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Acetylome Profiling Reveals Overlap in the Regulation of Diverse Processes by Sirtuins, Gcn5, and Esa1*⒮

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Although histone acetylation and deacetylation machineries (HATs and HDACs) regulate important aspects of cell function by targeting histone tails, recent work highlights that non-histone protein acetylation is also pervasive in eukaryotes. Here, we use quantitative mass-spectrometry to define acetylations targeted by the sirtuin family, previously implicated in the regulation of non-histone protein acetylation. To identify HATs that promote acetylation of these sites, we also performed this analysis in *gcn5* (SAGA) and *esa1* (NuA4) mutants. We observed strong sequence specificity for the sirtuins and for each of these HATs. Although the Gcn5 and Esa1 consensus sequences are entirely distinct, the sirtuin consensus overlaps almost entirely with that of Gcn5, suggesting a strong coordination between these two regulatory enzymes. Furthermore, by examining global acetylation in an *ada2* mutant, which dissociates Gcn5 from the SAGA complex, we found that a subset of Gcn5 targets did not depend on an intact SAGA complex for targeting. Our work provides a framework for understanding how HAT and HDAC enzymes collaborate to regulate critical cellular processes related to growth and division. *Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.043141, 162–176, 2015.*

Acetylation of histone tails serves as a regulator of eukaryotic transcription by neutralizing the positive charge on such tails and by serving as a conditional-binding interface for chromatin remodeling enzymes containing acetyllysine-binding bromodomains (8, 9). Mutation of histone acetyltransferase (HAT)[1] and deacetylase (HDAC) enzymes is associated with a plethora of disease states in humans including cancers and developmental defects. In particular, the sirtuin family of HDACS has been implicated in the regulation of various aspects of metabolic control. Much of the interest in sirtuins stems from a hypothesis based on early work in yeast, suggesting that they promote an increase in lifespan (10). Although the idea that sirtuins are regulators of longevity *per se* continues to generate controversy, it seems clear that these enzymes play important roles in pathways critical to aging well (*i.e.* healthspan).

The first non-histone acetylation substrates were identified over 15 years ago (11). Although recent efforts have demonstrated that acetylation is a frequent post-translational modification, little is known about the regulation of most of these marks (12–15). Moreover, although recruitment to chromatin is seen as the key step in acetylation of histone tails, little is known about the mechanism behind non-histone substrate selection. The connection of specific acetylation and deacetylation machineries to their target sites will provide a platform to dissect the regulation of targeting mechanisms and to understand the molecular consequences of acetylation within the cell.

In the budding yeast *Saccharomyces cerevisiae*, there are five sirtuin enzymes (16, 17). Of these, Sir2 (the founding member of the sirtuin class and the first to be connected to the regulation of lifespan), Hst1, and Hst2 have been associated with the deacetylation of non-histone substrates. Using a candidate approach, we previously identified the ribosomal protein gene transcription factor Ifh1 as a highly acetylated protein regulated both by Sir2 and Hst1 (1). Sir2 also mediates the deacetylation of the Pck1 enzyme to regulate gluconeogenesis (18). Finally, the acetylation of the Snf2 subunit of the SWI/SNF ATPase is increased slightly in the absence of *HST2* (19). Intriguingly, acetylation of Ifh1 is dramatically increased

[1] The abbreviations used are: HAT, Histone Acetyltransferase; WCE, Whole Cell Extract; HDAC, Histone Deacetylase; SILAC, Stable Isotope Labeling of Amino Acids in Cell Culture; DTT, Dithiothreitol; TFA, Trifluoroacetic acid; TCEP, *tris*(2-carboxyethyl)phosphine; PVDF, Polyvinylidene fluoride.

in strains lacking multiple sirtuins (1), suggesting that there is some overlap in function for these enzymes in the regulation of non-histone targets.

In this work, we set out to gain insights into the function of these three sirtuins. In a SILAC based mass-spectrometry approach, we identified over 52 proteins having acetylation sites regulated by sirtuin enzymes. These proteins functioned in a wide-variety of processes critical for cell growth and division and for the maintenance of homeostasis during stress. Analysis of the acetylome in strains mutated for HATs revealed distinct consensus sequences for Gcn5 and Esa1. Interestingly, the sirtuin consensus matched the Gcn5 consensus, suggesting a functional pairing of these enzymes. Our work suggests that control over key cellular events is regulated by intimate cooperation and cross-talk between multiple HAT and HDAC complexes.

<center>EXPERIMENTAL PROCEDURES</center>

Yeast Media and Cell Growth—Media used in these experiments was previously described (1). Strains listed in supplemental Table S5 were constructed using standard techniques. Where indicated, nicotinamide (Sigma) was used at a concentration of 20 mM in both overnight and diluted cultures. For temperature-shift experiments, cultures were incubated at 37 °C for 2 h.

Whole-cell Extract (WCE) Analyses—Six OD600 equivalents of cells in mid-log phase were lysed using a bead-beating protocol in 20% trichloroacetic acid. Precipitated protein was resuspended in SDS-PAGE sample buffer as previously described (1). 10–20 μl of protein was loaded on a 4–20% gradient gel (Biorad, Hercules, CA) and transferred to PVDF membrane. Membranes were blocked with 5% BSA (Sigma, St. Louis, MO) in 2× TBST (0.1% Tween) for 1 h before overnight incubation with anti-acetyllysine antibody from Cell Signaling Beverly, MA (Product Number 9441) at a dilution of 1:1000 in blocking solution overnight. Secondary antibody (Goat anti-Rabbit from BioRad) was used at a dilution of 1:10,000. Detection was with Western Lightning ECL from Perkin Elmer.

