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Snf1p Regulates Gcn5p Transcriptional Activity by Antagonizing Spt3p

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ABSTRACT

The budding yeast Gcn5p is a prototypic histone acetyltransferase controlling transcription of diverse genes. Here we show that Gcn5p is itself regulated by Snf1p and Spt3p. Snf1p likely controls Gcn5p via direct interaction. Mutating four residues in the Gcn5p catalytic domain, T203, S204, T211, and Y212 (TSTY), phenocopies *snf1* null cells, including Gcn5p hypophosphorylation, hypoacetylation at the *HIS3* promoter, and transcriptional defects of the *HIS3* gene. However, overexpressing Snf1p suppresses the above phenotypes associated with the phosphodeficient TSTY mutant, suggesting that it is the interaction with Snf1p important for Gcn5p to activate *HIS3*. A likely mechanism by which Snf1p potentiates Gcn5p function is to antagonize Spt3p, because the *HIS3* expression defects caused by *snf1* knockout, or by the TSTY *gcn5* mutations, can be suppressed by deleting *SPT3*. *In vitro*, Spt3p binds Gcn5p, but the interaction is drastically enhanced by the TSTY mutations, indicating that a stabilized Spt3p–Gcn5p interaction may be an underlying cause for the aforementioned *HIS3* transcriptional defects. These results suggest that Gcn5p is a target regulated by the competing actions of Snf1p and Spt3p.

THE *Saccharomyces cerevisiae* Gcn5p is one of the best-studied histone acetyltransferases. The structure, catalytic mechanism, and functions of Gcn5p are highly conserved through evolution (Roth *et al.* 2001). Gcn5p-mediated promoter histone H3 hyperacetylation is critical for transcriptional activation of multiple stress-related genes, including amino acid biosynthesis (Kuo and Allis 1998; Kuo *et al.* 2000), phosphate metabolism (Gregory *et al.* 1998; Vogelauer *et al.* 2000), sporulation (Burgess *et al.* 1999), and others (Chiang *et al.* 1996; Krebs *et al.* 1999; Verdone *et al.* 2002). In addition, Gcn5p contributes to global acetylation of histones H3 and H4 extending beyond the promoter region (Kuo *et al.* 2000), although the significance and mechanism of the global acetylation remains poorly understood. Under certain conditions, the H3 acetylation activity of Gcn5p spreads well into the open reading frame of several genes, and that acetylation is causally linked to H3 eviction on the path of the transcribing RNA polymerase II (Govind *et al.* 2007).

The structures of the catalytic domain of ciliate, yeast, and human Gcn5p have been solved by both NMR and X-ray crystallography (Clements *et al.* 1999; Lin *et al.* 1999; Rojas *et al.* 1999; Trievel *et al.* 1999). Kinetic and mutational studies identified residues essential for catalysis, acetyl coenzyme A binding, and histone substrate association (Kuo *et al.* 1998; Wang *et al.* 1998; Tanner *et al.* 1999; Langer *et al.* 2001; Poux and Marmorstein 2003). The yeast Gcn5p is the catalytic subunit of several chromatographically distinct complexes including SAGA, ADA (Grant *et al.* 1997), SALSA, and SLIK (Pray-Grant *et al.* 2002, 2005; Sterner *et al.* 2002a). All Gcn5p complexes except the smaller ADA complex share TBP-associated factors (TAFs) with TFIID (Grant *et al.* 1998). Indeed, SAGA and TFIID make overlapping contributions to the expression of yeast genes (Holstege *et al.* 1998; Lee *et al.* 2000; Huisinga and Pugh 2004). SAGA is recruited to the promoter by transcriptional activators (Kuo *et al.* 2000), and performs both HAT-dependent and HAT-independent functions (Roberts and Winston 1997; Sterner *et al.* 1999). The latter includes recruitment of TBP or the Swi/Snf complex (Dudley *et al.* 1999; Sterner *et al.* 1999; Belotserkovskaya *et al.* 2000; Lee *et al.* 2000; Larschan and Winston 2001, 2005; Bhaumik and Green 2002; Yu *et al.* 2003; Biswas *et al.* 2004; Qiu *et al.* 2004; Topalidou *et al.* 2004), regulation of histone H2B ubiquitinylation (Henry *et al.* 2003; Daniel *et al.* 2004; Wyce *et al.* 2004; Ingvarsdottir *et al.* 2005; Lee *et al.* 2005), stimulation of histone methylation (Pray-Grant *et al.* 2005; Govind *et al.* 2007), and H3/H4 eviction (Govind *et al.* 2007). Moreover, it appears that the histone acetyltransferase Gcn5p also possesses non-HAT functions. For example, microarray data showed that *gcn5*Δ cells displayed transcriptional defects in more genes than strains expressing a catalytically inactive mutant Gcn5p

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(Holstege *et al.* 1998; Huisinga and Pugh 2004). Tethering a catalytically inactive mutant of Gcn5p to subtelomeric regions is sufficient to counteract silencing (Jacobson and Pillus 2004). Consistent with these findings, a catalytically inactive mutant allele of Gcn5p, E173H, can be rescued by certain suppressors without changing the H3 hypoacetylation phenotype (Liu *et al.* 2005, and see below).

To date, very little is known about how Gcn5p itself may be regulated. The transcriptional coactivator function of Gcn5p depends critically on its stable association with Ada2p and Ada3p (Candau *et al.* 1997). These three proteins form a core for different Gcn5p-containing complexes. Upon associating with Ada2p and Ada3p, Gcn5p adopts the nucleosomal acetylation activity (Balasubramanian *et al.* 2002; Sterner *et al.* 2002b). Interestingly, Ada2p/Ada3p not only stimulate the enzymatic activity of Gcn5p, but also expand its *in vitro* substrate specificity from nearly exclusively K14 of free histone H3, to K18>K14>K9>K23 of H3 within oligonucleosomes (Sermwittayawong and Tan 2006). K36 of nucleosomal histone H3 is recently shown to be a conserved, novel target for the SAGA complex (Morris *et al.* 2007). The ability of Ada2/Ada3 to help Gcn5p expand its substrate specificity is reminiscent of, and possibly related to the observations that Mg^{2+} alone can convert an otherwise refractory nucleosomal array into a good acetylation substrate for recombinant Gcn5p (Tse *et al.* 1998). It is thus likely that transacting factors such as Ada2p/Ada3p and Mg^{2+} modulate the nucleosomal histone conformation in such a way that Gcn5p is less restrictive of selecting its substrate. Indeed, Ada2p possesses the chromatin-binding SANT domain (Aasland *et al.* 1996; Sterner *et al.* 2002b; Boyer *et al.* 2004). On the other hand, it remains possible that the molecular and enzymatic behaviors of Gcn5p may be modulated by its association with Ada2p, Ada3p, and even other proteins.

For transcriptional regulation, Gcn5p performs both synergistic and antagonistic functions with many factors, including Spt3p, Spt8p (Belotserkovskaya *et al.* 2000; Larschan and Winston 2001; Bhaumik and Green 2002; Yu *et al.* 2003; Helmlinger *et al.* 2008), TBP (Dudley *et al.* 1999; Barbaric *et al.* 2003), Swi/Snf complex components (Pollard and Peterson 1997; Syntichaki *et al.* 2000; Hassan *et al.* 2001, 2002), the histone H2A variant Htz1p (Santisteban *et al.* 2000), global chromatin regulators Nhp6p and Sin1p (Yu *et al.* 2003), histone deacetylases (Perez-Martin and Johnson 1998), and Snf1p (Lo *et al.* 2000, 2001). While some of these proteins may influence chromatin dynamics, which consequently affects Gcn5p functions, Snf1p and Spt3p appear to play more direct roles in regulating Gcn5p. For example, Gcn5p physically interacts with and is phosphorylated by Snf1p *in vitro* (Liu *et al.* 2005); overproduction of Snf1p rescues the *E173H* allele of Gcn5p selectively (Liu *et al.* 2005). Deleting *SPT3*, which encodes an integral component of the SAGA complex, partially rescues the transcriptional defects of *HO* in *gcn5*Δ (Yu *et al.* 2003). One explanation is that Spt3p inhibits TBP–TATA association (Belotserkovskaya *et al.* 2000; Yu *et al.* 2003) and that the Gcn5p action somehow stimulates TBP recruitment (Bhaumik and Green 2002). Conversely, transcriptional activation of *GAL* genes is enhanced by Spt3p (Eisenmann *et al.* 1992; Bhaumik and Green 2002). Spt8p and Spt3p can be crosslinked to TBP (Warfield *et al.* 2004; Sermwittayawong and Tan 2006; Mohibullah and Hahn 2008), consistent with the genetic evidence for a physical role of Spt3p for TBP recruitment *in vivo* (Eisenmann *et al.* 1992; Dudley *et al.* 1999; Larschan and Winston 2001; Yu *et al.* 2003; Laprade *et al.* 2007). It is thus likely that Gcn5p interacts with TBP in an indirect manner.

In the present work, the functional and physical relationships between Gcn5p, Snf1p, and Spt3p were characterized. Four previously uncharacterized residues within the catalytic domain of Gcn5p (T203, S204, T211, and Y212) are critical determinants for Snf1p-dependent Gcn5p phosphorylation both *in vitro* and *in vivo*. These residues are also important for the histone acetylation and transcriptional activation functions of Gcn5p. Interestingly, transcriptional defects resulting from alanine substitution of these residues or from deleting *SNF1* can be suppressed by knocking out *SPT3*. Physically, Gcn5p interacts with Spt3p *in vitro*. These findings suggest a new regulatory mechanism for transcriptional activation by Gcn5p.

