Histone H3 specific acetyltransferases are essential for cell cycle progression

LeAnn Howe,[1] Darryl Auston,[2] Patrick Grant,[3] Sam John,[1,5] Richard G. Cook,[4] Jerry L. Workman,[1] and Lorraine Pillus[2,6]

[1]Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802, USA; [2]Division of Biology, University of California, San Diego, California 92093, USA; [3]Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia 22908, USA; [4]Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030, USA

Longstanding observations suggest that acetylation and/or amino-terminal tail structure of histones H3 and H4 are critical for eukaryotic cells. For *Saccharomyces cerevisiae*, loss of a single H4-specific histone acetyltransferase (HAT), Esa1p, results in cell cycle defects and death. In contrast, although several yeast HAT complexes preferentially acetylate histone H3, the catalytic subunits of these complexes are not essential for viability. To resolve the apparent paradox between the significance of H3 versus H4 acetylation, we tested the hypothesis that H3 modification is essential, but is accomplished through combined activities of two enzymes. We observed that Sas3p and Gcn5p HAT complexes have overlapping patterns of acetylation. Simultaneous disruption of *SAS3*, the homolog of the *MOZ* leukemia gene, and *GCN5*, the *hGCN5/PCAF* homolog, is synthetically lethal due to loss of acetyltransferase activity. This key combination of activities is specific for these two HATs because neither is synthetically lethal with mutations of other MYST family or H3-specific acetyltransferases. Further, the combined loss of *GCN5* and *SAS3* functions results in an extensive, global loss of H3 acetylation and arrest in the G_2/M phase of the cell cycle. The strikingly similar effect of loss of combined essential H3 HAT activities and the loss of a single essential H4 HAT underscores the fundamental biological significance of each of these chromatin-modifying activities.

[*Key Words*: MOZ; MYST; GNAT; *S. cerevisiae*]

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The acetylation of specific lysine residues within the amino-terminal tails of core histones has been implicated in the regulation of transcription and other nuclear processes. The level of histone acetylation within a given region of the genome is maintained by varied recruitment of histone acetyltransferases (HATs) and deacetylases (HDACs). A number of acetyltransferases have been identified in *Saccharomyces cerevisiae* that acetylate histones in vitro (Sterner and Berger 2000), and in vivo (Kuo et al. 1998; Zhang et al. 1998). The majority of these HATs show discrete substrate specificities, including the *GCN5*-dependent SAGA, ADA, and HAT-A2 complexes (Grant et al. 1997; Ruiz-Garcia et al. 1997; Saleh et al. 1997; Eberharter et al. 1999), the *SAS3*-dependent NuA3 complex (John et al. 2000), the Nut1p containing mediator complex (Lorch et al. 2000), the *ESA1*-dependent NuA4 complex (Smith et al. 1998; Allard et al. 1999; Clarke et al. 1999), and Hpa2p, a protein that to date has only been characterized in recombinant form (Angus-Hill et al. 1999). Gcn5p and Hpa2p are members of the GNAT family of acetyltransferases, and acetylate preferentially Lys 14 of histone H3 in free histones in vitro (Kuo et al. 1996; Tse et al. 1998; Angus-Hill et al. 1999). When assayed as native HAT complexes, ADA and SAGA acetylate additional lysine residues within nucleosomes (Grant et al. 1999). The Mediator complex, with the GNAT-related subunit Nut1p, and the NuA3 complex, which has the MYST family acetyltransferase Sas3p as the catalytic subunit, also show specificity toward histone H3 in nucleosomes, but the particular lysines acetylated by these complexes is unknown (Grant et al. 1997; John et al. 2000; Lorch et al. 2000).

In contrast to the H3-specific HAT complexes, the NuA4 complex is the only identified *S. cerevisiae* HAT complex that acetylates primarily histone H4 within nucleosomes (Allard et al. 1999). Disruption of the gene encoding the NuA4 catalytic subunit, Esa1p, is a lethal event, suggesting that the acetylation of histone H4 is essential for viability (Smith et al. 1998; Clarke et al. 1999). In contrast, deletion of *GCN5*, *NUT1*, *HPA2*, or *SAS3* results in relatively weak or no detectable phenotypes (Georgakopoulos and Thireos 1992; Marcus et al.

[5]Present address: Laboratory of Receptor Biology and Gene Expression, National Cancer Institute, Bethesda, Maryland 20892, USA.
[6]Corresponding author.
E-MAIL lpillus@biomail.ucsd.edu; FAX (858) 534-0555.
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1994; Reifsnyder et al. 1996; Tabtiang and Herskowitz 1998; Angus-Hill et al. 1999). This contrasting difference in requirement for a H4-specific HAT, versus HATs that acetylate histone H3, may suggest an inherent difference in the functions of the H3 and H4 amino-terminal tails. However, concomitant deletion of the histone H3 and H4 amino terminal tails is lethal, suggesting that the two histone tails have, to some extent, an overlapping or mutually accomplished essential function (Megee et al. 1990; Morgan et al. 1991; Ling et al. 1996). Moreover, mutation of Lys 14 in histone H3, which serves as one of the major targets for Gcn5p containing complexes in vitro (Grant et al. 1999), confers a strong, synthetic growth defect in *gcn5*Δ cells, suggesting that acetylation of this site by other HATs is required for normal cell growth (Zhang et al. 1998). The lack of significant phenotypes in strains with deletion of *GCN5*, *HPA2*, *NUT1*, or *SAS3* alone may thus be explained by at least partially overlapping functions of one or more of these HATs. It was observed recently that simultaneous deletion of *GCN5* and *ELP3*, the catalytic subunit of the elongator HAT complex, results in a growth defect (Wittschieben et al. 2000). However, although Elp3p acetylates all four core histones in an in-gel assay, its substrate specificity on nucleosomal histones is not yet known. Thus, whether the *gcn5*Δ *elp3*Δ phenotype is due to loss of overlapping histone H3 HAT activities is not clear.

We sought to investigate the possibility that histone H3 acetylation is essential for cell viability by constructing double-mutant combinations of *GCN5*, *HPA2*, and *SAS3*, the genes encoding known H3 HATs. We show that the substrate specificity of the Sas3p-dependent NuA3 complex overlaps with Hpa2p and Gcn5p in terms of the specific lysines acetylated within histone H3. We show that concomitant disruption of *GCN5* and *SAS3* is uniquely synthetically lethal and that this lethality is due to loss of Sas3p and Gcn5p HAT activity. Finally, we show that loss of Gcn5p and Sas3p HAT activity results in a cell cycle defect and accumulation of the mutant cells in the G_2/M phase of the cell cycle. These results suggest that Gcn5p and Sas3p may have overlapping roles in histone H3 acetylation that are essential for cell cycle progression, and that these functions may parallel those of the essential role for histone H4 acetylation.