Immunoprecipitations—Immunoprecipitations have been described previously (1). Briefly, cells were lysed using a bead-beating protocol in 50 mM Tris HCl, pH 8, 150 mM NaCl, 5 mM EDTA, and 0.1 Tween. Immunoprecipitation was with Ab290 (AbCam, Cambridge, MA) against GFP for 2 h, with 25 μl protein-A conjugated beads (Life Technologies, Carlsbad, CA) being added for an addition hour. Beads were washed with lysis buffer 3×, followed by elution with 40–60 μl of SDS-PAGE sample buffer with 0.1 M DTT at 65 °C for 10 min. Supernatant was boiled prior to sample separation on a 4–20% gradient gel (BioRad) and Western blotting. GFP was detected using a monoclonal antibody (JL-8, Clontech) at 1:1000 dilution. Acetyllysine detection was with Immunechem (1:1000), Cell Signaling (9441, 1:1000), or Calbiochem, San Diego, CA (1:1000) as indicated for each blot. Secondary detection for acetyllysine blots was with protein-A HRP (Pierce) used at a dilution of 1:10,000. Molecular weight markers are estimated from the Kaleidoscope Precision Plus protein ladder from BioRad.

Cell Growth and Sample Preparation for SILAC Experiments—Cells were grown to mid-log phase in C-Lys media, with a dropout recipe that has been previously described (1), supplemented with heavy labeled lysine, 13C6,15N2, (Cambridge Isotope Laboratories, Tewksbury, MA) or unlabeled control lysine at a concentration of 0.06 mg/ml for greater than eight generations. Growth for eight generations gave a labeling efficiency of over 97%. Cell pellets were washed with water and flash frozen in liquid nitrogen. Cells pellets were combined and

lysed in a denaturing urea buffer (with 8 M urea, 0.1 M Tris-HCl, pH 8, 150 mM NaCl, 1 Roche mini protease inhibitor tablet without EDTA/10 ml, 10 mM sodium butyrate, and 10 mM nicotinamide) using 14 × 1.5 min bursts on a BioSpec mini bead-beater at room temperature. 2 ml screw-cap tubes used for lysis were pierced with an 18-gauge needle and spun in a swinging bucket centrifuge for 30 s at 1000 × *g* to collect extract. Extract was rotated end-over-end for 30 min at room temperature before clarification via centrifugation at 17,000 × *g* for 7 min followed by a second centrifugation at 17,000 × *g* for 2 min, both at room temperature. Extracts were quantitated using a BCA protein quantification kit (Pierce). 1 M TCEP (Sigma C4706–2) was added to final concentration of 4 mM to 10–20 mg of protein (~0.8 ml total volume) and incubated for 30 min at room temperature. 0.5 M iodo-acetamide (Sigma L1149–5G, prepared fresh in water) was added to a final concentration of 10 mM and incubated in the dark for 30 min. To quench excess iodoacetamide, 0.5 M DTT was added to a final concentration of 10 mM for another 30 min in the dark. Samples were diluted ~fourfold (to less than 2 M urea) with 0.1 M Tris, pH 8, and trypsin (Promega, Madison, WI, V511A, dissolved in 50 mM acetic acid) was added at a ratio of 1 mg trypsin to 100 mg total protein. Samples (~4 ml total volume of diluted sample) were incubated overnight at room temperature with rotation. After digestion, 10% TFA was added to a final concentration of 0.3–0.1% TFA, with pH of final solution <3. Insoluble material was precipitated by centrifugation and peptides were loaded onto a Sep Pak tC18 column that had been activated with 1 ml 80% ACN/0.1% TFA and equilibrated with 3 × 1 ml 0.1% TFA. Columns were washed with 5 × 1 ml 0.1% TFA and eluted with 1 ml 40% ACN/0.1% TFA prior to lyophilization over several days to completely remove TFA before storage at −20 °C. Dried peptides were dissolved in 800 μl buffer containing 0.1 M Tris HCl, pH 8, and 50 mM NaCl. pH was verified by spotting on indicator paper. To remove peptides that failed to redissolve, samples were spun at 10,000 × *g* for 2 min, with supernatant being transferred to a new microfuge tube. 25–50 μl of anti-acetyllysine matrix (Immunechem) was added for 2 h at 4 °C, with rotation. Beads were washed rapidly with 2 × 1 ml lysis buffer and 2 × 1 ml water at 4 °C. Beads were transferred to a fresh tube and peptides were eluted with 0.15% v/v TFA (two elutions with 60 μl for 10 min and one elution with 40 μl for 5 min) before storage at −20°C. Peptides were purified on a stage tip spin column wet with 40 μl 80% ACN/0.1% TFA and washed with 0.1% TFA twice. Eluate was passed through the tip in 40 μl increments and washed with 50 μl 0.1 TFA twice. Collection was with 40% ACN/0.1% TFA. Peptides were dried in a vacuum concentrator prior to analysis.

Mass-spectrometry—Samples were analyzed on a Thermo Scientific LTQ Orbitrap Elite MS system equipped with an Easy nLC-1000 HPLC and autosampler system that is capable of maintaining back pressures of up to 10,000 psi for high resolution chromatographic separations. The HPLC interfaces with the MS system via a nano-electrospray source.

Samples were injected onto a C18 reverse phase capillary column (75 μm inner diameter × 25 cm length, packed with 1.9 μm C18 particles). Peptides were then separated by an organic gradient from 5% to 30% ACN in 0.1% formic acid over 112 min at a flow rate of 300 nl/min. The MS continuously collected spectra in a data-dependent fashion over the entire gradient.