MATERIALS AND METHODS

Yeast strains and plasmids: Yeast strains, plasmid constructs, and oligonucleotide primers used in this work are listed in Tables 1–3. All genetic methods were performed according to standard procedures (Sherman 1991). Yeast transformation was done using the lithium acetate method (Gietz *et al.* 1992).

The *spt3*Δ strains were created by introducing a PCR fragment containing the KanMX6 cassette flanked by *SPT3* sequences outside the open reading frame (Wach *et al.* 1994). G418-resistant transformants were examined by genomic PCR to confirm the genotype.

The yeast construct pYL89 that expresses HA-tagged Spt3p was created by cotransforming *Xba*I-linearized pMK547 (Liu *et al.* 2005) (an *ARS CEN TRP1* plasmid with *ADH1* promoter and terminator flanking a multicloning sequence and an trimeric HA epitope tag), and a PCR-amplified *SPT3* open reading frame fragment, resulting in N′–HA-tagged Spt3p. To create pMK625 that expresses GST–HA–Spt3p in bacteria, the *Eco*RI–*Xho*I HA–SPT3 fragment of pYL89 was gel purified and inserted into the *Eco*RI and *Not*I sites of pGEX-5X-2 (GE Life Sciences) to create pMK625. The GST fusion significantly enhances the solubility of the fusion protein in *Escherichia coli* and is essential for obtaining sufficient quantity for *in vitro* assays. Following the QuikChange method (Roche), pMK625 was further modified using primers o415–o422 (Table 3) to create selective fragments of Spt3p.

2μ HA–GCN5 wild type, TSTY/4A, and HA–SNF1 constructs (pMK681, pMK681 TSTY/4A, and pMK682, respectively)

TABLE 1

Yeast strain list

Strains	Relevant genotype	Source
yMK839	*MAT***a** *trp1 leu2-3,112 ura3-52*	Kuo *et al.* (1996)
yMK842	*MAT***a** *trp1 leu2-3,112 ura3-52 gcn5*Δ*::hisG*	Kuo *et al.* (1996)
yMK986	*MAT***a** *trp1 leu2-3,112 ura3-52 gcn5E173H*	Liu *et al.* (2005)
yYL232	*MAT***a** *trp1 leu2-3,112 ura3-52 snf1*Δ*::LEU2*	Liu *et al.* (2005)
yYL515	*MAT***a** *trp1 leu2-3,112 ura3-52 spt3*Δ*::KanMX6*	This study
yYL516	*MAT***a** *trp1 leu2-3,112 ura3-52 gcn5E173H spt3*Δ*::KanMX6*	This study
yYL590	*MAT***a** *trp1 leu2-3,112 ura3-52 GCN5-8xmyc::*TRP1	This study
yYL591	*MAT***a** *trp1 leu2-3,112 ura3-52 SNF1-8xmyc::*TRP1	This study
yMK1422	*MAT***a** *trp1 leu2-3,112 ura3-52 gcn5*Δ*::hisG SNF1-8xmyc::TRP1*	This study
yYL622	*MAT***a** *trp1 leu2-3,112 ura3-52 SPT7-13xmyc::*TRP1	This study
yYL682	*MAT***a** *trp1 leu2-3,112 ura3-52 gcn5*Δ*::hisG spt3*Δ*::KanMX6*	This study
yYL683	*MAT***a** *trp1 leu2-3,112 ura3-52 snf1*Δ*::LEU2 spt3*Δ*::KanMX6*	This study
yYL782	*MAT***a** *trp1 leu2-3,112 ura3-52 gcn5TSTY/4A-8xmyc::TRP1*	This study
yYL783	*MAT***a** *trp1 leu2-3,112 ura3-52 gcn5TSTY/4A-8xmyc::TRP1 spt3*Δ*::KanMX6*	This study
yYL786	*MAT***a** *trp1 leu2-3,112 ura3-52 spt8*Δ*::URA3 snf1*Δ*::LEU2*	This study
yYL787	*MAT***a** *trp1 leu2-3,112 ura3-52 spt8*Δ*::URA3 GCN5-8xmyc::TRP1*	This study
yYL788	*MAT***a** *trp1 leu2-3,112 ura3-52 spt8*Δ*::URA3 gcn5 TSTY/4A-8xmyc::TRP1*	This study

were generated by cotransforming to yeast the *GCN5* and *SNF1* open reading frame PCR fragments flanked with sequences homologous to the *Not*I-linearized pMK595. A trimeric HA tag was consequently fused to the NH_3 terminus of Gcn5p and Snf1p.

To introduce *GCN5* mutants to the native locus, a *URA3* integrative construct, pMK284 (Liu *et al.* 2005) was used for site-directed mutagenesis. pMK284 bearing selective mutations of *GCN5* was linearized by *Ngo*MIV, and transformed into yMK986 possessing the *E173H* allele of *GCN5*. The *Eco*RI site spanning codons 169 and 170 had been eliminated upon creating the E173H mutation. Replacing the *E173H* allele with other mutations thus restored the *Eco*RI site, and the presence of which served as a screening criterion. After genomic PCR verification of the integration, yeast cells were selected by 5-FOA for *URA3* pop-out. Colonies formed on 5-FOA plates were grown for genomic PCR amplification using primers MHK234 and MHK232. PCR fragments bearing an *Eco*RI site were further sequenced for verification.

Pro-Q Diamond staining: For Pro-Q Diamond staining, 3×10^9 yeast cells expressing a TAP-tagged Gcn5p (expressed from pYL54 or pYL93) were collected from early log phase cultures. Whole cell lysates were prepared as previously described (Liu *et al.* 2005) except that 800 μl of PiPT buffer (50 mm potassium phosphate, pH 7.5; 140 mm potassium chloride; 0.1% Triton X-100; 1 mm DTT; 1 mm EDTA; 1 mm sodium orthovanadate; 10 mm sodium fluoride; complete protease inhibitor cocktail (Roche; 1 tablet/20 ml buffer); 1 mm PMSF) was used. Affinity purification was done by incubating 500 μl of whole cell lysates with 20 μl of IgG sepharose 6G beads (GE Life Sciences) at 4° for 1 hr. IgG beads were collected with gentle centrifugation and washed twice with PiPT buffer without protease inhibitors, followed by two more washes with PiPT buffer containing 500 mm KCl. If necessary, TEV digestion was conducted

TABLE 2

Plasmid list

Plasmid	Description	Source
pMK100	pRSET–Gcn5–6xHis	Kuo *et al.* (1996)
pFW32	YEp–*SNF1*	Winston and Minehart (1986)
pYL41	YEplac112–*SNF1*	Liu *et al.* (2005)
pYL42	pYEX-4T–GST–Snf1	Hahn and Thiele (2004)
pYL43	pYEX-4T–GST–Snf1K84R	Hahn and Thiele (2004)
pYL44	pYEX-4T–GST	Liu *et al.* (2005)
pYL54	pYEX-4T–Gcn5–TAP	Liu *et al.* (2005)
pYL55	pYEX-4T–Gcn5E173H–TAP	This study
pYL67	*8xmyc::TRP1* for tagging proteins with 8 myc repeats	Liu *et al.* (2005)
pYL72	pMK547Gcn5, 3xHA–Gcn5	Liu *et al.* (2005)
pYL89	pMK547Spt3, 3xHA–Spt3	This study
pYL90	pET21a–3xHA–Spt3	This study
pYL93	pYEX-4T–Gcn5TSTY/4A–TAP	This study
pYL98	pRSET–Gcn5TSTY/4A–6xHis	This study
pMK284	GCN5 integration construct	Liu *et al.* (2005)
pMK515	pET21–6xHis–Gcn5 protein	Liu *et al.* (2005)
pMK625	pGEX5–HA–SPT3 and derivatives	This study