Results

The NuA3 complex has a pattern of acetylation that overlaps with the Gcn5p-dependent HAT complexes and Hpa2p

Previously, it was shown that the yeast Gcn5p and Hpa2p proteins preferentially acetylate Lys 14 in the amino-terminal tail of histone H3 in free histones (Kuo et al. 1996; Tse et al. 1998; Angus-Hill et al. 1999). When Gcn5p is assayed from native complexes, its substrate specificity is expanded to include additional lysines within nucleosomal histones (Grant et al. 1999). The SAGA complex preferentially acetylates Lys 14, followed by Lys 18, and to lesser extents Lys 9 and Lys 23 within nucleosomes. The ADA complex is similar to SAGA, preferentially acetylating Lys 14 followed by Lys 18. We sought to determine the pattern of acetylation of another histone H3-specific HAT complex, NuA3. To this end, nucleosomes were incubated in vitro with the NuA3 HAT complex in the presence or absence of tritiated acetyl-CoA; the previously characterized ADA complex served as a positive control. After incubation, the nucleosomal histones were resolved by SDS-PAGE and subjected to either fluorography or immunoblotting for the different acetylated isoforms of histone H3 using site-specific antisera. Similar to the ADA complex, the NuA3 complex acetylated Lys 14 in an acetyl-CoA dependent fashion (Fig. 1A). However, unlike the Gcn5p containing HAT complexes, NuA3 did not acetylate Lys 9 or Lys 18, as shown by lack of immunostaining of NuA3 acetylated histones by antisera specific for Lys 9–Lys 18. To verify these data independently, nucleosomes were incubated in vitro with either the ADA or NuA3 HAT complexes in the presence of tritiated acetyl-CoA. Histone H3 was purified and subjected to parallel microsequencing and quantitation of radioactivity released at each step of degradation during microsequencing. The results confirmed that NuA3 acetylated Lys 14, and to a lesser extent, acetylated Lys 23 (Fig. 1B). Thus, the lysine specificity of the Sas3p-dependent NuA3 complex has similarities with, but is distinct from that of recombinant Hpa2p, Gcn5p, and the Gcn5p-dependent SAGA and ADA complexes, which all preferentially acetylate Lys 14 of histone H3.

Combined loss of Gcn5p and Sas3p HAT activity is synthetically lethal

The similar substrate specificity of NuA3 and Gcn5p containing HAT complexes raised the possibility that these complexes have overlapping functions in vivo. To test this hypothesis, we set out to construct the doubly mutant strain. Haploid *gcn5*Δ and *sas3*Δ deletion strains were mated, and the resulting diploids sporulated and dissected. We failed to recover viable *gcn5*Δ *sas3*Δ double mutants, suggesting that loss of both functions was synthetically lethal. This interpretation was confirmed by first transforming the diploid strain with a *URA3*-based plasmid carrying a wild-type *SAS3* gene. The transformed strain was then sporulated and dissected, and haploid spore products were identified with disruptions of both the *SAS3* and *GCN5* genes. These double-mutant strains all maintained the *SAS3* plasmid, and were unable to grow when subjected to negative selection for this *URA3*-based plasmid on medium containing 5-FOA (Fig. 2A). However, if simultaneously transformed with a non-*URA3*-based plasmid carrying a wild-type copy of the *GCN5* or *SAS3* genes, the double mutants grew on 5-FOA, but maintained the second plasmid. Together, these tests showed that simultaneous disruption of *GCN5* and *SAS3* is synthetically lethal.

To test whether deletion of *HPA2* is lethal in combination with loss of either *GCN5* or *SAS3*, the same strat-

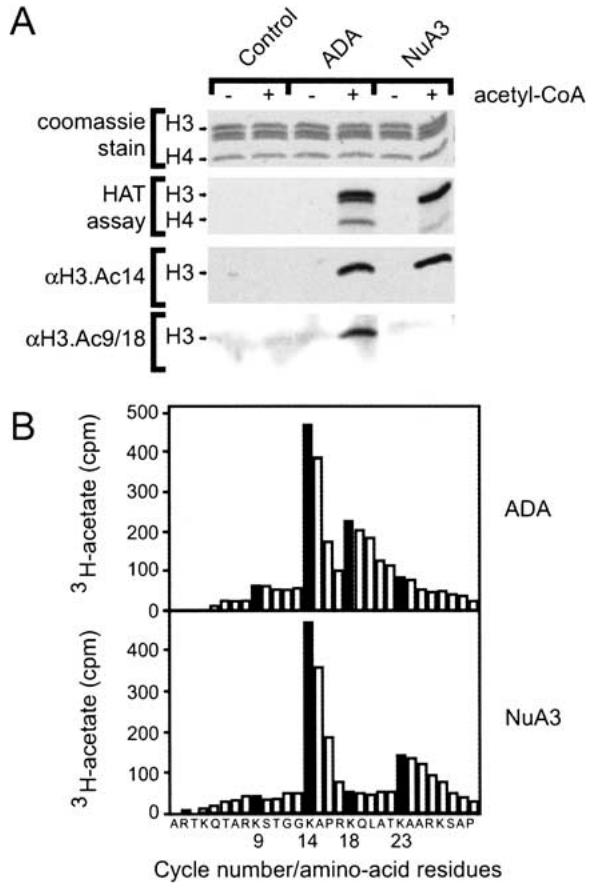

Figure 1. The NuA3 complex has overlapping site specificity with Gcn5p containing complexes for histone H3. (*A*) HAT assay fluorography and Western immunoblots of nucleosomes incubated alone (Control) or with purified ADA, or NuA3 complexes, in the presence (+) or absence (–) of radiolabeled acetyl-CoA. Western blots were probed with anti-H3.Ac14 or anti-H3.Ac9/18 antiserum. The positions of the individual histones are indicated. (*B*) HAT assays were performed using purified ADA or NuA3 HAT complexes and nucleosomes as substrate. Radiolabeled histone H3 was subsequently purified and subjected to microsequence analysis followed by direct determination of radioactivity at each position in the H3 polypeptide. The counts/min (cpm) for each cycle are plotted against the amino acid detected for that cycle. Potential acetylation sites are indicated.