Raw mass spectrometry data were analyzed using the MaxQuant software package (version 1.3.0.5)(Cox and Mann, 2008). Data were matched to the UniProt *S. cerevisiae* reference protein database (downloaded on 15/2/13, 6627 protein sequence entries). MaxQuant was configured to generate and search against a reverse sequence database for false discovery rate calculations. Variable modifications were allowed for methionine oxidation, lysine acetylation, and protein N terminus acetylation. A fixed modification was indicated for cys-

teine carbamidomethylation. Full trypsin specificity was required. The first search was performed with a mass accuracy of $\pm$ 20 parts per million and the main search was performed with a mass accuracy of $\pm$ 6 parts per million. A maximum of five modifications were allowed per peptide. A maximum of two missed cleavages were allowed. The maximum charge allowed was 7+. Individual peptide mass tolerances were allowed. For MS/MS matching, a mass tolerance of 0.5 Da was allowed and the top six peaks per 100 Da were analyzed. MS/MS matching was allowed for higher charge states, water, and ammonia loss events. Data were searched against a concatenated database containing all sequences in both forward and reverse directions with reverse hits indicating the false discovery rate of identifications. The data were filtered to obtain a peptide, protein, and site-level false discovery rate of 0.01. The minimum peptide length was seven amino acids. Results were matched between runs with a time window of 2 min for technical duplicates. An average of one in five peptides recovered was acetylated (with a range between 1:3 and 1:10). Annotated spectra available in Supplementary Materials.

Choice of Cut-offs—The minimum standard for regulation in all cases was considered to be twofold up- or down-regulated. For sirtuin and Esa1-regulated peptides, this value allowed us to include known non-histone substrates Ifh1 and Yng2, respectively, while still allowing for a pool of greater than 30 peptides of high-confidence for analysis. We applied a higher cut-off for our Gcn5 data set because, in theory, the null mutation should result in complete absence of acetylation marks on target lysines. The tenfold cutoff applied allowed us to include the known targets Rsc4 and Snf2, while allowing for a number of peptides in line with that obtained for the other mutant analyses.

Bioinformatics—

Acetylated Residue Conservation—A search database of proteins for homolog discovery was constructed from the UniProt reference proteomes(3). Homologs were identified for each acetylated protein in the data set presented in supplemental Table S2 using the BLAST algorithm(4). Orthologs were predicted from the set of homologs using the GOPHER algorithm(5). The ortholog protein sets were aligned using the MUSCLE multiple sequence alignment tool(6). The conservation of each acetylated residue was analyzed by investigating the column of the alignment at the position of the modification. The species range of each modification site was calculated using two metrics. A lysine is shared between yeast and another species if the residue in the column is a lysine in both species. A lysine is conserved between yeast and another species if the residue in the column is a lysine in both species and all species more closely related to yeast.

Acetylated Residue Mapping—The complete yeast proteome consisting of 6621 protein was retrieved from the UniProt resource (March 2014) (3). All peptides were returned from the MS analysis containing internal acetylated lysines were mapped onto the yeast proteome and the position of each acetylated residue was noted to create a data set of unique acetylated sites. 3967 unique acetylated peptides mapped to 3686 unique acetylated sites (allowing each peptide to map to a single site on the proteome) and 4277 redundant acetylated sites (allowing each peptide to map to a multiple site on the proteome). Finally, acetylated residues were annotated with functional and structural information (see detailed information in supplemental Table S3).

Acetylation Site Consensus—An alignment of 11-mer peptide centered on the acetylation site was created for each data set of acetylated peptides. Sequence logos were created from these alignments using the pLogo motif consensus visualization tool(7). The amino acid frequency for each position of the alignment was compared: 1) to the corresponding position of a background data set of all acetylated sites, and 2) to the background amino acid frequency of the yeast proteome. The statistical significance of the over- or under-representation of each amino acid at each position was calculated.

GO-term Analyses—Functional GO-term analyses was carried out using DAVID (http://david.abcc.ncifcrf.gov/) with *S. cerevisiae* as the background and with otherwise default settings. Localization analyses were carried out using the GO-Slim tool at yeastgenome.org. Complex analysis was carried out using the Complexome tool (http://complexome.holstegelab.nl/index.php?framesrc=Dataset/complex.cgi).

RESULTS

The HST1, HST2, and *SIR2*-regulated Acetylome—Analysis of yeast whole-cell extracts (WCEs) with an antibody generated against acetylated lysine revealed an increase in acetylated species in strains deleted for yeast sirtuins *SIR2, HST1,* and *HST2* (Fig. 1*A*, left, hereafter referred to as "sirtuins"). These data are reminiscent of previous observations that multiple sirtuin enzymes cooperate to deacetylate the ribosomal protein transcription factor Ifh1 (1, 20). In contrast, we observed little effect in our WCE analyses of deleting the genes encoding sirtuins Hst3 and Hst4, which regulate the deacetylation of histone H3 at lysine 56 (21, 22). To identify other proteins whose acetylations are regulated by sirtuin enzymes, we compared the acetylation landscape of *sir2Δ hst1Δ hst2Δ* triple mutant cells to isogenic counterparts using stable isotopic analysis of amino acids in cell culture (SILAC, Fig. 1*A*, right). Differential labeling of sirtuin mutant and control cell populations with heavy or light labeled lysine was followed by denaturing lysis and cleavage of extracts with trypsin. Relative levels of heavy and light labeled acetylated peptides enriched with anti-acetyllysine antibodies were quantified using mass spectrometry (see "Materials and Methods"). As expected, the heavy/light (sirtuin Δ/wild-type) ratio for most acetylated peptides detected was equal to ~one (Fig. 1*B*). A small fraction of acetylated peptides were enriched in the sirtuin mutant strain by a factor of twofold or greater (Fig 1*B*). Biological replicates (three replicates, including one label-swap experiment, wherein the heavy and light labeled strains were switched), were used to refine our data set. We classified up-regulated peptides as high-confidence hits and candidate sirtuin substrates if they were up-regulated in the sirtuin mutant strain in all three replicates by a factor of twofold or greater (See Materials and Methods and supplemental Table S1 for details and scoring). These 71 peptides contained a minimal number of 67 unique acetylation sites from 52 proteins and showed significant consistency in their level of enrichment in our sirtuin mutant strains between experiments (Fig. 1*C* and data not shown). Contained within this data set were peptides from known sirtuin substrates, including Ifh1 and histone H3 (Fig. 1*C*), suggesting that our approach can identify sirtuin target proteins. No peptides from sirtuin target Pck1 (18) were observed in our data sets. Although they did not meet the stringent twofold cut-off employed here, peptides containing Hst2-regulated Snf2 acetylation sites (19) were up-regulated an average of 1.7-fold in