TABLE 3

List of oligos

Name	Sequence	Description
GCN5AAs	GACTATGTTAGAAATGCCGCGAACATAAAATATTTTTTG	*GCN5* T203A/S204A, sense
GCN5AAas	CAAAAAATATTTTATGTTCGCGGCATTTCTAACATAGTC	*GCN5* T203A/S204A, antisense
GCN5T203As	CTTAAAAGACTATGTTCGAAATGCCTCGAACATAAAATATT	*GCN5* T203A, sense
GCN5T203Aas	AATATTTTATGTTCGAGGCATTTCGAACATAGTCTTTTAAG	*GCN5* T203A, antisense
GCN5S204As	CTTAAAAGACTATGTTCGAAATACCGCGAACATAAAATATTT	*GCN5* S203A, sense
GCN5S204Aas	AAATATTTTATGTTCGCGGTATTTCGAACATAGTCTTTTAAG	*GCN5* S203A, antisense
T211As	CATAAAATATTTTTTGGCGTACGCAGATAATTACGCT	*GCN5* T211A, sense
T211Aas	AGCGTAATTATCTGCGTACGCCAAAAAATATTTTATG	*GCN5* T211A, antisense
T211/Y212As	CATAAAATATTTTTTGGCCGCGGCAGATAATTACGCT	*GCN5* T211A/Y212A, sense
T211/Y212Aas	AGCGTAATTATCTGCCGCGGCCAAAAAATATTT TAT G	*GCN5* T211A/Y212A, antisense
Y212As	CATAAAATATTTTTTGACTGCAGCAGATAATTACGCT	*GCN5* Y212A, sense
Y212Aas	AGCGTAATTATCTGCTGCAGTCAAAAAATATTTTATG	*GCN5* Y212A, antisense
spt3KX	GATCGGCGGAAACGAAAAGTAAAAAGTAAGGTTGAAGACAC TCTGGCTTCGTACGCTGCAGGTCG	Replacing *SPT3* with KanMX
spt3KXas	CATACCAGAAGGAAACCCATGCACCTCCATGATGAAATTATA CCATAGGCCACTAGTGGATCTG	Replacing *SPT3* with KanMX
MHK232	GCGATTTCGAAATCGTTC	Genomic PCR to verify *gcn5* mutant integration
MHK234	CTGAGAGAATAGGAGG	Genomic PCR to verify *gcn5* mutant integration
MK80	CTGTGGGAAAAACTTATC	*HIS3* oligo for ChIP, −1 nucleosome
MK80as	AAAGGACTGTGTTATGAC	*HIS3* oligo for ChIP, −1 nucleosome
MK81	TGAGCAGGCAAGATAAAC	*HIS3* oligo for ChIP, +1 nucleosome
MK81as	CCACCCTTTAAAGAGATC	*HIS3* oligo for ChIP, +1 nucleosome
MK100-1	CGGTAACATCGTTATGTCCG	*ACT1* oligo for ChIP
MK100as	ACGATAGATGGACCACTTTCG	*ACT1* oligo fpr ChIP
YLK92	CTGCTCAGTGCGGCCGCTCTAGCTCTAGAATGATGGACAAG CATAAG	3xHA–Spt3 construction
YLK93	TGCAGGTCGACGGTATCGGGGGATCCACTATTACATGATAAT TGGTTTAG	3xHA–Spt3 construction
YLK94	TCCGAATTCGGCGGCCGCATCTTTTACCCATAC	HA–Spt3 PCR from pYL89
YLK95	CTACTCGAGCTACATGATAATTGGTTTAGAACTGAG	HA–Spt3 PCR from pYL89
YLK107	GTACAATCAATCAATCAATCATCACATAAAATGTTCAGCGAATTG ACCATGGCAATTCCC	Replacing Gcn5–TAP with HA-Spt3–TAP in pYL54
YLK108	GACGGCTATGAAATTCTTTTTCCATCTTCTCTTTTCCATGGATGG TTTAGAACTGAGTC	Replacing Gcn5–TAP with HA-Spt3–TAP in pYL54
HIS3 5′RT	AGCTTTGCAGAGGCTAGCAG	*HIS3* RT–PCR 5′ primer
HIS3 3′RT	GCGAGGTGGCTTCTCTTATG	*HIS3* RT–PCR 3′ primer
PGK1 5′RT	TCATTGGTGGTGGTGACACT	*PGK1* RT–PCR 5′ primer
PGK1 3′RT	GCAACACCTGGCAATTC	*PGA1* RT-PCR 3′ primer
o415	GCGAGTGGCACTGGAAATCCTTAAGGGTAGAGGTGGTGAAGATG ATTTGAAAAAAGC	SPT3(1–107) sense
o416	GCTTTTTTCAAATCATCTTCACCACCTCTACCCTTAAGGATTTCCA GTGCCACTCGC	SPT3(1–107) anti-sense
o419	GGCCGCTCTAGCTCTAGAATGATGGACAAGCTTCCTGGGGCAGG TGGTGAAGATGATTTG	SPT3(107–337) sense
o420	CAAATCATCTTCACCACCTGCCCCAGGAAGCTTGTCCATCATTCTA GAGCTAGAGCGGCC	SPT3(107–337) anti-sense
o421	GCCGCTCTAGCTCTAGAATGATGGACAAGCTTAATAATGACGACAAT GATGATATGGATG	SPT3(156–337) sense
o422	CATCCATATCATCATTGTCGTCATTATTAAGCTTGTCCATCATTCTA GAGCTAGAGCGGC	SPT3(156–337) anti-sense

according to Rigaut *et al.* (1999). In most cases, the IgG-bound materials were directly boiled in 1× SDS–PAGE loading dye and resolved by electrophoresis. Alternatively, some of the beads were treated with λ phosphatase (New England Biolab) prior to boiling and electrophoresis.

Following SDS–PAGE, the gels were fixed with 100 ml of 50% methanol and 10% acetic acid (v/v) for 1 hr to overnight. The residual methanol and acetic acid was removed by washing with 50 ml deionized water for 10 min with gentle agitation. After repeating the wash three times, the gel was

incubated with 50 ml of Pro-Q Diamond phosphoprotein gel staining solution (Molecular Probes) for 1 hr, with gentle agitation in dark. To reduce the background and nonspecific staining, the gel was treated with destaining solution [50 mm NaOAc, pH 4.0; 20% acetonitrile (v/v)] for 30 min with three repeats, and twice deionized water wash at room temperature for 5 min each. The staining was detected by Molecular Imager FX-PRO Plus (BioRad), and stained by Coomassie Blue R250.

Recombinant protein expression and purification: Protocols for purification of His-tagged Gcn5p (amino acid residues 19–348), GST–Snf1p, and for *in vitro* phosphorylation of Gcn5p by Snf1p were as previously described (Liu *et al.* 2005). To express GST–HAx3–Spt3p (pMK625) and its truncated fragments, BL21 Codon-Plus strain was transformed with pMK625 and derivatives. Two hundred ml LB-Amp cultures grown to 0.6 OD_{600} were induced with 0.5 mm IPTG at 37° for 3 hr. Cells were then pelleted and suspended in 6 ml lysis buffer (50 mm NaPi, pH 7.5, 150 mm NaCl, 1 mm PMSF), and frozen at −80°. After two freeze-and-thaw (4°) cycles, cell slurry was sonicated on ice for 20 sec, seven times, with ice-water chilling in between each homogenization. Lysates were clarified by centrifugation at 10,000 × *g* for 15 min at 4°. One hundred fifty microliters of 1:1 glutathione beads slurry (Sigma) were added to the lysates and incubated under gentle agitation for 2 hr at 4°. Beads were pulse-spin collected and washed twice with 10 ml lysis buffer, and once with 1.5 ml lysis buffer. Bound proteins were eluted with 50 mm reduced glutathione (Sigma) in 150 μl lysis buffer at 4° for 30 min. A second elution was conducted exactly as the first one, and the two eluates were combined and stored at −80°.

Gcn5p–Spt3p interactions: The interaction between Gcn5p and Spt3p was tested by the Farwestern approach. Pulldown assays using immobilized Gcn5p or Spt3p suffered from high background binding of both proteins to the matrix nonspecifically (data not shown). For Farwestern assays, His-tagged Gcn5p or Hmt1p, 0.5 μg each, were resolved by SDS–PAGE and blotted to PVDF membrane by standard blotting methods. The membrane was first blocked by 10% nonfat milk in TTBS (50 mm Tris–HCl, pH 7.5, 150 mm NaCl, and 0.1% Tween-20) for 0.5–1 hr at room temperature, followed by three washes of 100 ml of TTBS. All subsequent steps, except the final development, were carried out at 4°. For Spt3p–Gcn5p interaction, the membrane was incubated with 4 ml of TTBS containing protease inhibitors, 0.1% gelatin, and about 5 μm of GST or GST–HA–Spt3p recombinant proteins. Alternatively, crude bacterial lysates containing the Spt3 derivatives or GST were used for the binding. No clear difference was seen between the use of a highly purified Spt3 or crude bacterial lysates. The binding reaction was gently rocked at 4° overnight, followed by three 10-min TTBS washes. To detect the presence of HA-tagged Spt3p on the membrane, the 12CA5 monoclonal anti-HA antibodies (Roche), 1:1000 dilution, was incubated with the membrane (in TTBS supplemented with 0.1% gelatin) for 2 hr, followed by three TTBS washes and secondary antibody incubation (HRP-conjugated goat anti-mouse Ab, BioRad, 1:7500 dilution) (2 hr), and final washes (three TTBS washes, 10 min each). The Lumi-Light Western Blotting kit (Roche) was used for the development.

RNA preparation and RT–PCR: Yeast cells were grown in appropriate selective media until OD_{600} reached 0.5. Cells were then collected by centrifugation (5000 × *g*, 5 min, 4°) and transferred to either YPD or synthetic minimal medium supplemented with required nutrients and 40 mm 3-AT for *HIS3* induction. Cell cultures were further incubated at 37° for 2–3 hr before harvesting for RNA preparation. Procedures for RNA preparation were described previously (Liu *et al.* 2005). Ten micrograms of total RNA was treated with 10 units of DNaseI (Roche) in 100 μl (50 mm Tris-HCl, pH 7.5, 5 mm $MgCl_2$), and incubated at 37° for 1 hr. cDNA was synthesized following the instruction of ImpronII reverse transcriptase kit (GE Life Sciences) using 30 ng of poly(dT) primer. Semi-quantitative PCR reactions were conducted in 50 mm KCl, 10 mm Tris-HCl (pH 9.0, 25°), 1% Triton X-100, 2 mm $MgCl_2$, 0.1 mm each dNTP, 0.5 μM each primer, and 1.25 units Taq DNA polymerase (Promega), and appropriately diluted DNA templates. PCR parameters were (94°, 4 min; 50°, 4 min; 72°, 30 sec) for 2 cycles; (94°, 45 sec; 50°, 45 sec; 72°, 30 sec) for 24 cycles; and 72°, 3 min. PCR products were resolved in polyacrylamide gels followed by ethidium bromide staining. Chromatin IP was conducted according to Kuo and Allis (1999).