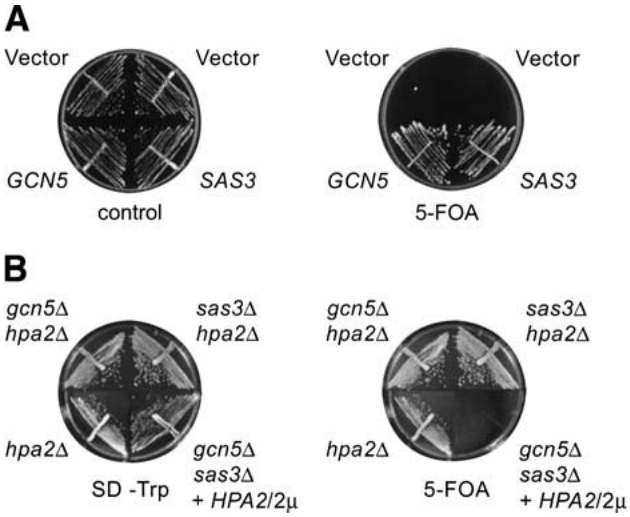

Figure 2. Deletion of *SAS3* is synthetically lethal with *gcn5*Δ. (*A*) Yeast strain YJW134 (*MAT***a** *his3*Δ*200 leu2*Δ*1 ura3-52 trp1-63 gcn5*Δ*::HIS3 sas3*Δ*::HIS3MX6*, pJW214) was transformed with vector alone or plasmids encoding wild-type (*GCN5*, *SAS3*) Gcn5p or Sas3p, and the resulting transformants plated on either synthetic complete medium (control) or synthetic complete with 5-FOA. (*B*) Yeast strains LPY5686 (*MAT*α *ade2-1 his3 leu2 lys2*Δ*0 trp1*Δ*1 ura3 gcn5*Δ*::HIS3 hpa2*Δ*::KANMX*), LPY5679 (*MAT*α *ade2-101 his3 leu2 lys2 trp1*Δ*1 ura3 sas3*Δ*::HIS3 hpa2*Δ*::KANMX*), LPY5680 (*MAT*α *ade2-1 his3 leu2 lys2 trp1*Δ*1 ura3 hpa2*Δ*::KANMX)*, and LPY5529 (*MAT*α *ade2-101 his3*Δ*200 leu2 lys2*Δ*801 trp1*Δ*1 ura3-52 sas3*Δ*::HIS3 gcn5*Δ, pLP640 pLP1399) were streaked on synthetic complete medium or synthetic complete with 5-FOA.

egies were used. We found that, unlike the *gcn5*Δ *sas3*Δ deletions, both *gcn5*Δ *hpa2*Δ, and *sas3*Δ *hpa2*Δ strains were viable (Fig. 2B; and Table 1). Thus, combined loss of two H3 HATs was compatible with viability and the synthetic lethality observed for the *gcn5*Δ *sas3*Δ double mutant was specific for these genes. Furthermore, increased gene dosage of *HPA2* did not rescue the *gcn5*Δ *sas3*Δ synthetic lethality (Fig. 2B), suggesting that the requirement for one of these two HATs is not simply a requirement for histone H3 HAT activity.

We also constructed double mutants with *gcn5*Δ and other MYST family acetyltransferases, *ESA1* and *SAS2*. Using a *SAS2* deletion strain and a temperature-sensitive *esa1* strain, we found that mutation of either *SAS2* or *ESA1* in a *gcn5*Δ or *sas3*Δ background was compatible with viability (Table 1). Thus the synthetic lethality between *gcn5*Δ and *sas3*Δ is not a general property of members of the MYST family combined with *gcn5* or GNAT mutants.

Synthetic lethality in gcn5Δ sas3Δ *mutants is due to loss of HAT activity*

To determine whether the *gcn5*Δ *sas3*Δ synthetic lethality is due to loss of Sas3p HAT activity, we asked

Table 1. *Mutant combinations tested*

Mutant Combination	Results
*gcn5*Δ *sas3*Δ	dead
*gcn5*Δ *hpa2*Δ	viable
*sas3*Δ *hpa2*Δ	viable
*gcn5*Δ *esa1L254P*	viable
*sas2*Δ *sas3*Δ *esa1L254P*	viable
*gcn5*Δ *sas2*Δ	viable
*sas3*Δ *sas2*Δ	viable
*sas3*Δ *spt20*Δ	viable
*sas3*Δ *ahc1*Δ	viable
*sas3*Δ *ahc1*Δ *spt20*Δ	viable
*sas3*Δ *ada2*Δ	viable
*sas3*Δ *ada3*Δ	viable
*sas3*Δ *ada2*Δ *ada3*Δ	viable

whether Sas3p HAT activity was required for viability in the absence of Gcn5p. To this end, plasmids expressing Sas3p with alanine substitutions within the acetyl CoA-binding domain ($GYG_{429–431}$ and $LM_{434–435}$) were shuffled into the *gcn5*Δ *sas3*Δ strain and tested for ability to substitute for wild-type *SAS3* by rescuing growth on 5-FOA. Previously, it has been shown that these mutants rescue the formation of the NuA3 complex in a *sas3*Δ strain, but Sas3p with alanine substitutions of $GYG_{429–431}$ did not rescue the HAT activity of the NuA3 complex (John et al. 2000). Figure 3 shows that although sas3p-$LM_{434–435}$ rescued the synthetic lethality of the *gcn5*Δ *sas3*Δ strain, sas3p-$GYG_{429–431}$ could not, indicating that loss of Sas3p HAT activity is responsible for the synthetic lethality of a *gcn5*Δ *sas3*Δ double mutant.

To investigate the requirement for Gcn5p HAT activity, a similar experiment was performed using plasmids expressing Gcn5p with alanine substitutions within the HAT domain ($LKN_{120–122}$ and $KQL_{126–128}$). In previous studies, both point mutants were shown to rescue intact SAGA and ADA complexes, but only gcn5p-$LKN_{120–122}$ is capable of rescuing the HAT activity of these complexes (Wang et al. 1998, Grant et al. 1999). A *gcn5*Δ *sas3*Δ strain harboring plasmid-borne gcn5p-$LKN_{120–122}$ was viable on 5-FOA, whereas cells containing gcn5p-$KQL_{126–128}$ were not, showing that the loss of Gcn5p-acetyltransferase activity is responsible for the synthetic lethality of *gcn5*Δ with *sas3*Δ.

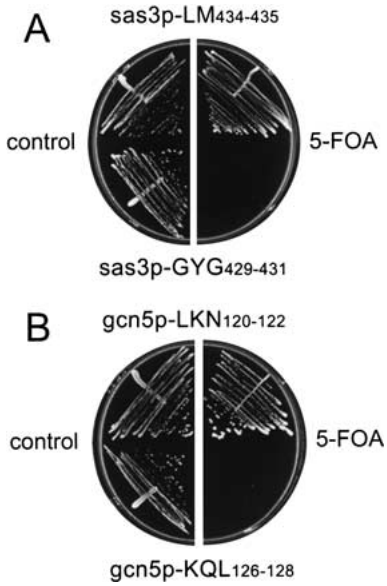

Figure 3. Synthetic lethality of the *gcn5*Δ *sas3*Δ strain is due to loss of HAT activity. Yeast strain YJW134 (*MAT**a** his3*Δ*200 leu2*Δ*1 ura3-52 trp1*Δ*63 gcn5*Δ*::HIS3 sas3*Δ*::HIS3MX6*, pJW214) was transformed with plasmids encoding alanine substitution mutations in Sas3p (*A*, sas3p-$LM_{434–435}$ and sas3p-$GYG_{429–431}$) or Gcn5p (*B*, gcn5p-$LKN_{120–122}$ and gcn5p-$KQL_{126–128}$) and plated directly on either synthetic complete medium (control) or synthetic complete with 5-FOA.