A

B

C

Fig. 1. **A quantitative approach to identify sirtuin regulated acetylations in yeast.** *A*, Experimental design. Analysis of WCEs prepared from the indicated strains with an antibody against acetyl-lysine indicated that strains mutated for *hst1Δ*, *hst2Δ*, and *sir2Δ* have an up-regulation of acetylations on multiple non-histone substrates. To examine these and other acetylations, a sirtuin triple mutant strain was grown in heavy labeled amino acids in parallel with a wild-type strain grown in light-labeled amino acids. Cell pellets were combined prior to lysis and cleavage with trypsin. Acetylated peptides were purified with an antibody against acetylated lysine and analyzed by mass spectrometry. Acetylated peptides were visualized as paired peaks and a sirtuin Δ::wild-type ratio was calculated for each species. *B*, Sample data from a single SILAC experiment. Normalized heavy::light (sirtuin Δ::wild-type) ratios were plotted for each peptide (where this ratio could be calculated) from a single experiment. Included here are data from two technical replicate MS runs. Acetylated species were considered enriched if the sirtuin::wild-type ratio was greater than 2. *C*, Consistency in high-confidence sirtuin-regulated species. The sirtuin::wild-type ratios from forward and reverse labeled control experiments. Each data point represents a unique acetylated peptide from a single protein. Paralogs were counted as one protein. Peptides with two sites were considered to have only one unique "regulated" site (only one of the sites is guaranteed to be differentially regulated), unless other high-confidence peptides suggested otherwise.
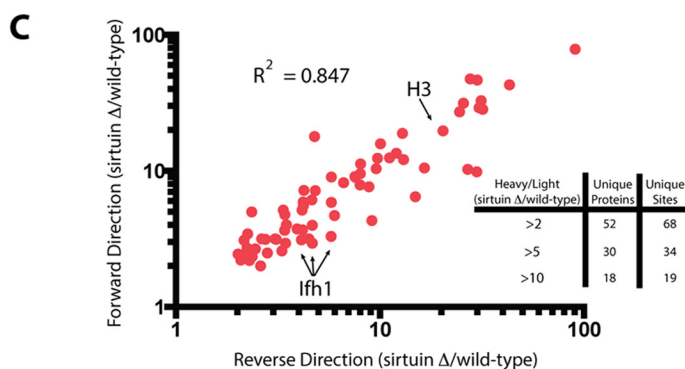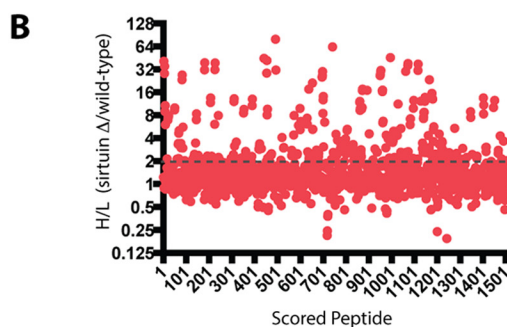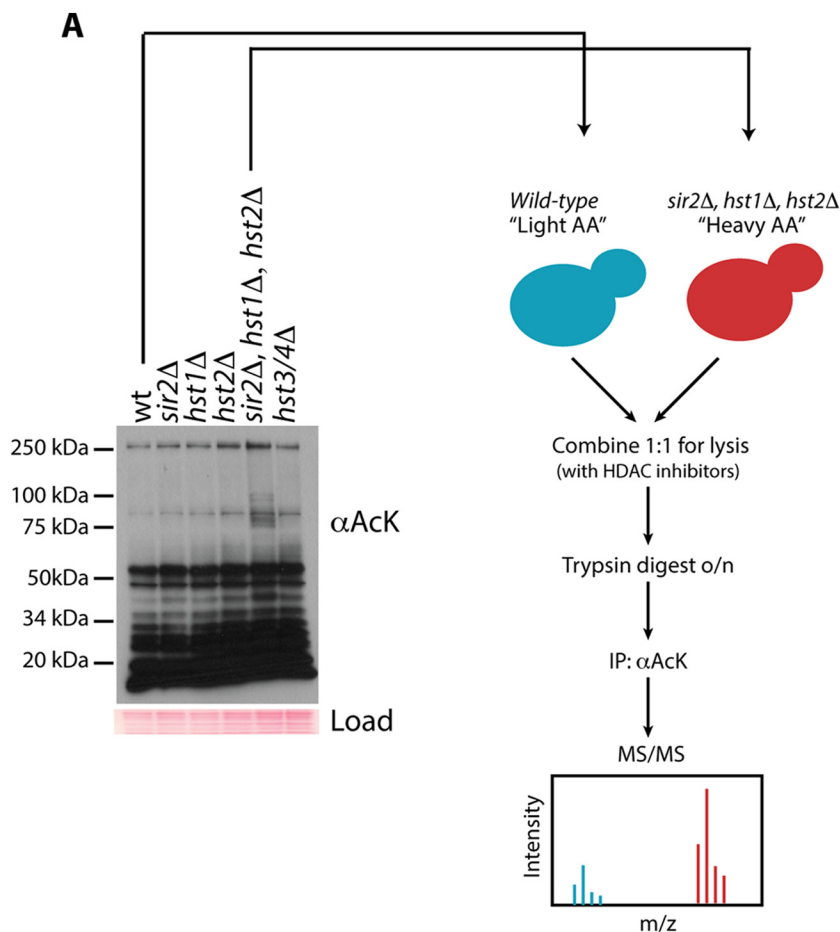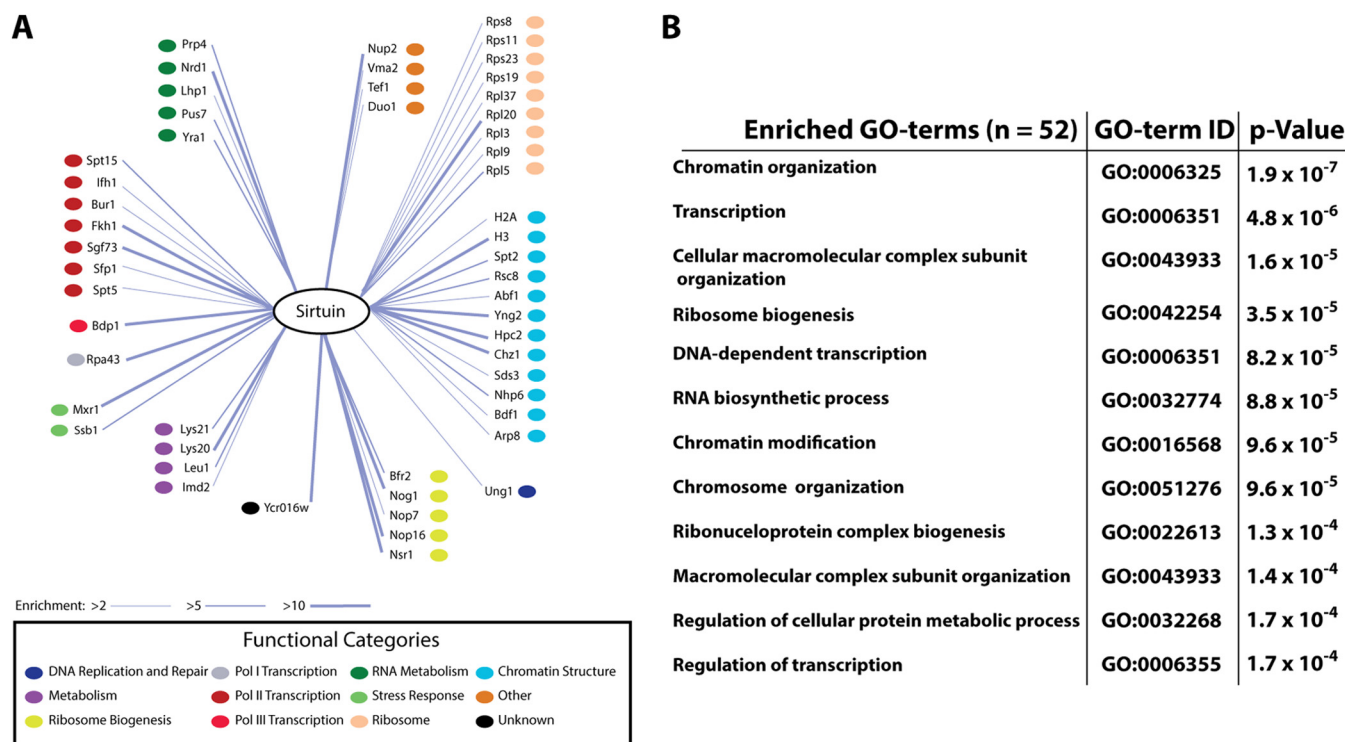
Fig. 2. **Functional analysis of sirtuin-regulated acetylation sites.** *A*, Proteins with one or more high-confidence sirtuin-regulated sites were manually grouped into the functional categories shown. *B*, Enrichment of gene ontology (GO) terms was carried out using DAVID for the high-confidence hits in the sirtuin triple-mutant data set, with *S. cerevisiae* as the background and with default settings. The top 12 enriched biological process-associated GO terms are shown, with *p* values as indicated.