RESULTS

Gcn5p is a phosphoprotein: We previously reported that Snf1p phosphorylated Gcn5p *in vitro*, and that these two proteins were copurified from yeast whole cell lysates (Liu *et al.* 2005). These results suggested that Gcn5p was regulated by Snf1p via direct interaction and/or phosphorylation. To test this hypothesis, we first examined whether Snf1p controlled Gcn5p phosphorylation *in vivo*. We used the phosphate-specific Pro-Q Diamond fluorescence dye to assess the *in vivo* phosphorylation status of Gcn5p. Gcn5p was fused to the tandem affinity purification tag (TAP) consisting of (from the carboxyl end) the protein A fragment (PrA), endopeptidase TEV cleavage site, and the calmodulin binding protein (CBP) (Rigaut *et al.* 1999). Constitutively expressed Gcn5p–TAP was partially purified from yeast whole cell lysate by IgG beads, followed by SDS–PAGE and Pro-Q Diamond staining. Gcn5p was positively stained (Figure 1A, lane 2), and this staining was diminished in λ phosphatase-treated samples (compare lanes 2 and 3 in Figure 1A). The identity of Gcn5p was further verified by treating the IgG matrix-bound materials with the TEV protease (lane 1) that liberated Gcn5p from the IgG beads and concomitantly caused a faster mobility on SDS–PAGE. We thus conclude that Gcn5p is phosphorylated *in vivo*.

To assess the role of Snf1p in Gcn5p phosphorylation *in vivo*, we examined the effects of varying the Snf1p dosage on Gcn5p phosphorylation (Figure 1A, lanes 5–7). Compared with the wild-type strain, *snf1*Δ cells produced a much lower phosphostaining intensity of Gcn5p (lane 5). In contrast, introducing a multicopy *SNF1* plasmid to yeast (lane 7) caused significantly stronger Gcn5p phosphorylation. Thus, the *in vivo* phosphorylation of Gcn5p correlates positively to the dosage of Snf1p.

To explore further the relationship between Snf1p and Gcn5p, we attempted to map the amino acid residues of Gcn5p important for its phosphorylation. By comparing the Gcn5p catalytic domain to the consensus sequence shared by the yeast Snf1p and other AMP-activated protein kinases (AMPK), we found that residues T203, S204, and T211 resembled the AMPK consensus (Φ-x-K/R-x-x-S/T-x-x-x-Φ, Φ = hydrophobic residues) (Kuchin *et al.* 2000) (^{200}VRNT*S*NIKYFLT*YADNYA).

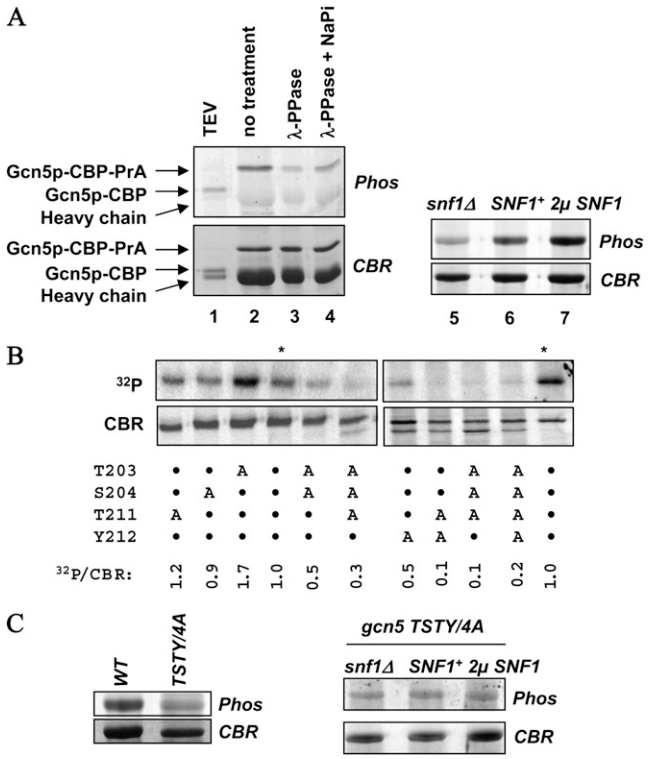

FIGURE 1.—Gcn5p is a phosphorylated protein. (A) *SNF1* dosage-dependent Gcn5p phosphorylation *in vivo*. TAP-tagged Gcn5p was purified from yeast and stained with the phosphate-specific fluorescence dye, Pro-Q Diamond. After capturing the fluorescent images, protein gels were stained with Coomassie Blue R250 (CBR). Phos, Pro-Q Diamond staining. (B) Mapping amino acid residues important for Gcn5p phosphorylation in vitro. Recombinant Gcn5p bearing the indicated alanine mutations was treated with yeast Snf1p in the presence of [γ-^{32}P]ATP, followed by SDS–PAGE, CBR staining, and autoradiography. The radiolabeling efficiency was assessed by PhosphoImager. Asterisks indicate wild-type Gcn5p reaction products. The relative phosphorylation status was obtained by calculating the ^{32}P-to-CBR staining intensities, with the latter acquired by National Institutes of Health (NIH) Image analysis. Two sets of samples were analyzed separately, each with the wild-type Gcn5p as the normalization standard. A, Ala substitution; •, residues unaltered. (C) The TSTY/4A quadruple mutant is hypophosphorylated (left; both from *SNF1*$^+$ background) and insensitive to changes in Snf1p dosage in vivo (right). TAP-tagged Gcn5p bearing the TSTY/4A mutations was purified from strains with the indicated *SNF1* dosage and stained by Pro-Q Diamond and Coomassie Blue.

Of these three residues, T211 is conserved among the GNAT family members (NEUWALD and LANDSMAN 1997; DYDA *et al.* 2000), while T203 and S204 are present primarily in fungal homologs. The catalytic function of any of these residues has not been tested. In addition, T211 and the adjacent hydroxyl amino acid, Y212, are spatially equivalent to the suspected active center of Hat1p, E255, and D256 (STERNGLANZ and SCHINDELIN 1999), raising an interesting possibility that phosphorylation at or around T211 may influence the HAT activity of Gcn5p. We thus created and purified a series of mutant Gcn5p in *E. coli* for [γ-^{32}P]ATP labeling by Snf1p *in vitro*. The wild-type Gcn5p was phosphorylated by Snf1p strongly (Figure 1B, asterisk-marked lanes). Single alanine substitutions of T203, S204, or T211 had only modest effects. We repetitively observed stronger phosphorylation of the T203A mutant. The biochemical basis is unclear. Significantly, the Y212A single mutation reduced phosphorylation to ~50% of the wild-type level, suggesting that Y212 was a critical residue for Snf1p to recognize Gcn5p for its phosphorylation. More severe defects were seen in two double mutants (T203A/S204A and T211A/Y212A), the T203A/S204A/T211A triple mutant (referred to as TST/3A hereafter), and the quadruple TSTY/4A mutant. We thus conclude that these four residues are collectively critical determinants for Gcn5p phosphorylation *in vitro* by Snf1p, and that Y212 is probably the most important among the four.

To examine whether T203, S204, T211, and Y212 were also important for Gcn5p phosphorylation *in vivo*, we introduced the TSTY/4A quadruple mutations to the TAP-tagged *GCN5* construct and purified Gcn5p for Pro-Q Diamond staining. Contrary to the wild-type Gcn5p in which its phosphorylation correlated well with the dosage of Snf1p (Figure 1A), the TSTY/4A mutant showed weak Pro-Q Diamond staining intensity (Figure 1C) even in the presence of a multicopy *SNF1* plasmid. Deleting *SNF1* failed to cause further reduction of Gcn5p phosphorylation. We thus surmise that Snf1p is likely the major kinase that targets or depends on the TSTY region for Gcn5p phosphorylation *in vivo*. However, as shown below, we believe that Gcn5p phosphorylation *per se* plays a less important role in transcriptional activation than does the interaction with Snf1p *in vivo*.

TSTY mutants affect Gcn5p functions *in vivo*: To test whether the TSTY residues were also important for the transcriptional activation function of Gcn5p, we created several mutants and integrated them into the *GCN5* locus. The expression status of *HIS3* was assessed by comparing the cellular resistance to 3-amino-triazole (3-AT), and by reverse-transcription PCR (RT–PCR) for quantifying the *HIS3* mRNA levels. When expressed from the native *GCN5* promoter, single alanine substitution of the conserved T211 and Y212 did not cause a discernible *HIS3* expression defect; neither did T203A/S204A nor T203A/S204A/T211A mutants (Figure 2A left, rows 1–4 and 6). In contrast, the T211A/Y212A (dubbed TY/2A hereafter) and TSTY/4A mutants caused clear 3-AT hypersensitivity (rows 5 and 7) that also correlated with the diminishment of *HIS3* transcription, as shown by RT–PCR (Figure 2A, right panel). The severity of the *HIS3* expression defects was similar to the E173H mutant that targeted the catalytic center of Gcn5p (LIU *et al.* 2005).

