

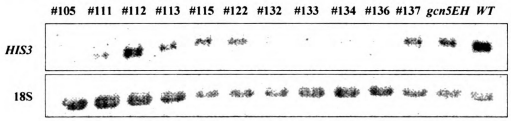

#3 #4 #31 #34 #52 #58 #61 #93 #98 #99 #102 #103 WT gcn5EH
HIS3
18S
1.4 1.0 0.6 0.7 1.4 0.3 0.6 0.5 1.1 1.6 1.8 2.0 2.3 1

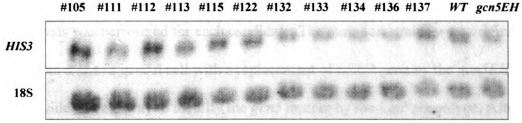
B

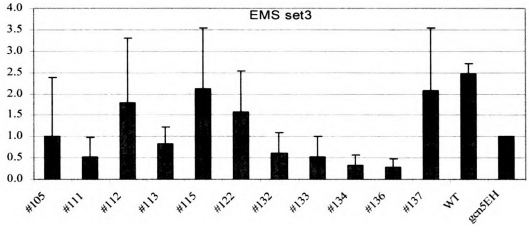

#3 #4 #31 #34 #52 #58 #61 #93 #98 #99 #102 #103 WT gcn5EH
HIS3
18S
2.6 2.0 1.9 1.7 3.5 0.8 1.6 1.4 4.0 5.3 4.7 1.8 2.9 1


EMS Set2
HIS3/18S
6
5
4
3
2
1
0
#3
#4
#31
#34
#52
#58
#61
#93
#98
#99
#102
#103
WT
gcn5EH

Set3


#105 #111 #112 #113 #115 #122 #132 #133 #134 #136 #137 gcn5EH WT
HIS3
18S
0.03 0.2 0.7 0.6 1.1 0.9 0.3 0.2 0.2 0.2 1.0 1.0 2.3


#105 #111 #112 #113 #115 #122 #132 #133 #134 #136 #137 WT gcn5EH
HIS3
18S
2.0 0.9 2.9 1.1 3.1 2.3 1.0 0.9 0.5 0.4 3.1 2.6 1.0


EMS set3
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
#105 #111 #112 #113 #115 #122 #132 #133 #134 #136 #137 WT gcn5EH

RNA analysis results indicate there are some false correlations between *HIS3* expression and *HIS3-lacZ* expression. However, 18 candidates repeatedly show the enhanced *HIS3* expression in both assays.

Sporulation and isolation of the mutations

EMS mutagenesis randomly introduces point mutations into yeast genome. Although the condition of EMS treatment is optimized to make most cells have only one hit with the mutagen, multi-mutations does happen inevitably. To distinguish the single mutation, spore analysis was performed for each candidate. The mutation strains were originally derived from a *MATa gcn5Δ* cell with a replacement of *LEU2* gene by the *gcn5EH* gene, which provide an easy way to identify the *gcn5* mutant by the Leu$^-$ phenotype. After crossing with a wild type *MATα* strain, the diploid strains carry the heterozygous of *bgr* alleles were subjected for sporulation. The spore analyses were performed by either random spore enrichment or tetrad- dissection. The isolated spores were patched on YPD plate to let cells growing for overnight then replica-plate to following plates:

SC-Leu *GCN5* genotype indicator

SC-Ura *HIS3-lacZ* reporter indicator

3-AT *bgr* allele indicator

YPD plates pre-spread with 227a or 70a, for matting type test.

Phenotypes on each plate were recorded and listed in Table 2.

Table 2 Summary of Spore Analyses

Mutant Number	Total number of Leu$^-$ spores analyzed	3AT R:S*	*P* value for 2:2 segregation
#1	40	18:22	> 0.5
#2	53	25:28	> 0.7
#3	33	15:18	> 0.7
#4	25	11:14	> 0.5
#15	30	16:14	> 0.7
# 18	25	13:12	> 0.7
#29	29	20:9	*<0.05*
#31	33	17:16	> 0.7
#32	236	119:117	> 0.9
#34	24	10:14	> 0.3
#35	161	81:80	> 0.95
#37	27	18:9	> 0.05
#38	195	94: 101	> 0.5
#52	210	115:95	> 0.1
#61	N/D^		
#68	221	110:111	> 0.95
#74	114	52:62	> 0.3
#79	243	109:124	> 0.3
#93	23	17:6	*< 0.05*
#98	204	104:100	> 0.7
#99	256	125:131	> 0.7
#102	242	123: 119	> 0.7
#103	26	18:8	*< 0.05*

Mutant Number	Total number of Leu⁻ spores analyzed	3AT R:S*	*P* value for 2:2 segregation
#105	175	90:85	> 0.7
#111	185	100:85	> 0.2
#112	209	79:130	*< 0.001*
#113	240	119:121	> 0.9
#115	236	125:111	> 0.3
#122	246	131:115	> 0.3
#132	33	23:10	*< 0.05*
#133	34	25:9	*< 0.01*
#134	38	30:8	*< 0.001*
#136	31	15:16	> 0.7
#137	235	123:112	> 0.3