our sirtuin mutant strain (supplemental Table S2). Eighty-eight additional acetylated peptides were up-regulated in one of two forward experiments and the reverse-labeled experiment and were defined as medium-confidence hits (supplemental Table S1). Medium-confidence acetylated peptides, like their high-confidence counterparts, showed similar sirtuin mutant/wild-type enrichments in experiments where they were detected, but simply were not observed (in either the heavy or light labeled states) in the third replicate experiment (supplemental Table S1).

Proteins with one or more sites regulated by sirtuin enzymes are diagrammed in Fig. 2*A*. These proteins function in diverse biological processes and include proteins known to function in the same complex or pathway. For example, acetylated peptides from both the Bur1 kinase and its target Spt5 (23, 24), a regulator of transcription elongation, were classified as high-confidence hits in our analyses (supplemental Table S1 and Fig. 2*A*). We also identified a number of ribosomal components from both the small and large subunit, as well as assembly factors for both subunits as having sirtuin-regulated sites (Fig. 2*A*), and enrichment in these functional groups was statistically significant (Fig. 2*B*). High and medium confidence sirtuin-regulated proteins also included multiple members of the NuA4, DASH, V-ATPase, Rpd3L, RSC, SWR1, SAGA, TFIID, and the U4/U6 × U5 tri-snRNP complexes (25).

Overlap of HAT and HDAC Function—To determine how sirtuins may cooperate with different HATS, we used SILAC to examine the acetylome of cells mutated for *GCN5*, *ESA1*, or *HAT1* in our sirtuin triple-mutant background (Fig. 3*A*, right). Performing our analyses in the sirtuin mutant background allowed us to detect sirtuin-regulated acetylations of interest more easily, while still allowing for the evaluation of non-sirtuin-regulated sites. For analysis of the essential HAT Esa1, we employed a previously characterized temperature sensitive allele, *esa1*-L254P (26). Cells carrying this mutant showed down-regulation of H4 acetylation after 2 h of incubation at 37°C (supplemental Fig. S1) and also failed to form colonies after overnight growth at this nonpermissive temperature (data not shown).