Histone H3 is an in vivo substrate of Sas3p

Although both Sas3p and Gcn5p-dependent HAT complexes acetylate histones in vitro, it had not been established whether Sas3p acetylated histones in vivo. There is a significant amount of genetic and biochemical evidence suggesting that Gcn5p acetylates histones in vivo (Howe et al. 1999), so it was tempting to speculate that, as suggested by the *gcn5*Δ *sas3*Δ synthetic lethality, that Sas3p did as well. To answer this question directly, we wished to determine whether loss of Sas3p resulted in a decrease in bulk histone acetylation in vivo. In preliminary experiments comparing *SAS3* wild-type and *sas3*Δ strains, we saw no difference in acetylation of bulk histones when analyzed by Western blotting with antibodies specific for acetylated histone H3 Lys 9 and Lys 14 (data not shown). However, Gcn5p is present in at least three different HAT complexes, which may contribute significantly to histone H3 acetylation in vivo. To decrease this substantial background, we wished to assay levels of histone acetylation in cells in which *GCN5* was deleted. This was made possible by constructing a series of conditional *sas3* mutant strains whose acetylation could be assayed in *gcn5*Δ null strains.

A library of *SAS3*-encoding plasmids that had been mutagenized with hydroxylamine was screened for plasmids that rescued the *gcn5*Δ *sas3*Δ synthetic lethality at a permissive temperature (26°C) but not at an elevated temperature (37°C) [Fig. 4A, *gcn5*Δ *sas3-C357Y/P375A*(YJW135)]. One such plasmid was rescued, sequenced, and determined to encode a protein with two amino acid substitutions, a cysteine residue at amino acid position 357 was replaced by tyrosine, and a proline at position 375 was replaced by alanine. Sas3p is a member of the MYST family of proteins that share a conserved acetyl CoA-binding site, and a C2HC zinc finger domain that is required for Sas3p histone HAT activity in vitro (Reifsnyder et al. 1996; Takechi and Nakayama 1999). The C357Y and P375A mutations in *SAS3* map to a highly conserved region between these two motifs. In the crystal structure of the MYST family protein Esa1p, this conserved cysteine maps within the α2 helix, and is buried in the interior of the protein, which may indicate an important role in protein folding and stability (Yan et al. 2000). The conserved proline at position 375 is located within a loop structure of Esa1p, which connects the α2 helix with the β7 strand. A second *SAS3* temperature-sensitive allele was created directly by substituting the cysteine 323 residue within the putative zinc finger domain with alanine, thereby creating a strain that we observed to grow well at 26°C, but not at 37°C [Fig. 4A, *gcn5*Δ *sas3-C323A*(YJW138)]. Finally, a third temperature-sensitive allele was created by deleting the 193 carboxy-terminal amino acids [Fig. 4A, *gcn5*Δ *sas3–638*Δ (YJW137)].

Histones were isolated from each of these temperature-sensitive strains after growth at the nonpermissive temperature and subjected to Western blot analysis. These blots show that there was a substantial decrease in histone H3 acetylation when compared with strains con-

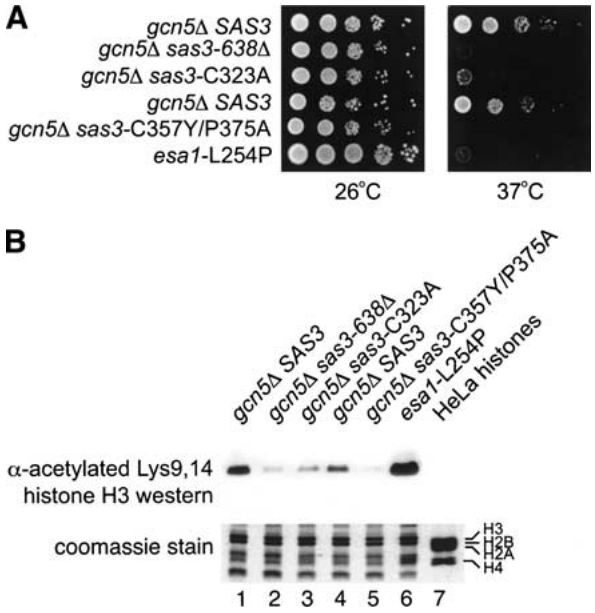

Figure 4. Sas3p is required for histone H3 acetylation in vivo. (*A*) *gcn5*Δ *sas3* temperature sensitivity was observed upon plating *gcn5*Δ *SAS3* [YJW136 (*MAT**a** his3*Δ*200 leu2*Δ*1 ura3-52 trp1*Δ*63 gcn5*Δ*::HIS3 sas3*Δ*::HIS3MX6*, pLP0640)], *gcn5*Δ *sas3-638*Δ [YJW137 (*MAT**a** his3*Δ*200 leu2*Δ*1 ura352 trp1*Δ*63 gcn5*Δ *::HIS3 sas3*Δ*::HIS3MX6*, pLP1364)], *gcn5*Δ *sas3-C323A* [YJW138 (*MAT**a** his3*Δ*200 leu2*Δ*1 ura3-52 trp1*Δ*63 gcn5*Δ*::HIS3 sas3*Δ*::HIS3MX6*, pLP1398)], *gcn5*Δ *SAS3* [YJW134 (*MAT**a** his3*Δ*200 leu2*Δ*1 ura3-52 trp1*Δ*63 gcn5*Δ*::HIS3 sas3*Δ*::HIS3MX6*, pJW214)], and *gcn5*Δ *sas3-C357Y/P375A* [YJW135 (*MAT**a** his3*Δ*200 leu2*Δ*1 ura3-52 trp1*Δ*63 gcn5*Δ*::HIS3 sas3*Δ*::HIS3MX6*, pJW216)] on YPD and growing at 26 and 37°C for 3 d. (*B*) Histones were isolated from the wild-type and temperature-sensitive strains shown in *A* after growth for 12 h at 37°C, and resolved either on 18% SDS-PAGE and stained with Coomassie brilliant blue, or resolved on a 12% SDS-PAGE, transferred to nitrocellulose and probed with anti-acetylated histone H3 (Lys 9 and Lys 14) antiserum. Purified HeLa core histones were run in parallel.

taining wild-type Sas3p (Fig. 4B, cf. lane 1 with 2 and 3, and lane 4 with 5). In fact, there is a clear correlation between the severity of the temperature sensitivity and the levels of histone H3 acetylation at the nonpermissive temperature. Such decreases in histone H3 acetylation were not seen when comparing a strain with temperature-sensitive mutation in Esa1p (esa1-L254P), a histone H4-specific acetyltransferase (Fig. 4B, lane 6). These results show that Sas3p is responsible for at least a portion of global histone H3 acetylation in vivo.

Loss of SAS3 is compatible with disruption of Gcn5p-dependent HAT complexes

Gcn5p is present in multiple HAT complexes in yeast, including SAGA, ADA, and HAT-A2, each of which contains both unique and shared protein subunits (Grant et al. 1997; Pollard and Peterson 1997; Ruiz-Garcia et al. 1997; Eberharter et al. 1999; Belotserkovskaya et al. 2000). We sought to determine whether the essential function of *GCN5* in the absence of *SAS3* was mediated uniquely through one of these complexes. To test this possibility, we constructed double mutants between *sas3*Δ and genes encoding noncatalytic components of these Gcn5p-dependent complexes.