* R: resistant; S: sensitive; ^ N/D: not determined; italic and underlined: rejected by Chi-Square test

By the statistical analyses of the above data, we noticed most of the mutations (27 out of 34) fulfill the hypothesis of 2:2 segregation for the 3-AT resistance phenotypes, which implies there is only one extra mutation besides *gcn5E173H* in these strains. For some of the candidates, like #133 and #134, which exhibit apparent differences for supporting the one mutation hypothesis, we think it's still early to make conclusion from them because we haven't obtained enough spores yet. The only candidate that is significant different is #112, which gives 79 3-AT resistant and 130 sensitive spores. The apparent deviation can not be explained the linkage between *leu2::gcn5EH* and the *bgr* allele, because that will make the spores with Leu⁻ /3-AT^r dominate the population. Nor it can be simply explained by phenotypes require the existence of two mutations, since that will show an 1:3 segregation pattern in stead of 1:2. Before further evidence coming up, we hypothesis

that, there are two mutations in candidate #112, the lethality of one mutation is suppressed by the other to accomplish the *bgr* phenotypes.

Dominance Test

Dominant/recessive is an important feature of a mutation. Knowing the dominancy of the mutations will be helpful for understanding the nature of the mutation, as the dominant mutations are always gain-of-function mutations. Also, the dominant mutations and recessive mutations will be treated with different strategies for mutation identifications. Based on the above thinking, I did the dominance tests for the 18 candidates that repeatedly showed the suppression of *HIS3* expression (Figure 2). The mutants were backcrossed to the parental *gcn5* mutant strain. The resultant diploid strains contain two copies of *gcn5 E173H* allele and heterozygous at the *bgr* loci. The diploid strain will show *bgr* phenotypes (3-AT resistant) if the mutation is dominant. If the diploid cell shows *gcn5* phenotype (3-AT sensitive), it indicates that the mutation is recessive.

Fig. 3A Strategy of Dominance Test

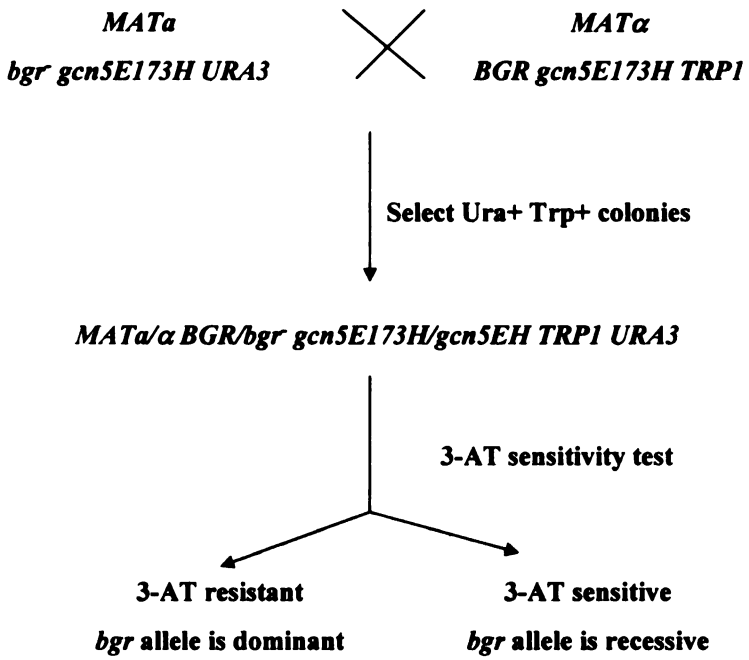


Table 3 Summary of Dominance Test

Dominant	Recessive
#3	#52
#32	#74
#35	#98
#38	#103
#68	#111
#79	#115
#99	
#105	
#112	
#113	
#122	
#137	

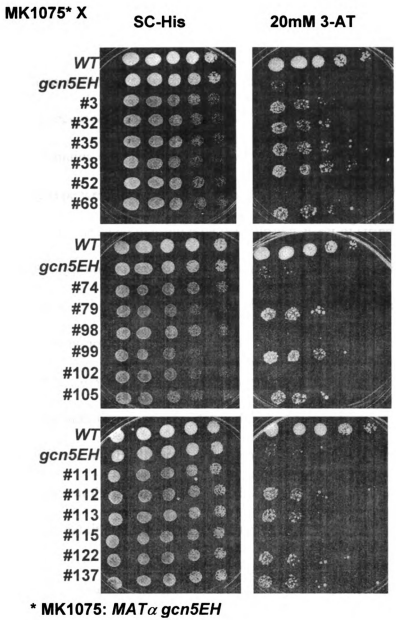
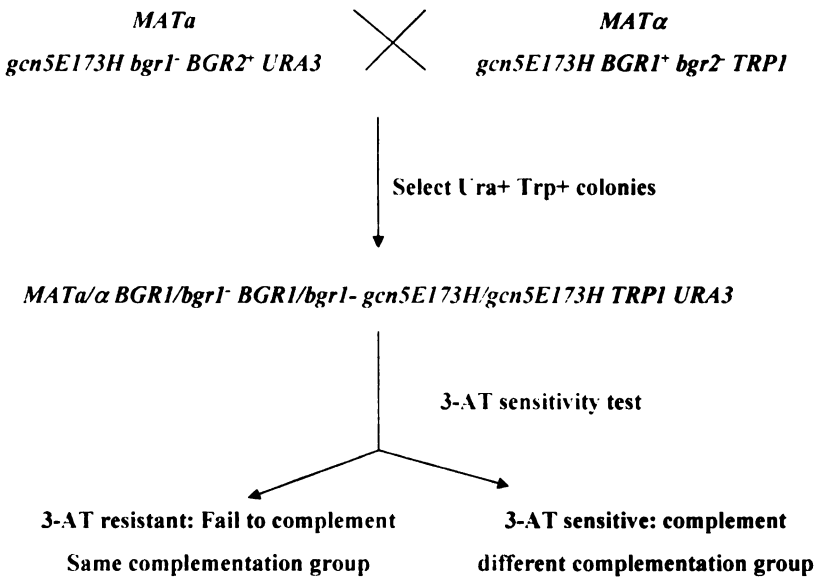