For all three HAT mutants, we focused on acetylated peptides that were under-represented in our SILAC experiments relative to the control strain (Fig. 3*B*–3*D*). Acetylated peptides down-regulated in the three HAT mutants showed consistency in the level of that down-regulation across multiple experiments (Fig. 3*E*). We classified 39 Gcn5-regulated sites from 34 proteins as high-confidence hits (down-regulated tenfold or more in *gcn5Δ* strains in three independent experiments, see Materials and Methods for details). Proteins with regulated acetylated sites included previously characterized substrates Snf2 (19), and H3 (27, 28). Lysine 25 of Rsc4, the first non-histone Gcn5 substrate in yeast (29), was found in
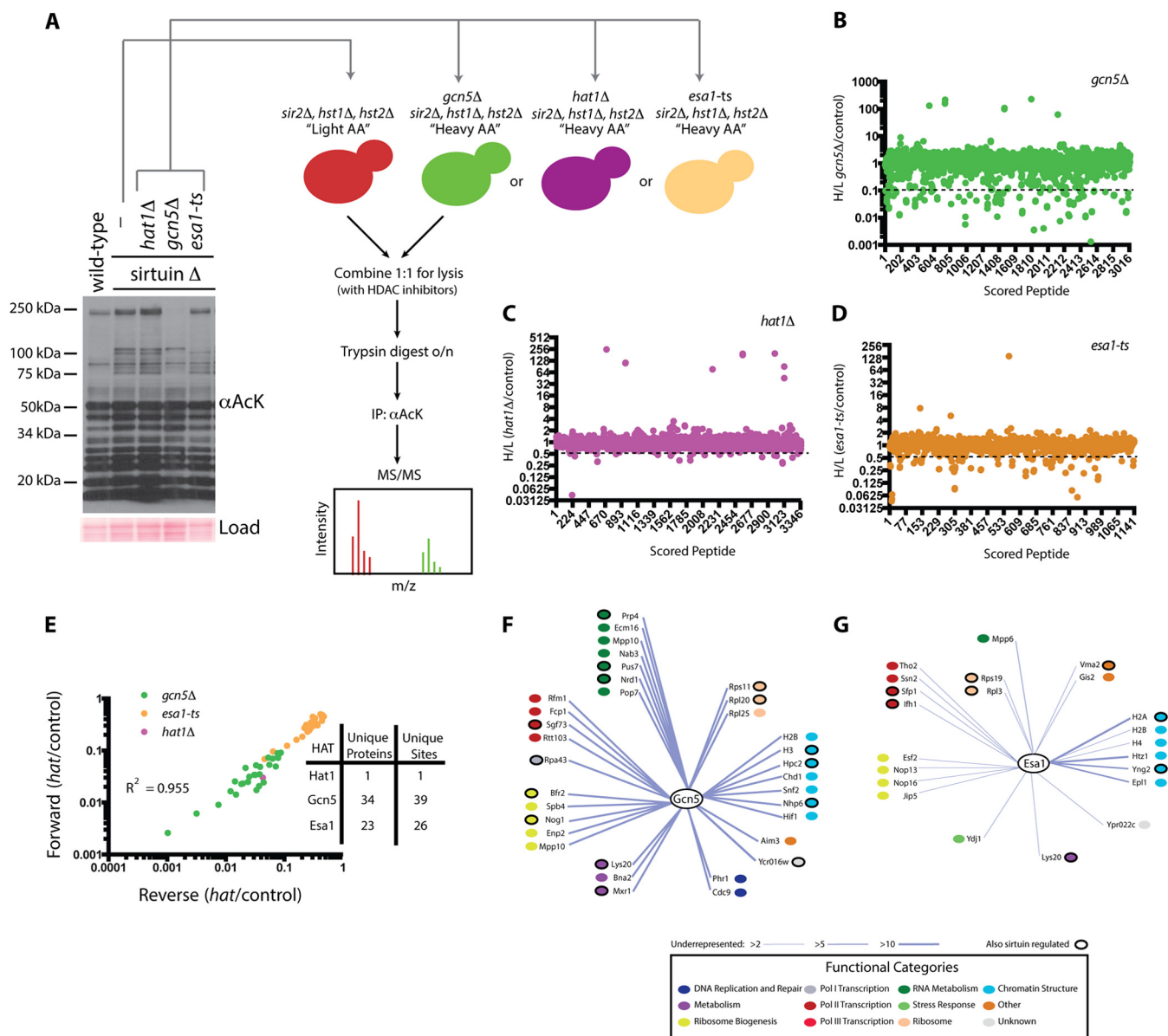
FIG. 3. **Acetylome profiling of HAT mutants.** *A*, Experimental Design. WCE analysis of the indicated strains indicated that Gcn5 and Esa1 contribute to acetylation of non-histone substrates in a sirtuin mutant background. Strains mutated for these enzymes or for *HAT1* were processed as described in Fig. 1, with the sirtuin mutant strain being used as the control. *B–D*, Examples of single SILAC experiments for HAT mutant strains. HAT mutant::control ratios for each acetylated species measured in repeated technical MS runs from a single experiment. For *esa1-ts* and *hat1Δ* experiments, peptides were considered under-represented at ratios of <0.5. For *gcn5Δ* experiments, a threshold of < 0.1 was used. *E*, Average of high-confidence down-regulated peptides identified in both forward and reverse experiments for the indicated mutants. Site and protein numbers listed here are minimum numbers. Homologous proteins were counted as one protein. Peptides with two sites were considered to have only one unique "regulated" site (only one of the sites is guaranteed to be differentially regulated), unless other high-confidence peptides suggested otherwise. *F–G*, Proteins with high-confidence Gcn5 and Esa1-regulated sites were manually grouped into functional categories as shown. For the Esa1 data set, Htb1 and Htb2 isoforms (having slightly different sequences) were both recovered but are both included under H2b in this figure.