To determine whether *sas3* is synthetically lethal with disruption of the SAGA complex, we first evaluated genetic interactions with *SPT20*, which encodes a component of the SAGA complex. Because *spt20*Δ strains possess the collective phenotypes associated with loss of other nonessential SAGA components, it is interpreted that loss of this protein is highly disruptive to SAGA function (Marcus et al. 1996; Roberts and Winston 1996, 1997; Sterner et al. 1999). By use of methods parallel to those described above, the *sas3*Δ *spt20*Δ double mutants were readily recovered (Table 1), suggesting that loss of Sas3p is not synthetically lethal due to activities overlapping with SAGA.

To test for synthetic lethality between Sas3p loss and disruption of the ADA complex, interactions with *AHC1* were examined. Currently, Ahc1p is the only known protein component that is unique to the ADA complex, and the ADA complex fails to purify from *ahc1*Δ strains using standard protocols (Eberharter et al. 1999). We observed that concomitant disruption of *SAS3* and *AHC1* is neither synthetically lethal (Table 1), nor does it cause any obvious growth defects on rich or minimal medium (data not shown), suggesting that disruption of the ADA complex alone is not responsible for the inviability of a *gcn5*Δ *sas3*Δ mutant.

In addition to Gcn5p, the SAGA, ADA, and HAT-A2 complexes all share Ada2p and Ada3p as subunits. Experimental evidence suggests that these common components are required to potentiate Gcn5p HAT activity (Syntichaki and Thireos 1998). Catalytically active ADA, SAGA, or HAT-A2 complexes fail to purify from strains in which *ADA2* and/or *ADA3* have been deleted (Grant et al. 1997; Sendra et al. 2000). Furthermore, phenotypes associated with deletions of *GCN5* are indistinguishable from deletions of *ADA2* and *ADA3* (Georgakopoulos et al. 1995). Thus, both in vitro and in vivo evidence supports the idea that Ada2p and Ada3p are required for the function of Gcn5p. To determine whether the *gcn5*Δ *sas3*Δ synthetic lethality is due to the loss of any or all of the known Gcn5p-containing HAT complexes, we disrupted *SAS3* in combination with either *ADA2* or *ADA3*. We found that *ada2*Δ *sas3*Δ, *ada3*Δ *sas3*Δ, and *ada2*Δ *ada3*Δ *sas3*Δ mutant combinations are viable (Table 1) and that the phenotypes of these strains recapitulate those of *ada2* or *ada3* single mutants.

The observation that disrupting *ADA2* and *ADA3* in combination with *sas3*Δ did not parallel the synthetic lethality of a *gcn5*Δ *sas3*Δ double mutant was surprising. This result raised the possibility that deletions of *ADA2* or *ADA3* are not as disruptive to the respective HAT complexes as originally proposed, and that subcomplexes exist in these mutants that can carry out at least a partial function of the HAT complex. To address this possibility, we analyzed the effects of *GCN5* and *ADA2*

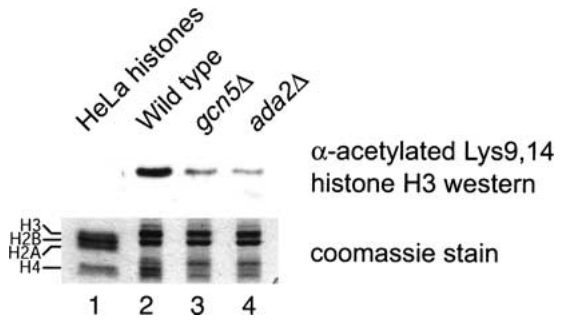

Figure 5. Gcn5p requires Ada2p for its catalytic function in vivo. Histones were isolated from PSY316 *GCN5*, PSY316 *ada2*Δ, or PSY316 *gcn5*Δ cells and resolved either on 18% SDS-PAGE and stained with Coomassie brilliant blue, or resolved on a 12% SDS-PAGE, transferred to nitrocellulose and probed with anti-acetylated histone H3 (Lys 9 and Lys 14) antiserum. Purified HeLa core histones were run in parallel.

deletions on the level of histone H3 acetylation in vivo. It was shown earlier that *GCN5* is required for the acetylation of several sites in H3 in vivo (Zhang et al. 1998). To test whether *ADA2* is also required for Gcn5p function, we isolated histones from *gcn5*Δ and *ada2*Δ cells and analyzed the level of histone acetylation by protein immunoblotting for acetylated histone H3. Consistent with the earlier results, loss of Gcn5p resulted in a significant decrease in the levels of histone H3 acetylated at Lys 9 and Lys 14 (Fig. 5, cf. lanes 2 and 3). Loss of Ada2p mimicked this effect (Fig. 5, lane 4), as levels of histone H3 acetylation in *ada2* cells were similar to those of *gcn5* cells. These in vivo results support prior models that Gcn5p does require Ada2p for much of its catalytic function, thereby leaving the viability of *sas3*Δ *ada2*Δ strains as somewhat puzzling. It is possible that alternative or residual Gcn5p-dependent HAT complexes are still present in *ada2*Δ and *ada3*Δ strains, and that even the minimal levels of histone H3 acetylation provided by these are sufficient to maintain cell viability. However, an additional intriguing possibility is that Gcn5p has further, formerly unsuspected, functions that are independent of its characterized HAT complexes, and these may contribute to the overlapping essential functions shared with Sas3p.

Mutation of GCN5 *and* SAS3 *disrupts cell cycle progression*

It was shown previously that, in the absence of *GCN5*, an increased proportion of cells accumulate in the G_2/M stage of the cell cycle (Zhang et al. 1998), indicating that Gcn5p contributes to normal cell cycle progression. To determine wheter *SAS3* also contributes to normal cell cycle control, we evaluated the *SAS3* conditional mutants in temperature-shift experiments. Mutant *gcn5*Δ strains containing either wild-type or temperature-sensitive alleles of *SAS3* were grown at the permissive temperature, after which a portion of each culture was removed and shifted to the restrictive temperature. When grown at a permissive temperature (26°C) and analyzed for DNA content, both the wild-type and mutant *SAS3* cells were found to have comparable profiles typical of normally cycling asynchronous cultures (Fig. 6). When shifted to 37°C, there was a slight increase in the proportion of *gcn5*Δ *SAS3* cells found in the G_2/M stage of the cell cycle, consistent with previous observations (Zhang et al. 1998). In contrast, when the temperature-sensitive *gcn5*Δ *sas3* strains were grown at the elevated temperature, the majority of cells were found to accumulate with 2C DNA content. This observation suggests that *SAS3*, like *GCN5*, normally contributes to cell cycle progression, and that disrupting both activities together disrupts this control.