We further used chromatin immunoprecipitation to see whether the *HIS3* transcriptional defects were coupled to histone H3 hypoacetylation. The TSTY/4A mutations reduced H3 K14 acetylation at the *HIS3* promoter (compare lanes 2 and 4, Figure 2B). Hypoacetylation at

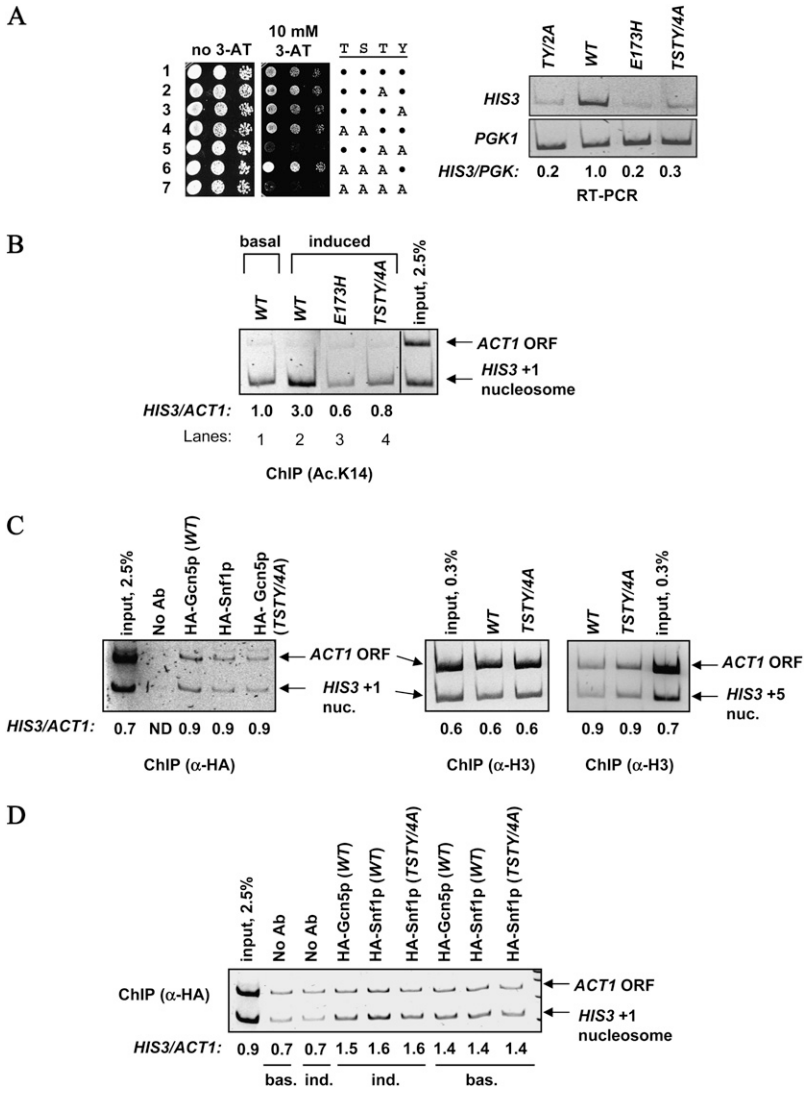

FIGURE 2.—Residues critical for Gcn5p phosphorylation are also important for transcriptional activation and promoter acetylation. (A) *HIS3* expression defects are caused by TY/2A and TSTY/4A mutants. Left panel, cellular sensitivity to 3-AT was assessed by spotting serially diluted yeast strains to 10 mM 3-AT plates. Right panel, semiquantitative RT–PCR comparing *HIS3* and *PGK1* expression. The intensity of each band was quantified by NIH Image, and normalized to the wild-type samples. RT–PCR and ChIP experiments in this and subsequent figures were from at least two independent experiments. Although absolute values varied somewhat among different experiments, trends were highly reproducible (data from representative experiments are shown). All mRNA analyses and ChIP studies, unless otherwise noted, were conducted under amino acid starvation conditions that activated *HIS3* expression. (B–D) *gcn5* TSTY/4A mutant causes H3 hypoacetylation but does not affect the recruitment of Gcn5p, Snf1p, or histone H3 occupancy at the *HIS3* promoter. Chromatin immunoprecipitation with the indicated antibodies was conducted using yeast cells harvested from minimal medium that induced *HIS3* transcription or from the YPD medium that allowed basal expression. Semiquantitative multiplex PCR was used to compare the amount of *HIS3* promoter and the *ACT1* open reading frame associated with the indicated antigens. The relative immunoprecipitation efficiency, expressed as *HIS3*-to-*ACT1* ratio, was quantified by NIH Image. Note that panel C only shows ChIP results from induced cultures. ND, not determined.

another lysine of H3, K18, also was prominent in the TSTY/4A strain (data not shown). Neither the recruitment of Gcn5p nor the histone H3 occupancy was appreciably affected at the *HIS3* promoter (Figure 2C). Similarly, Snf1p remained associated with the *HIS3* promoter in both wild-type and *gcn5* TSTY/4A background (Figure 2, C and D). Together, these data revealed new residues in the catalytic domain of Gcn5p that are important for its HAT and transcriptional activation activities. Of the four residues, T211 and Y212 appear to be more important than T203 and S204. On the other hand, double alanine substitutions of T203 and T204 caused ~50% reduction of the *in vitro* phosphorylation of Gcn5p (Figure 1B), suggesting that these two residues may perform a hitherto unidentified function *in vivo*. With respect to *HIS3* regulation, however, the TSTY/4A and TY/2A mutants displayed comparable phenotypes (*e.g.*, Figure 2A and see below).

Snf1p is important for Gcn5p functions *in vivo*: The above data reveal that the TSTY region of Gcn5p is important for the well-established histone acetylation and transcriptional activation activities of Gcn5p and for the Snf1p-dependent phosphorylation of Gcn5p *in vivo*. Snf1p has been shown to phosphorylate multiple chromatin proteins, including histone H3 (Lo *et al.* 2000, 2005) and possibly the Srb/mediator complex components (Kuchin *et al.* 2000) (although our previous work ruled out the involvement of histone H3 phosphorylation in *HIS3* transcriptional activation; see Liu *et al.* 2005). It is tempting to speculate that Gcn5p phosphorylation is critical for the transcriptional activation of *HIS3*. On the other hand, if Snf1p is indeed the responsible kinase, these two proteins have to be engaged in a physical interaction, transiently or stably, on chromatin or in the nucleoplasm. It is therefore also possible that the physical interaction between Gcn5p and Snf1p plays a more direct role in activating *HIS3* transcription. For example, Gcn5p may provide a docking site for Snf1p to get access to another protein(s) that is important for transcriptional activation. In this case, Gcn5p phosphorylation is a functionally redundant byproduct of this contact with Snf1p.

To see whether phosphorylation of Gcn5p or its association with Snf1p was more important for *HIS3* activation, we examined the effects of overexpressing Snf1p in the TSTY/4A mutant. The Pro-Q Diamond staining (Figures 1C) showed that the TSTY/4A quadruple mutations quantitatively eliminated Snf1p-dependent Gcn5p phosphorylation. If phosphorylation of Gcn5p by Snf1p is critical for *HIS3* activation, then the phosphodeficient *gcn5* TSTY/4A mutant should remain hypersensitive to 3-AT even in the presence of a 2μ *SNF1*. In contrast, if the Gcn5p–Snf1p association is more important, increasing the concentration of Snf1p may augment its interaction with the TSTY/4A mutant of Gcn5p, thus upregulating *HIS3* expression without causing Gcn5p hyperphosphorylation. Results in Figure 3 conform to the latter hypothesis. When transformed with a 2μ plasmid bearing the wild-type *SNF1* gene, TSTY/4A mutant cells exhibited enhanced resistance to 3-AT (Figure 3A), consistent with about a twofold increase in *HIS3* transcription (Figure 3B). Intriguingly, the 2μ *SNF1* plasmid also rescued the *E173H* alleles of *GCN5 (*Figure 3A). This suppression is in contrast with our previous findings that overexpressing Snf1p did not rescue the *HIS3* expression defects caused by the complete knockout or another catalytically inactive allele of Gcn5p, *F221A* (Liu *et al.* 2005). The allele specificity of the *SNF1* high-copy suppressor is in agreement with the notion that Gcn5p and Snf1p interact directly. As the TSTY/4A phosphodeficient mutant was rescued by 2μ *SNF1* without exhibiting discernible increase in its phosphorylation (Figure 1C), it is likely that Gcn5p phosphorylation either has a minor effect in *HIS3* expression, or that the normal function of Gcn5p phosphorylation at or near the TSTY region is dispensable if there is sufficient amount of Snf1p available.

In addition to restoring *HIS3* transcription, 2μ *SNF1* also suppressed the histone H3 hypoacetylation phenotype (Figure 3C). Chromatin IP using antibodies against histone H3 acetylated at K14 demonstrated that both the TY/2A and TSTY/4A mutant alleles resumed their ability to acetylate H3 at the *HIS3* promoter in the presence of the multicopy *SNF1* gene. These results suggest that one of the likely molecular mechanisms underlying the TSTY/4A and TY/2A phenotypes is a weakened Gcn5p–Snf1p interaction, resulting in the crippled HAT activity of Gcn5p (see below and Figure 5B) and consequently the compromised activation of *HIS3* gene.