two of three experiments and was classified as a medium confidence hit (supplemental Table S1). Intriguingly, we also identified acetylation sites at lysines 87, 410, and 423 of Rsc4 and on other components of the RSC chromatin remodeling complex, although these sites were not regulated by Gcn5 (supplemental Tables S2 and S3). Rsc4 acetylation at K25 and

Snf2 acetylation at K1494/K1498 promote intramolecular binding with C-terminal bromodomains, preventing the interaction of this domain with acetylated histone residues (19, 29). The yeast genome encodes an additional eight proteins with bromodomains, and our analysis also identified acetylation sites on seven of these factors. Of these seven (Gcn5, Spt7,

Sth1, Rsc1, Rsc2, Bdf1, and Bdf2), only acetylations on SAGA components Spt7 and Gcn5 appear to be dependent on Gcn5 (supplemental Tables S1 and S2). Similarly, only Bdf1's acetylations were regulated by sirtuin enzymes (supplemental Table S1). Multiple HAT and HDAC activities may converge on bromodomain containing proteins to differentially regulate their intra- and intermolecular interactions. Our high-confidence Gcn5 data set was also included proteins involved in ribosome biogenesis, DNA metabolism, and transcription (Figs. 3*F* and supplemental Fig. S1*B*). Twenty-three potential Esa1 targets (down-regulated twofold or more in all three experiments, see Materials and Methods) included previously characterized substrates Yng2 (30) and H4 (31), as well as proteins involved in ribosome biogenesis, chromatin remodeling, and transcription (Fig. 3*G* and supplemental Fig. S1*B*). In contrast to the Esa1 and Gcn5 data sets, only one acetylation site on a single protein, ribosome assembly factor Nop53, was reproducibly under-represented in *hat1Δ* cells in both forward and reverse SILAC experiments (Fig. 3*E* and supplemental Table S2). Nop53 had other acetylation sites that were *HAT1*-independent (supplemental Table S2), which may mask the contribution from the single *HAT1*-regulated site and account for our inability to detect a change in Nop53 acetylation in *hat1Δ* mutant cells via Western blot (supplemental Fig. S2*A*). Indeed, a benefit of our approach is that we were able to evaluate the contribution of HATS and HDACS to individual acetylation sites on proteins that may be coregulated (directly or indirectly) by multiple enzymes.

Overlap of Gcn5, Esa1, and Sirtuin Function—To determine if proteins with sites regulated by sirtuins, Gcn5 and Esa1 showed localization to specific cellular compartments, we used GO-slim analysis (yeastgenome.org) to assign localizations to high-confidence proteins identified in our SILAC experiments (Fig. 4*A*). All three data sets were enriched for proteins showing localization to the nucleolus and chromosome subcompartments, as predicted from the functional assignment of these proteins to processes related to ribosome biogenesis and chromosome structure and function (Fig. 2 and Fig. 3). We found significant overlap between the sirtuin data set and the Gcn5 and Esa1 data sets at the protein and acetylation site level (Fig. 4*B*). This observation was especially striking for Gcn5, 40% of whose putative targets were also found to be sirtuin regulated. In contrast, no high-confidence protein had the same acetylation site(s) regulated by both Gcn5 and Esa1, suggesting that these enzymes may prefer to target lysine residues within distinct amino acid contexts (Fig. 4*B*). The very high-degree of overlap in sirtuin and Gcn5-regulated sites suggested that these enzymes may share similar consensus sequences. Our Gcn5 data set showed striking enrichment for small amino acids serine and alanine at the −2 position, positively charged amino acids (R/K) at the +1 position, and proline at the +2 position (Fig. 4*C*). As noted by Rojas *et al.*, the crystal structure of Tetrahymena Gcn5 bound to a histone H3 peptide shows that there exists a hydrophobic pocket in Gcn5 that can accommodate proline at the +2 position (32). In agreement with the observed overlap with potential Gcn5 substrates and acetylation sites, the sirtuin data set was also enriched for serine and alanine at the −2 position, although glycine, preferred in the Esa1 consensus sequence, was also tolerated (Fig. 4*C*). The sirtuin and Gcn5-dependent consensus sequences are strikingly similar (Fig. 4*C*) at other positions as well, highlighting the functional relationship between these two opposing enzymes. In particular, the selection for serine at the −2 position in these sequences suggests the possibility for cross-regulation with phosphorylation. Putative Esa1 targets showed less specificity at the −2 position and, instead, were enriched at the −1 position for glycine and alanine (Fig. 4*C*). We note that this consensus sequence very closely matches the sequences surrounding known acetylation sites on the glycine-rich H4 tail. Although we cannot exclude the possibility that our consensus sequences are affected by the acetyllysine antibody used in our experiments, distinct Gcn5 and Esa1 consensus sequences were also observed when data was normalized against amino acid frequencies surrounding acetylation sites identified in this work and when both high and medium confidence sites were used for this analysis (supplemental Fig. S1*C*). This later observation strongly suggests that our medium confidence sites are also strong candidates for direct regulation by Gcn5 or by Esa1.

In further support of Gcn5 mediating its effects directly, we observed only very minor changes in protein levels of high-confidence regulated hits in a strain lacking *GCN5* (supplemental Fig. S3*F*), suggesting that indirect effects on transcription are not generally responsible for the decrease in acetylated species observed in this strain. Similarly, 20 of 23 proteins having *esa1*-sensitive acetylations also have other acetylation sites that were not regulated in our *esa1-ts* experiments, suggesting a level of regulation extending beyond pathways affecting overall protein abundance.