Fig. 3. Dominance test. BGR candidates were backcrossed and the resultant diploid strains were grown in YPD media to log phase. The harvested cultures were 5-fold serial diluted and spotted on the SC-His plates with or without 20mM 3-AT. The pictures were taken after 3 days of incubation at 37°C. The results were repeated 3 times.

Complementation test

Complementation tests were done as described by Fasser and Winston,1988 (3). The isolated recessive *bgr*⁻ mutants with *MATa* matting type were transformed with YCplac33, which contains *URA3* gene as selective marker. Similarly, the *bgr*⁻ alleles with *MATα* matting type were transformed with YCplac22 to introduce *TRP1* marker. Then the strains with the same matting type were grown as set of stripes on plates dropped out uracil or tryptophene respectively. Then the two sets of stripes with different matting types were replica-plated perpendicularly onto one YPD plate. After the incubation at 30°C for 6 hours, the grid of *MATa* and *MATα* mutants were replica-plated onto the plate in the absence of both uracil and tryptophene to select the diploid strains. The complementation groups were defined by 3-AT sensitivity test. The diploids with the crossing between the different complementation groups, or in another word, the mutation(s) at the different genes, will sensitive to 3-AT since the heterozygosity of the *bgr* genes exhibits the dominant wild type phenotypes.



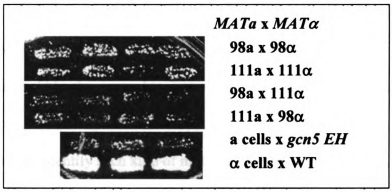


Fig. 4. Complementation test. The recessive mutants with isolated *MATa* haploid and *MATα* haploid were matted to each other in pairwise. The resultant diploid cells were patched onto an YPD plate and grown for overnight. Then the patches were replica-plated to 3-AT plate and incubated at 37°C. The crossing with wild type strains were used as positive control. The diploid strains from backcrossing were also tested to ensure the recessive feature of the mutations. Self-crossing diploids are as expected to be resistant to 3-AT. However, neither 98a x 111α nor 111a x 98α grew well in the presence of 3-AT. Here "98a" represent the *MATa* isolation of EMS mutant #98.

The same experiments as shown in Fig. 4 were done or will be done for all the 6 recessive *bgr* mutants. So far, we identified at least two complementation groups, #98 and # 111. And the *MATa* form of #115 complement both #98 and #111, which indicates #115 mutation may represents another group of suppressors. Also the #52 and the #74 mutation are not in the same complementation group. Whether the #52 and #74 mutations overlap with #98 group or #111 group are still under investigating.

GCN5 deletion study

One group of expected suppressors is the mutations restore the HAT activity of Gcn5p. These mutations could be the reversal mutation of *gcn5E173H*, i.e. histidine 173 – to - glutamic acid, or an extra mutation within or at other loci of the *gcn5* gene that enhances the HAT activity. To identify such kind of mutations, we performed the *GCN5* deletion studies. By removing the entire open reading frame of *gcn5*, any factors altering the Gcn5 activity are eliminated. For instance, we got *gcn5* deletion from 7 *bgr* candidates, which are #3, #35, #52, #68, #99, #102, and #103. The 3-AT test indicate all of them still possess the suppression power, which suggests these mutations indeed bypassed the requirement of Gcn5.

Future plan: mapping the *bgr* mutations

Other than finishing the above experiments, we are more interested in where do those mutations happen. For dominant and recessive mutation, we decide to apply different approaches to mapping the mutations.

To those dominant mutants, we will make yeast DNA libraries from those strains. The clones in the libraries contain the DNA fragments that contribute the BHR phenotype will rescue the 3-AT sensitivity phenotype after being transformed to the parent strain carrying the *gcn5* mutation. To those recessive mutants, a yeast genomic DNA library

will be transformed to the mutant strains. The clones that complement the *bgr* phenotypes will be sequenced to identify the mutated genes.

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