***GCN5*, *SNF1*, and *SPT3* interact genetically to control *HIS3* expression:** *GCN5* genetically interacts with many regulators for appropriate control of transcription (see the Introduction for details). Overexpressing Snf1p may reinforce the relationship of Gcn5p with a positive partner, or neutralizes a negative regulator that opposes Gcn5p. We focused our studies on Spt3p, because Gcn5p and Spt3p perform antagonistic functions at

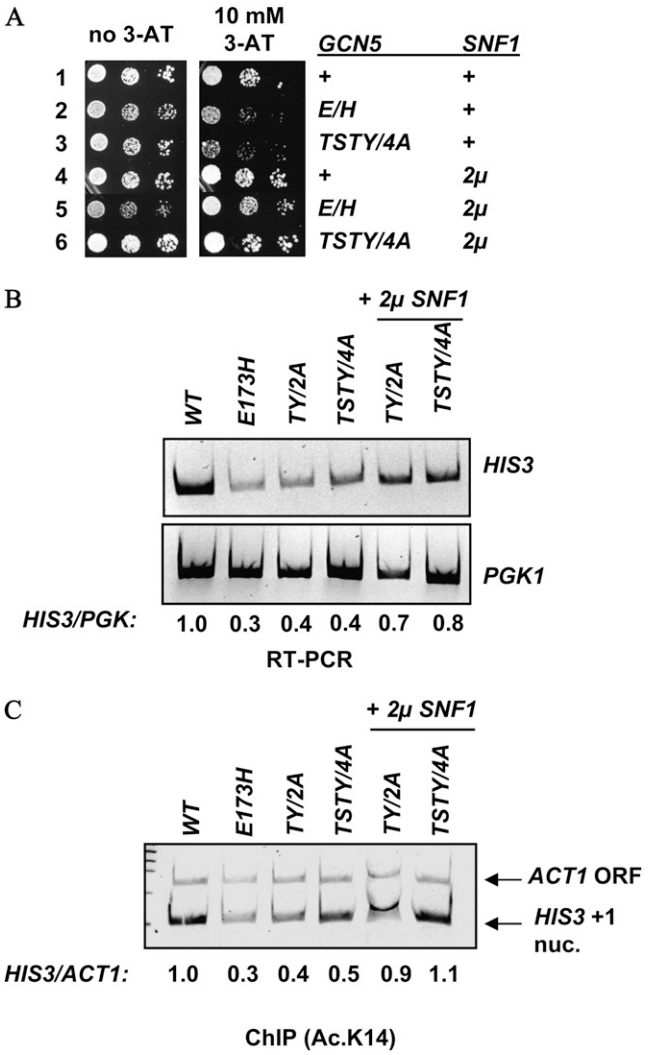

Figure 3.—*gcn5* TSTY/4A is suppressed by overexpressing Snf1p. (A) Cellular sensitivity to 3-AT. +, wild type without extra copies of *SNF1*; 2μ, multicopy plasmid introduced. (B) RT–PCR analysis of *HIS3* expression. (C) 2μ *SNF1* rescues the H3 hypoacetylation phenotype associated with *gcn5* TSTY/4A. Shown are ChIP results. TY/2A: T211A Y212A; TSTY/4A: T203A, S204A, T211A, and Y212A.

certain well-characterized genes. For example, deleting *SPT3* rescues the transcriptional defects of *HO* caused by either a HAT-deficient, or a complete knockout allele of *GCN5* (Yu *et al.* 2003). The Gcn5p-dependent basal expression of *HIS3* is increased in *spt3*Δ cells (Sterner *et al.* 1999). We suspected that the negative effect by Spt3p may be one of the targets for Snf1p in facilitating Gcn5p functions.

To delineate the genetic relationship between *GCN5* and *SPT3*, we deleted the latter in different *gcn5*$^-$ background and examined the expression status of *HIS3*. Figure 4A shows that Spt3p indeed was an allele-specific regulator of Gcn5p. Deleting *SPT3*, while imposing no discernible effect on 3-AT resistance (row 6), suppressed the TSTY/4A mutant (compare rows 3 and 8). However, the *E173H* (row 7) and complete

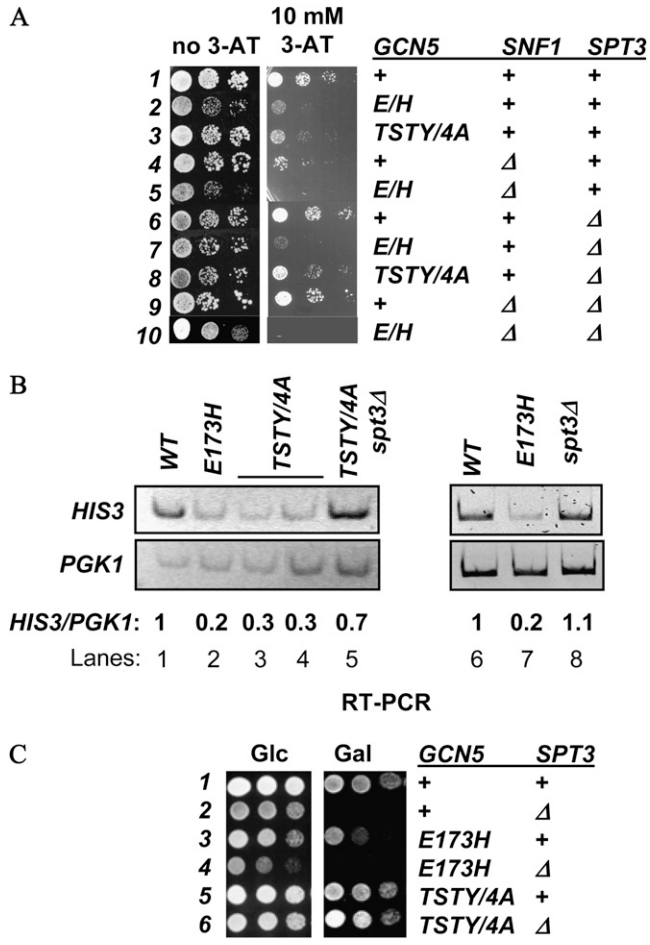

FIGURE 4.—*SPT3* is a negative regulator of *GCN5*. Deleting *SPT3* rescues the 3-AT hypersensitivity (A) and *HIS3* transcriptional defects (B, shown are RT–PCR results). (C) *gcn5* TSTY/4A suppresses the *gal*$^-$ phenotype caused by *SPT3* deletion. Yeast cells were serially diluted and spotted to glucose (Glc) or galactose (Gal) medium.

knockout (data not shown) mutants were not rescued by deleting *SPT3*. Semiquantitative RT–PCR assays confirmed the suppression of *HIS3* transcriptional defects (compare lanes 3, 4, and 5, Figure 4B). To understand further the *GCN5*–*SPT3* genetic interactions, we examined cellular growth on galactose, as *spt3*Δ cells were reported by several groups to be unable to use galactose as the sole carbon source (DUDLEY *et al.* 1999; LARSCHAN and WINSTON 2001; BHAUMIK and GREEN 2002). *spt3*Δ cells in our hands also were Gal$^-$ (row 2, Figure 4C), whereas *gcn5* TSTY/4A cells grew well on the galactose medium (compare rows 1, 3, and 5). Importantly, the *gcn5* TSTY/4A *spt3*Δ double mutant cells displayed clear *Gal*$^+$ growth (row 6). Thus, *gcn5* TSTY/4A was the suppressor for the galactose auxotroph phenotype of *spt3*Δ cells. These results demonstrate that the antagonistic relationship between Gcn5p and Spt3 was maintained in cellular responses to both amino acid starvation (*e.g.*, *HIS3* activation) and galactose utilization. Similar to the observation that *spt3*Δ allele was unable to suppress the *HIS3* expression defects caused by the *E173H* allele (row 7, Figure 4A), this *gcn5* mutant cannot rescue the Gal$^-$ phenotypes of *spt3*Δ cells (row 4, Figure 4C), further supporting the notion that the genetic interactions between *GCN5* and *SPT3* were dependent on the target genes as well as the alleles of these two regulators.

That 2μ *SNF1* and *spt3*Δ were common suppressors of the TSTY/4A allele of *GCN5* prompted us to look more deeply into the interrelationship of these three. To this end, we combined different *gcn5* alleles with *snf1*Δ or *spt3*Δ and analyzed *HIS3* expression and acetylation. Yeast cells lacking Snf1p were defective in *HIS3* activation (Figure 5A, lane 3; LIU *et al.* 2005). Consistently, the *HIS3* promoter became hypoacetylated in the *GCN5*$^+$ *snf1*Δ strain (Figure 5B, lane 3). Since the level of Gcn5p remained unchanged in the absence of Snf1p (Figure 5C), the HAT action of Gcn5p at the *HIS3* promoter clearly depended on a functional Snf1p, a notion consistent with the genetic and physical interactions between Gcn5p and Snf1p (see Figures 1–3), as well as the discovery that Snf1p was present at the *HIS3* promoter (Figure 2C, left panel). Furthermore, the *GCN5*–*SNF1* genetic interaction appeared to involve *SPT3* because the *HIS3* transcriptional defect of *snf1*Δ cells was effectively reverted by deleting *SPT3* (Figure 5A, compare lanes 3 and 4), suggesting that a key function of Snf1p was to antagonize a negative activity of Spt3p. This antagonism required a certain function(s) of Gcn5p, for *snf1*Δ *spt3*Δ cells became sensitive to 3-AT if *GCN5* was replaced with the E173H allele (rows 9 and 10, Figure 4A). Intriguingly, deleting *SPT3*, though rescuing the *HIS3* transcriptional defect of *snf1*Δ cells, did not suppress the hypoacetylation phenotype (lane 5, Figure 5B). Similarly, *GCN5*$^+$ *spt3*Δ cells were hypoacetylated at the *HIS3* promoter but exhibited near normal expression of *HIS3* (Figure 4B, lane 8). Together, these results suggest that a histone H3 acetylation-independent function of Gcn5p is responsible for *HIS3* activation in the *snf1*Δ *spt3*Δ background, and that this activity of Gcn5p may be suppressed by Spt3p under normal conditions.