Discussion

The packaging of DNA into chromatin is thought to be largely repressive to nuclear processes such as transcription. Numerous multiprotein complexes exist within nuclei to modulate the nucleosomal environment, including those with histone acetyltransferase and chro-

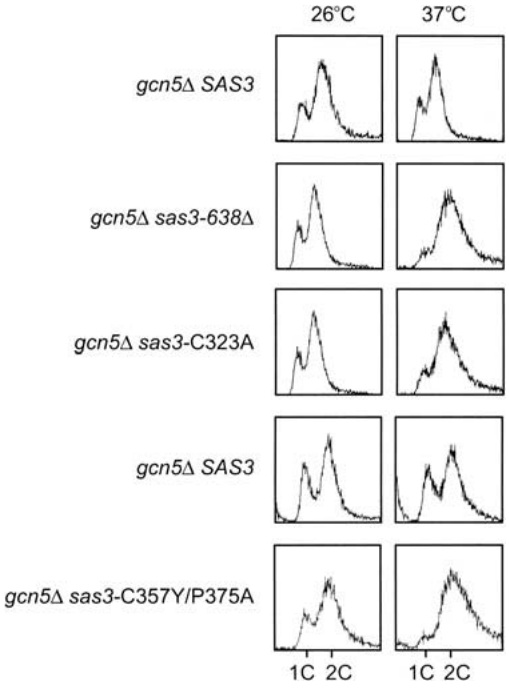

Figure 6. Flow cytometric analysis of *gcn5*Δ *sas3* strains. Exponentially growing cultures of *gcn5*Δ strains containing either wild-type *SAS3* [YJW136 (*MAT**a** his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn5Δ::HIS3 sas3Δ::HIS3MX6*, pLP0640) and YJW134 (*MAT**a** his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn5Δ::HIS3 sas3Δ::HIS3MX6*, pJW214)], or temperature-sensitive alleles of *SAS3* [YJW137 (*MAT**a** his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn5Δ::HIS3 sas3Δ::HIS3MX6*, pLP1364), YJW138 (*MAT**a** his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn5Δ::HIS3 sas3Δ::HIS3MX6*, pLP1398), and YJW135 (*MAT**a** his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn5Δ::HIS3 sas3Δ::HIS3MX6*, pJW216)] were incubated at either permissive (26°C) or nonpermissive (37°C) temperatures, stained with propidium iodide and subjected to flow cytometry. The position of cells with a 1C or 2C DNA content is marked.

matin remodeling activities (Sterner and Berger 2000; Vignali et al. 2000). In this work, we evaluated the effect of impairing one of these activities, that is, the ability to acetylate histone H3 on cell viability. Our results show that Sas3p and Gcn5p-dependent HAT complexes have overlapping patterns of acetylation on histone H3 in vitro, and that simultaneous deletion of *SAS3* and *GCN5* is synthetically lethal due to loss of Sas3p and Gcn5p acetyltransferase activities. The essential nature of these combined activities is specific, because loss of viability does not result if *GCN5* or *SAS3* are disrupted in combination with other acetyltransferases such as Hpa2p, Esa1p, and Sas2p. We also show that Sas3p acetylates histones in vivo.

Surprisingly, Sas3p's essential overlapping function with Gcn5p may be independent of any of the known Gcn5p-containing HAT complexes. Finally, we observe that *gcn5 sas3* double mutants arrest in the G_2/M phase of the cell cycle, suggesting that histone H3 acetylation is essential for normal cell cycle progression. The fact that loss of these specific histone H3 HATs recapitulates the effects of loss of the histone H4 specific HAT, Esa1p, supports two key conclusions. First, the results extend earlier models drawn from mutation of the amino terminus of H3 and show that H3 acetylation itself may be essential for viability. Second, it is now clear that two distinct HAT activities can individually support the essential requirement for H3 acetylation or that of other essential shared substrates.

Histone H3 HATs are essential but histone H3 tails are not

The actual role of nucleosomal histone acetylation is unclear. This neutralization of charge was originally thought to weaken histone–DNA interactions, but there is only limited experimental evidence to this effect (García-Ramirez et al. 1992; Libertini et al. 1988; Mutskov et al. 1998; Norton et al. 1989). Histone acetylation may mediate protein–protein interactions required for transcriptional regulation. For example, high levels of histone acetylation negatively influence the interaction of Tup1p, a transcriptional repressor, with the tails of histones H3 and H4 (Edmondson et al. 1996). Moreover, histone acetylation enhances retention of the chromatin-remodeling complex, SWI/SNF, to nucleosome arrays in vitro (Hassan et al. 2001).

Incomplete knowledge of the functional consequences of histone acetylation makes understanding its essential role for cell viability still somewhat elusive. Cells with deletions of the amino-terminal tail of histone H3 are viable, except in combination with deletion of the histone H4 tail (Megee et al. 1990; Morgan et al. 1991; Ling et al. 1996). These observations suggest that either the structure of the amino-terminal tails or their modifications may play an essential role in cells. For example, one possibility raised previously is that the H3 tail itself is not essential, but the ability to neutralize the charged tail residues is. This may be unlikely, considering that mutation of H3 Lys 14 to glycine, arginine, or glutamine causes similar phenotypes in a *gcn5*Δ strain suggesting that it is not simply loss of charge that is important (Zhang et al. 1998). However, these data must be interpreted with caution, as it has been shown that histone acetylation alters the secondary structure of the histone tails, and thus, substitutions with neutral amino acids may not accurately mimic acetylation, and may, in fact, have secondary effects on tail structure (Wang et al. 2000). A second possibility is that, although the NuA3, SAGA, and ADA complexes show a preference for acetylation of histone H3 in vitro (Grant et al. 1999), they may have expanded specificities in vivo. The overexpression of Gcn5p in vivo does result in increased acetylation of histone H4, and deletion of *GCN5* causes decreased acetylation of this histone at specific sites (Kuo et al. 1996; Zhang et al. 1998). Thus, if the substrate specificity of the NuA3 complex in vivo includes histone H4 as well as H3, the *gcn5*Δ *sas3*Δ synthetic may parallel the loss of viability that occurs upon simultaneous deletion of the H3 and H4 tails. A third possibility is that, although both Gcn5p and Sas3p acetylate histones in vivo, they may also be required to acetylate nonhistone substrates. The acetylation of Sin1p by Gcn5p-dependent HATs has been shown in vitro, and mutation of *SIN1* suppresses *gcn5*Δ transcriptional defects of an *HO:lacZ* reporter gene (Pollard and Peterson 1997; Perez-Martin and Johnson 1998; Yu et al. 2000). The systematic identification of nonhistone substrates for both *SAS3* and *GCN5*-encoded activities is largely unexplored, but is likely to hold significant clues to their essential roles.

Multiple roles for multiple H3-directed HAT complexes

Numerous yeast proteins have been identified that specifically acetylate histone H3, including Gcn5p, Sas3p, Hpa2p, and Nut1p (Sterner and Berger 2000). Gcn5p is the catalytic subunit of several distinct HAT complexes (Grant et al. 1997), and the possibility that Sas3p, Hpa2p, and Nut1p also exist in multiple complexes has not been excluded. Why cells maintain many complexes with overlapping specificities is not yet well understood, but may be considered in the context of at least three possibilities. One possibility is for the maintenance of a histone code, through which multiple, specific, histone modifications act together to effect unique downstream functions (Paro 2000; Strahl and Allis 2000). It has been shown previously that incorporation of Gcn5p into native complexes expands the specificity of this enzyme to additional lysines beyond its primary H3–Lys 14 target (Grant et al. 1999). Thus, the incorporation of HAT proteins into larger complexes may permit the cell to acetylate all of the lysines of histone H3 in various unique and dynamic patterns.