Gcn5p interacts with Spt3p: We next sought to test the possible molecular basis underlying the Gcn5p–Spt3p functional connection. Given the extensive genetic interactions between *GCN5* and *SPT3* in the regulation of multiple genes (see the Introduction), we suspected that direct association might exist between these two proteins. Since both Gcn5p and Spt3p are components of the SAGA and the SLIK/SALSA complexes, it would be difficult to detect the *in vivo* physical interaction between Gcn5p and Spt3p. Instead, we expressed Gcn5p and Spt3p in *E. coli* and tested whether these two proteins could interact directly *in vitro*. Wild type and two different mutant Gcn5p, E173H and TSTY/4A, were expressed as Hisx6-tagged proteins, resolved, and blotted to PVDF membrane. HA–Spt3p (Figure 6A, right panel) was then used as the probe to

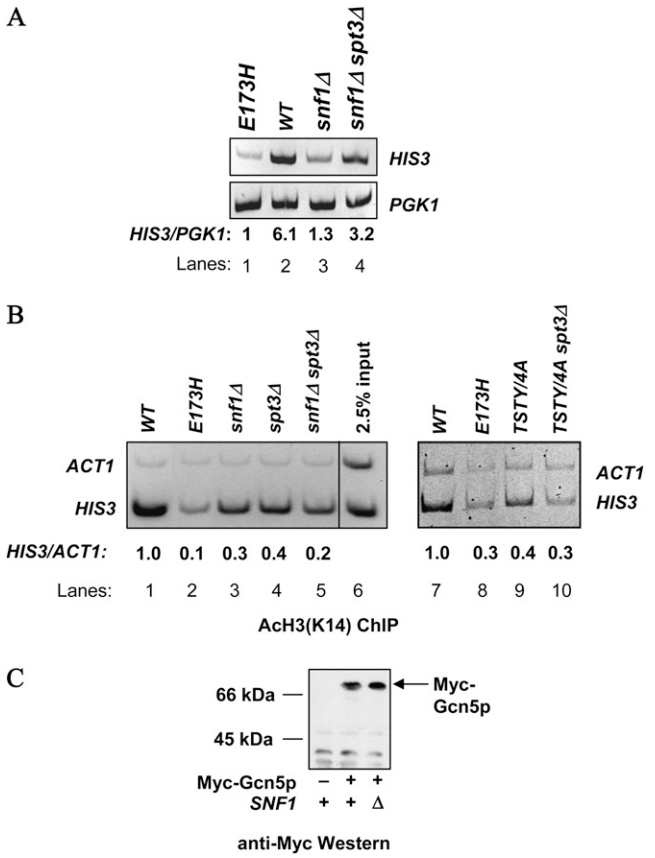

FIGURE 5.—Deleting *SPT3* suppresses *HIS3* transcriptional defects caused by *snf1*Δ. (A) RT–PCR shows suppression of *HIS3* transcriptional defects in *snf1*Δ cells, but (B) histone H3 remains to be hypoacetylated at the *HIS3* promoter, as shown by ChIP analyses. (C) The steady state level of Gcn5p is not affected by deleting *SNF1*. Myc-tagged Gcn5p was expressed from its native chromosomal locus in *SNF1*$^+$ or *snf1*Δ background. Yeast whole cell extracts were prepared and probed with anti-Myc antibodies in Western blotting assays.

bind the immobilized Gcn5p. The anti-HA antibodies were used under a standard Western blotting condition to examine the relative amounts of HA–Spt3p bound by different Gcn5p.

Figure 6A shows that the interaction between Gcn5p and Spt3p could be readily detected by the Farwestern approach. Intriguingly, the TSTY/4A and TY/2A (data not shown) Gcn5p trapped HA–Spt3p even more strongly than did the wild-type counterpart. The E173H allele (center lane, Figure 6A, left), also displayed enhanced affinity for Spt3p, although the enhancement seen in the E173H allele was substantially weaker than the TSTY/4A mutant. These biochemical data demonstrated the intrinsic affinity between Gcn5p and Spt3p and that certain mutations of Gcn5p may either increase the affinity or stabilize the association with Spt3p.

Homologs of Spt3p are found in fungi, insects, worm, and mammals (our BLAST search results; data not shown). Spt3p shares significant homology with the histone fold domains of two TBP-associated factors, TAF11 (a.k.a. $TAF_{II}28$) and TAF13 (a.k.a. $TAF_{II}18$) (BIRCK *et al.* 1998) (Figure 6B). We were interested in knowing which of the two histone fold domains of Spt3p, if separable, was critical for Gcn5p association. To this end, we prepared three GST–HA fusion fragments of Spt3p for *E. coli* production and *in vitro* binding assays (Figure 6B).

The Spt3 (1–107) and (156–337) fragments contained respectively the N′ and C′ histone fold domains; Spt3 (107–337) also included the bridging sequence in between. Comparable amounts of these three fragments were purified from *E. coli* and used as the probe in binding assays (Figure 6C). While Spt3 (1–107) showed very weak, if any, interaction with either allele of Gcn5p, both (107–337) and (156–337) fragments interacted positively with the TSTY/4A mutant. No significant interaction was seen with either the BSA internal control (data not shown), or another unrelated Hisx6-tagged protein, Hmt1p. We repetitively observed that the (156–337) fragment had the strongest affinity, suggesting that the N′ histone fold domain and the linker sequence may interfere with the Gcn5p–Spt3p association. We conclude that the C′ TAF11 histone fold domain contains the major interface for Gcn5p interaction, and that the TSTY/4A mutations of Gcn5p significantly enhance the association with the TAF11 histone fold domain of Spt3p.

DISCUSSION

Snf1p and Spt3p are respectively positive and negative regulators of Gcn5p: We report here a Gcn5p–Snf1p–Stp3p regulatory network that is critical for the transcriptional activation of *HIS3* (Figure 7). Snf1p is an activator of Gcn5p for both the promoter acetylation and transcriptional activation of *HIS3*. Conversely, Spt3p is an inhibitor that becomes more potent in *gcn5* TSTY or *snf1*Δ mutant cells, leading to transcriptional defects in these mutants. Since the *spt3*Δ suppressor only rescues the *HIS3* transcriptional defect but not the promoter hypoacetylation phenotype (lane 5, Figure 5A), we suspect that Gcn5p exerts an H3 acetylation-independent function inhibited by Spt3p. This inhibition likely results from a direct interaction between Gcn5p and Spt3p. As to Snf1p, in addition to potentiating the HAT function of Gcn5p, it may dampen the negative effect of Spt3p. A probable scenario regarding the TSTY/4A quadruple mutant is that these mutations augment or stabilize the interaction with Spt3p, hence enhancing the repressive strength of Spt3p. Overproducing Snf1p may effectively compete against Spt3p for the same or overlapping binding site on Gcn5p. The observed interdigitating relationships among these three proteins demonstrate a delicate system that balances the action of the conserved histone acetyltransferase Gcn5p. However, given the multitude of genetic inter-

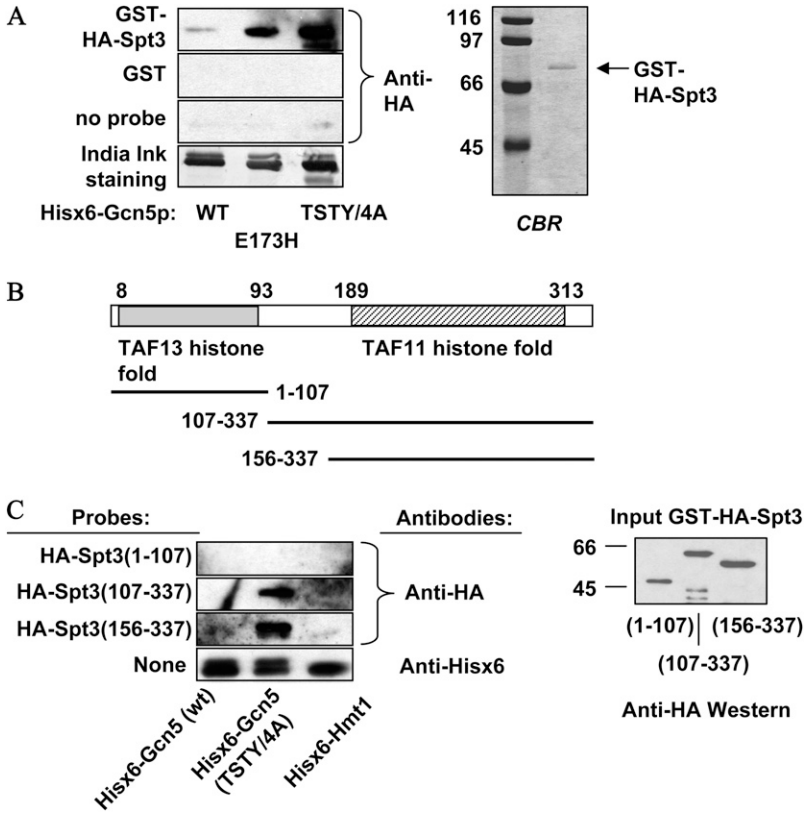

FIGURE 6.—Gcn5p and Spt3p interact directly in vitro. (A) Full-length Spt3p was double tagged by GST and HA and was purified from *E. coli* for Gcn5p binding in the Farwestern. Bacterially expressed, Hisx6-tagged wild-type and mutant Gcn5 proteins were resolved and immobilized on PVDF membrane and incubated with soluble GST–HA–Spt3p. Anti-HA antibodies were then used to quantify the relative amount of Spt3p trapped by different Gcn5p (top three panels, left). A fourth membrane strip was stained by India ink for Gcn5p loading control. Right panel: CBR staining of the purified Spt3p used in the Farwestern assays. (B) Schematics of Spt3p histone fold domains and the three fragments used for Farwestern assays. (C) The carboxyl histone fold domain of Spt3p is sufficient for Gcn5p interaction. Left: Farwestern results. Three different Spt3p fragments were expressed as GST- and HA-double tagged probes. The fragments used in each assay are listed on the left of each Western strip. Anti-His tag Western results (bottom strip, left) revealed comparable amounts of Hisx6–Gcn5p and Hisx6–Hmt1p. Right: Anti-HA Western blotting showing the relative amounts of the three GST–HA–Spt3p derivatives used in the Farwestern tests.

actions between Gcn5p and other transcriptional and chromatin regulators, it is possible that Snf1p also negotiates with additional factors, such as histone deacetylases, in the control of Gcn5p. Further genetic tests and screens may yield clues for this hypothesis.