A second possibility is that different HAT complexes may function in regulating different levels or stages of transcription or other nuclear processes. It has been shown previously that the Gcn5p-dependent SAGA complex interacts directly with transcriptional activators and can target histone acetylation to promoter regions, suggestive of a role in mediating transcriptional

initiation (Utley et al. 1998; Vignali et al. 2000). A similar role has been suggested for Nut1p, a Mediator complex-associated HAT (Lorch et al. 2000). In contrast, the Sas3p-dependent NuA3 complex interacts with Spt16p (John et al. 2000), a component of the FACT complex, which enhances transcription elongation through chromatin templates in vitro (Orphanides et al. 1998, 1999). This activity and the fact that *spt16 sas3*Δ mutants show enhanced sensitivity to 6-azauracil, a phenotype correlated with transcriptional elongation defects, may point to a role for the NuA3 complex in enhancing elongation (John et al. 2000).

A third explanation for the requirement of multiple H3 HAT complexes is that the various complexes mediate different levels of histone acetylation throughout the genome. Several studies have shown that Gcn5p is responsible for high levels of promoter-specific acetylation, as well as lower, global levels of acetylation in surrounding, transcribed and nontranscribed regions (Kuo et al. 1996, 2000; Krebs et al. 1999; Parekh and Maniatis 1999; Vogelauer et al. 2000). Whether these regions are modified by direct and targeting mechanisms, or whether there are more passive gradients of activity starting from a single point-source is unknown. Perhaps incorporation of Gcn5p into multiple complexes is required to mediate its effects in both promoter-targeted acetylation as well as for maintaining low levels of more uniform acetylation in surrounding regions. Certainly the possibilities considered above are not mutually exclusive. It is likely that the multiple H3 HAT complexes may have synergistic and/or interdependent activities.

Overlapping, essential roles for Gcn5p and Sas3p?

A central, challenging question that remains is to define the essential biological activities demanding function of either *GCN5* or *SAS3*. Our results show that both Gcn5p and Sas3p are responsible for a significant portion of global histone H3 acetylation in yeast. However, we observe that cells can tolerate large decreases in histone acetylation without affecting cell viability, suggesting that maintenance of high levels of acetylation throughout the genome may not be the essential function of these HATs. Thus, although a decrease in histone acetylation may have deleterious consequences, we instead favor the possibility that only threshold levels of histone H3 acetylation, mediated by either Gcn5p or Sas3p complexes, are required for viability. In strains with gene deletions that are highly disruptive to these HAT complexes, such as *sas3*Δ *ada2*Δ, this threshold may be mediated by residual amounts of Gcn5p-dependent HATs, or by Gcn5p functioning as part of an unidentified, Ada2p-independent complex, or independently of other known interacting proteins. Of course, although Gcn5p and Sas3p both acetylate histones in vivo, the possibility that their essential function requires acetylation of a nonhistone substrate must also be considered.

One consequence of conditional loss of Sas3p and Gcn5p activity is that cells arrest with a replicated 2C DNA content. There are a number of reasons why this failure in cell cycle progression after S-phase may occur. For example, it is possible that these HATs are required for the regulation of transcription of cell cycle-specific genes that promote the cell cycle after DNA replication. Or, these HATs may only be required for transcription of essential genes during mitosis. It has already been shown that *GCN5* is required during mitosis for the expression of certain genes, presumably due to the condensed state of mitotic chromatin (Krebs et al. 2000). Another possibility is that Sas3p and Gcn5p serve a nontranscription-related function.

It is noteworthy that the *sas3 gcn5*Δ arrest at nonpermssive temperatures is reminiscent of that observed for conditional mutants of the H4 HAT Esa1p. This arrest is specifically dependent on the *RAD9* checkpoint gene. Whether the *sas3 gcn5*Δ mutants share this checkpoint dependency is not yet known. Interestingly, the *sas3 gcn5*Δ arrest is also similar to that of temperature-sensitive mutants of *POB3*, which normally functions in DNA replication (Schlesinger and Formosa 2000). Pob3p is a component of the CP/FACT/DUF complex (Wittmeyer and Formosa 1997; Orphanides et al. 1998; Okuhara et al. 1999), and Sas3p has already been shown to interact with another component of this complex, Spt16p (John et al. 2000). Sas2p, a MYST family member like Sas3p, is implicated in DNA replication (Ehrenhofer-Murray et al. 1997). Furthermore, there are data suggesting that other chromatin-modifying complexes contribute to DNA replication. For example, mutations in components of the SWI/SNF complex affect the replication of plasmids containing specific yeast origins of replication (Flanagan and Peterson 1999). Further, the MYST family protein Hbo1p was identified in mammalian cells as interacting with Orc1p, suggesting a role for this HAT in DNA replication, possibly by acetylating a nonhistone regulator of initiation (Iizuka and Stillman 1999). Thus, Sas3p and Gcn5p may be directly required for some aspect of DNA replication, although presumably not bulk replication.

Identifying additional genomic targets or nonhistone substrates of Gcn5p and Sas3p will be critical for understanding the essential combined nature of their activities. This will be especially important considering that the hGCN5/PCAF and MOZ human homologs of *GCN5* and *SAS3* are being increasingly implicated for their roles in both normal development and cancer (for reviews, see Jacobson and Pillus 1999; Roth et al. 2001). It seems likely that these enzymes together contribute to fundamental aspects of cellular and organismal function.

Materials and methods

Yeast strains, plasmids, and genetic methods

Strains used in this study were created by use of standard yeast manipulations (Ausubel 1987; Adams 1997) and are described in Table 2. HAT complexes were purified from yeast strain CY396 (Peterson et al. 1994) as described previously (Eberharter et al. 1998). Several *gcn5*Δ *sas3*Δ strains were constructed in different genetic backgrounds. For example, the *gcn5*Δ *sas3*Δ strain, YJW134, was created by crossing a *sas3* null allele [YJW121 (John et al. 2000)] with a *gcn5*Δ strain [FY1370 (Roberts and Winston 1997)]. The resulting diploid was transformed with a