Intriguingly, a low-resolution electron microscopy study showed that Gcn5p and Spt3p are spatially segregated by ~14 nm within the purified SAGA complex (Wu *et al.* 2004). We do not believe that the electron microscopy image of a static SAGA complex and our model are mutually exclusive. We hypothesize that the interactions between Gcn5p, Snf1p, and Spt3p are transient but are critical for regulating the biochemical activities of the SAGA complex, *i.e.*, chromatin modification and transcription activation. Given that SAGA is responsible for mostly stress-related transcription (Lee *et al.* 2000; Huisinga and Pugh 2004), it seems possible that SAGA exists as an inactive form prior to its engagement in transcriptional activation. In its inactive state, the SAGA complex positions Spt3p in a way that Gcn5p is inhibited. Stresses, such as nutrient deprivation and heat shock by which many genes are activated in a SAGA-dependent fashion (Huisinga and Pugh 2004), segregate Gcn5p and Spt3p and consequently instigate the chromatin modification and transcriptional activation activities of SAGA. This scenario is consistent with the observations that deleting *SPT3* enhances the basal expression of *HIS3* in rich medium in a *GCN5*-dependent manner (Sterner *et al.* 1999; Belotserkovskaya *et al.* 2000). A recent report by Winston and colleagues (Helmlinger *et al.* 2008) that Gcn5 and Spt8 proteins in the *Schizosaccharomyces pombe* SAGA complex play opposing roles in the control of proliferation-to-sexual differentiation switch also points to a dynamic interrelationship between SAGA subunits. It is worth noting that in our hands, *spt3*Δ and *spt8*Δ cells exhibited equivalent responses to genetic manipulations of *GCN5* or *SNF1* (data not shown). As to the Gcn5p activator Snf1p, it has been shown that the kinase activity of Snf1p is repressed when cells are grown in glucose-rich medium (Hardie *et al.* 1998) and becomes activated after brief centrifugation or wash of yeast cells (Smith *et al.* 1999). We suspect that Snf1p was inadvertently activated during the purification of SAGA and related Gcn5p complexes, hence awakening the nucleosomal HAT activity of Gcn5p. Indeed, Berger and colleagues (Belotserkovskaya *et al.* 2000) first reported that the composition and chromatographic behaviors of the SAGA complex were changed upon amino acid starvation.

Gcn5p phosphorylation by Snf1p: It is interesting that the TSTY/4A phosphorylation-deficient mutant can be rescued by Snf1p overexpression (Figure 3). This suppression can be due to one of two reasons. While the Pro-Q Diamond phosphostaining did not detect an obvious phosphorylation change of the TSTY/4A mutant when Snf1p was overexpressed, we cannot rule out that a key, but quantitatively minor phosphor-

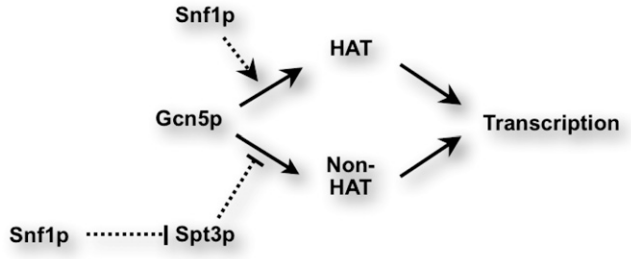

Figure 7.—Model for Gcn5p, Snf1p, and Spt3p relationship for *HIS3* transcriptional control. Dotted lines represent activating (with arrow) or inhibiting (with end bar) functions of Snf1p and Spt3p revealed in this work. See discussion for details.

ylated Gcn5p species can be augmented when the dosage of Snf1p increases. Alternatively, Snf1p may use Gcn5p as the docking site to get access to other target proteins, such as the Srb/mediator complex (Kuchin *et al.* 2000). In this case, Gcn5p phosphorylation is functionally redundant. Overproducing Snf1p may compensate for the crippled affinity for the TSTY/4A mutant Gcn5p, thus restoring *HIS3* activation. Consistently, the complete knockout allele of *GCN5* is insensitive to Snf1p overproduction for *HIS3* activation (Liu *et al.* 2005).

Of the four residues tested, T211 and Y212 clearly play critical roles in both Gcn5p phosphorylation and in *HIS3* activation. T203 and S204 appear to have an auxiliary function for phosphorylation (data not shown). When we mutated any of the four residues to aspartic acid, a commonly used phosphomimetic amino acid to assess the effect of constitutive phosphorylation, yeast cells became hypersensitive to 3-AT, suggesting a severe loss of Gcn5p function (data not shown). Intriguingly, all these phosphomimetic mutants were totally insoluble when expressed in bacteria and could not acetylate histones in the in-gel activity tests (data not shown). It is therefore possible that constitutive phosphorylation of Gcn5p is detrimental and causes a structural catastrophe. Phosphorylation of Gcn5p is thus likely a strictly regulated event. Protein phosphatases, such as Glc7p known to interact genetically with Snf1p (McCartney and Schmidt 2001; Hedbacker and Carlson 2008), may be part of the Gcn5p regulatory circuit as well.

It is also interesting that both Gcn5p and Snf1p appear to be associated with the *HIS3* locus relatively constitutively (Figure 2D). This observation is consistent with our Ada2p ChIP results (M.-H. Kuo, unpublished data) and the notion that the enzymatic activities of Gcn5p and Snf1p are subjected to regulation (see above). On the other hand, the stable association of Gcn5p with *HIS3* seems to contradict the observations that Gcn5p-dependent H3 hyperacetylation of the *HIS3* promoter is triggered by amino acid starvation (Kuo *et al.* 1998) and that the SAGA complex is recruited to several loci including *GAL1* and *ARG1* (Henry *et al.* 2003; Govind *et al.* 2007) under certain induced conditions. One explanation is that the HAT activity of Gcn5p, instead of its recruitment, is upregulated in response to amino acid starvation at the *HIS3* locus. Alternatively, two different Gcn5p-containing complexes may each be responsible for the basal and induced H3 acetylation.

Spt3p and Gcn5p regulation: It remains to be seen how Spt3p inhibits Gcn5p at the biochemical and molecular level. One possibility is that Spt3p and Snf1p compete for an overlapping motif of Gcn5p for transcriptional regulation. *In vitro*, we did not observe a clear effect on the HAT activity of Gcn5p in the presence or absence of Spt3p (data not shown), suggesting that Spt3p does not directly influence the HAT action in a highly refined biochemical system. Similarly, we did not observe strong evidence for Spt3p acetylation by Gcn5p *in vitro*. To our surprise, the H3 hyperacetylation of the *HIS3* promoter is lost in the *spt3*Δ strain (Figure 5A), even though *HIS3* activation appears to be normal. Equally intriguing is that the *snf1*Δ *spt3*Δ strain exhibits a similar H3 hypoacetylation trait (Figure 5B). These results clearly demonstrate that the HAT activity of Gcn5p is not indispensable under certain conditions. Evidence presented in this work (*e.g.*, Figure 5B) led us to speculate that Spt3p represses a non-H3 acetylation activity of Gcn5p. In the absence of Spt3p, this activity of Gcn5p is upregulated to an extent that the canonical H3 hyperacetylation is masked or no longer needed. Indeed, the H3 K14Q mutation, which mimics a constitutively acetylated state, triggers upregulation of a Gcn5p-driven reporter gene, but deleting *GCN5* perturbs such enhancement (Zhang *et al.* 1998). The TSTY/4A mutant of Gcn5p may preserve this mystic, acetylation-independent function that is rendered active upon the removal of Spt3p.

Another function of Gcn5p is to evict histones H3 and H4 within the open reading frame during transcription (Govind *et al.* 2007). H3/H4 eviction depends on the HAT activity of Gcn5p (Govind *et al.* 2007). We have been using *HIS3* as the model to understand how Gcn5p activates transcription. *HIS3* is a small gene, with only five positioned nucleosomes covering the entire open reading frame. Probably because of the highly compact nature of this gene, we did not observe clear H3 eviction during *HIS3* activation (Figure 2C). Consistently, the quadruple TSTY/4A mutant, though causing H3 hypoacetylation, does not affect histone H3 occupancy at the *HIS3* locus. It will be interesting to examine whether H3 eviction is impaired by the TSTY/4A mutant at other longer Gcn5p target genes, and, if so, whether manipulating *SNF1* and *SPT3* can modulate this function of Gcn5p.

Finally, phenotypic comparison between two alleles of *gcn5*, E173H and TSTY/4A, further suggests molecular distinction of these mutants, even though both alleles cause H3 hypoacetylation and *HIS3* transcriptional

defects. For example, while TSTY/4A is rescued by overexpressing Snf1p and by deleting *SPT3*, the E173H allele does not respond to *spt3*Δ. Furthermore, the hypoacetylation phenotype of the TSTY/4A mutant can be suppressed by 2μ *SNF1*, but the E173H mutant remains hypoacetylated at *HIS3*. We attribute these differences to the facts that E173 is the active center for the HAT action, and that the TSTY residues are outside the active center (Tanner *et al.* 1999; Trievel *et al.* 1999) and may be important for maintaining a certain conformational isoforms of Gcn5p. Allele-specific suppression displayed by these two mutants underscores the value of genetic dissection, and provides clues for further examination that will likely lead to a better understanding of how Gcn5p performs its chromatin modification and transcriptional regulation functions.

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