Table 2. *Strains used in this study*

Strain	Genotype
CY396	*MATα swi2::HIS3, HO-LacZ, SW12-HA-6HIS::URA3*
LPY2562	*MAT***a** *ade2-101 his3-Δ200 hmra::URA3 leu2-Δ1 lys2-801 sas2Δ::TRP1 trp1-Δ63 ura3-52 sas3Δ::HIS3*
LPY2563	*MAT***a** *ade2-101 his3-Δ200 hmra::URA3 leu2-Δ1 lys2-801 sas2Δ::TRP1 trp1-Δ63 ura3-52 sas3Δ::HIS3*
LPY5297	*MAT***a** *his3 leu2 ura3 ahc1Δ::kanMX sas3Δ::HIS3*
LPY5298	*MATα his3 leu2 ura3 ahc1Δ::kanMX sas3Δ::HIS3*
LPY5678	*MAT***a** *ade2-101 his3 leu2 lys2 trpaΔ1 ura3Δ::HIS3 hpa2Δ::KANMX*
LPY5679	*MATα ade2-101 his3 leu2 lys2 trp1Δ1 ura3 sas3Δ::HIS3 hpa2Δ::KANMX*
LPY5686	*MATα ade2-1 his3 leu2 lys2Δ0 trp1Δ1 ura3 gcn5Δ::HIS3 hpa2Δ::KANMX*
LPY5687	*MAT***a** *his3 leu2 lys2Δ0 trp1Δ1 ura3 gcn5Δ::HIS3 hpa2Δ::KANMX*
LPY5688	*MATα ade2-101 his3Δ200 leu2 lys2Δ801 trp1Δ1 ura3-52 sas3Δ::HIS3 gcn5Δ* pLP640 pLP1399
PSY316	*MATα ade-101 his3-200 leu2-3,112 lys2 ura3-52*
PSY316 *ada2Δ*	*MATα ade-101 his3-200 leu2-3,112 lys2 ura3-52 ada2Δ*
PSY316 *gcn5Δ*	*MATα ade-101 his3-200 leu2-3,112 lys2 ura3-52 gcn5Δ*
YJW134	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn5Δ::HIS3 sas3Δ::HIS3MX6* pJW214
YJW135	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn5Δ::HIS3 sas3Δ::HIS3MX6* pJW216
YJW136	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn5Δ::HIS3 sas3Δ::HIS3MX6* pLP0640
YJW137	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn5Δ::HIS3 sas3Δ::HIS3MX6* pLP1364
YJW138	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn5Δ::HIS3 sas3Δ::HIS3MX6* pLP1398
YJW516	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 lys2-128δ ada2Δ::HIS3 sas3Δ::HISMX6*
YJW517	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 lys2-128δ ada3Δ::HISMX6 sas3Δ::HIS3MX6*
YJW518	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 lys2-128δ ada2Δ::HIS3 ada3Δ::HISMX6 sas3Δ::HISMX6*
YJW519	*MAT***a** *his3Δ200 leu2Δ1 ura3-52 lys2-128δ ahc1Δ::HISMX6 sas3Δ::HISMX6*
YJW520	*MATα his4-917 leu2Δ1 ura3-52 lys2-173R2δ spt20Δ sas3Δ::HISMX6*
YJW521	*MAT***a** *his4-917 leu2Δ1 ura3-52 lys2-173R2δ spt20Δ ahc1Δ::HISMX6 sas3Δ::HISMX6*

URA3-based centromeric plasmid expressing *SAS3* from its endogenous promoter (pJW214). The strain was sporulated and dissected to generate haploids carrying deletions of both *GCN5* and *SAS3*. Plasmid shuffle experiments were performed by transforming YJW134 with either *LEU2*-based centromeric plasmids encoding wild-type and HAT domain mutants of Sas3p [pS3FLG, pS3FLGM1, pS3FLGM3 (John et al. 2000)], or *TRP1*-based centromeric plasmids expressing wild-type or HAT domain mutants of Gcn5p [pJW215, (Wang et al. 1998)]. These transformants were then plated on 5-FOA. Plasmid pJW214 (*SAS3*/*URA3*/CEN) consists of the *SAS3*-coding region and 1 kb of upstream sequence, cloned into pRS416 with a FLAG-CYC terminator cassette (John et al. 2000). Plasmid pJW215 (*GCN5*/*TRP1*/CEN) was created by inserting the *GCN5*-coding region and 998 bp of upstream sequences into YCplac33 with a HA-CYC terminator cassette (Eberharter et al. 1999). Plasmid pLP1399 (*HPA2*/*TRP1*/2μ) contains the *HPA2*-coding region, 380 bp of upstream, and 222 bp of downstream sequences inserted into the *TRP1* vector pFL455. Plasmid pLP0640 (*SAS3*/*URA3*/CEN) consists of the *SAS3*-coding region and 1300 bp of upstream and 100 bp of downstream sequences inserted into pRS316. Plasmid pLP1364 (*sas3-638*Δ/*URA3*/CEN) was created by digesting pLP0640 with *Eco*RI and *Hin*dIII, blunt-ending, and religating the vector fragment. Plasmid pLP1398 was created by excising a 750-bp *Bsp*EI/*Cla*I fragment from a pGEX-4T-1 plasmid encoding GST-*sas3*-C323A (Takechi and Nakayama 1999) and ligating to *Bsp*EI and *Cla*I cut pLP0640.

HAT assays, Western immunoblotting, microsequence analysis

Assays to determine the site specificity of NuA3 were performed as described (Grant et al. 1999). Bulk yeast histones were prepared essentially as described previously (Waterborg 2000), with the exception that following elution from the Bio-Rex 70 resin, the histones were dialyzed into deionized water, lyophilized, and resuspended in 1× Laemmli SDS sample buffer. The level of histone acetylation was analyzed by Western blot (Zhang et al. 1998) using antibodies specific for acetylated Lys 9 and Lys 14 in histone H3 (Upstate Biotechnology).

Generation of conditional mutant alleles of SAS3

Ten micrograms of pS3FLG (John et al. 2000) was resuspended in 500 μL of 1 mM hydroxylamine/45 mM NaOH and incubated for 23 h at 37°C. Following the addition of 50 μg BSA, the DNA was ethanol-precipitated twice, transformed into strain YJW134, and plated on synthetic complete medium lacking leucine. After 4 d of growth at 30°C, colonies were replica plated in duplicate onto medium containing 5-FOA and incubated at 20 and 37°C. One thousand colonies were screened and five null mutations and one temperature-sensitive mutation (YJW135) were recovered The plasmid bearing the temperature-sensitive mutation (pJW216) was isolated and the entire ORF sequenced. Temperature-sensitive strains YJW137 and YJW138 were created by transforming YJW134 with pLP1364 and pLP1398, respectively.

Flow cytometry

Wild-type and temperature-sensitive strains were grown to a cell density of 0.5–0.1 × 10^7 cells/mL in YPD for 12 h at 37°C. Three milliliters of culture were harvested by centrifugation at 1000*g* and resuspended in 2 mL of cold, 70% ethanol. After incubation overnight, cells were washed twice with 50 mM sodium citrate (pH 7.4), resuspended in 1 mL of 0.1 mg/mL RNaseA/50 mM sodium citrate (pH 7.4), and incubated for 3 h at 37°C. The cells were stained by the addition of 1 mL of 8 μg/mL propidium iodide and incubated for 2 h at 4°C. Following a brief sonication, the cells were counted on a Coulter XL-MCL single laser flow-cytometer.

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