Specificity in Sirtuin Function—Our data set suggests that the SAGA complex, containing the Gcn5 HAT, is highly acetylated (supplemental Table S2). Sgf73 acetylation, which was found to be dependent on Gcn5 as previously described (33), was also up-regulated following nicotinamide treatment (Fig. 5*A* and supplemental Fig. S2*B*). Although other members of the SAGA complex are also highly reactive with our acetyllysine antibodies in immunoblot experiments, only Sgf73 was sensitive to sirtuin inhibition by nicotinamide, as predicted by our SILAC experiments (Fig. 5*B*). Acetylation was also increased in strains lacking *HST1*, *HST2*, and *SIR2* individually, suggesting that each of these three sirtuin HDACs contributes to regulating one or more Sgf73 acetylation sites (Fig. 5*C*). This finding is reminiscent of Ifh1 acetylation, which is also reversed by multiple sirtuins (1). Sgf73 acetylation was visible only at background levels in strains mutated for genes encoding SAGA HAT submodule components Ada3 and Sgf29, but was unaffected by the absence of SAGA interactor Chd1 (Fig.
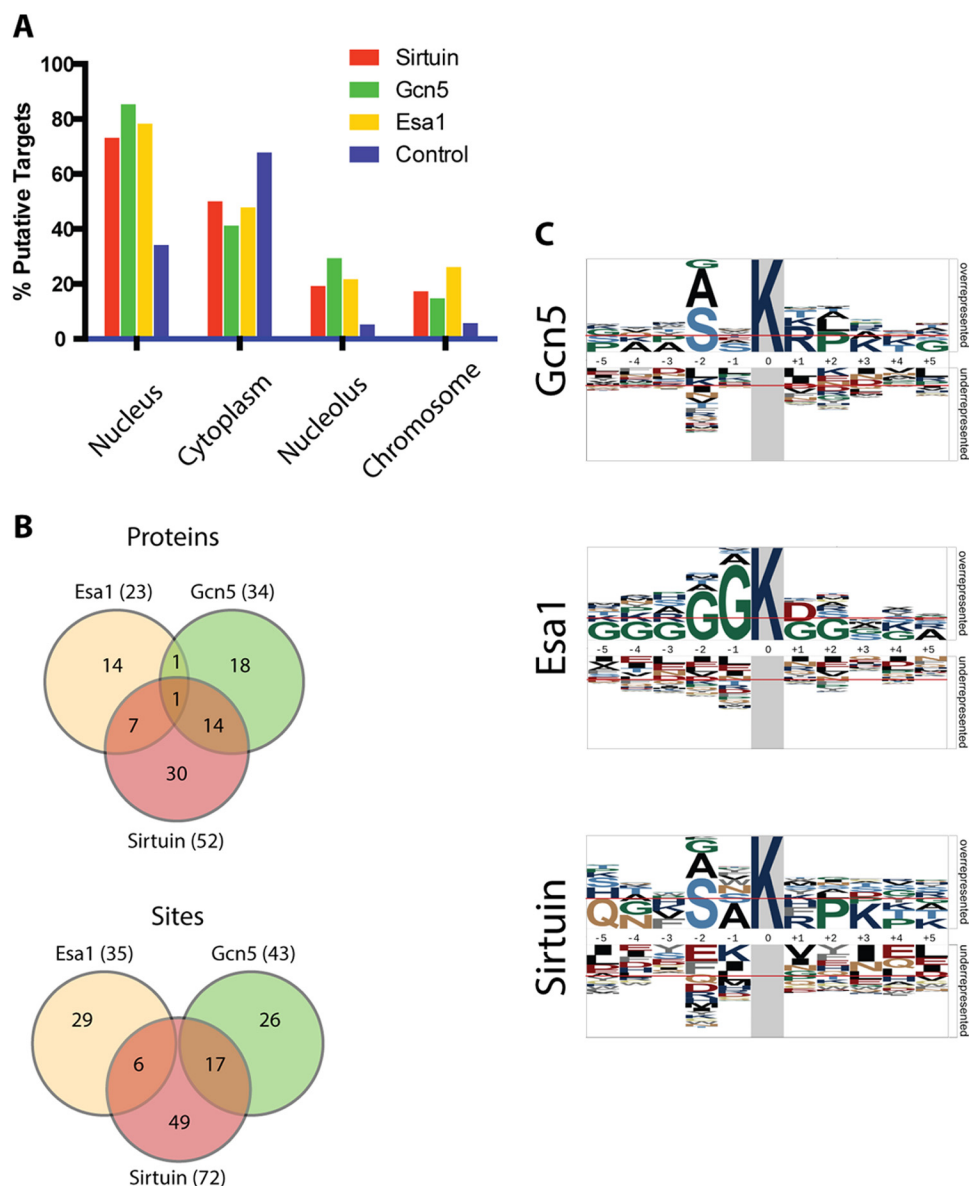
FIG. 4. **Analysis of sites targeted by sirtuin, Gcn5 and Esa1 enzymes.** *A*, Localization of proteins with regulated acetylations. Proteins with one or more high confidence sirtuin, Gcn5 or Esa1-regulated sites were analyzed for localization to the indicated cellular compartments using Yeast GO-Slim analysis (yeastgenome.org) with the default settings. The percentage of each protein set that localizes to an indicate compartment is plotted. As a control, the percentage of total yeast proteins that localize to each compartment is shown. *B*, Overlap of protein substrates and acetylation sites identified in sirtuin Δ, *gcn5*Δ, and *esa1-ts* data sets. *C*, Frequencies of amino acid residues surrounding high-confidence regulated acetylated lysine residues for each HAT or HDAC enzyme. The height of each amino acid represents its over or under representation at the given positions. The red line indicates significant enrichment. The residue coloring reflects the shared physico-chemical properties of certain sets of amino acids.

5E). Sgf73 serves to bind the histone deubiquitylation (DUB) module to SAGA (34) (Fig. 5*D*). The Ubp8 component of this submodule reverses the Bre1-dependent monoubiquitylation of histone H2B, a modification that has roles in the regulation of transcription (35, 36). Interestingly, deletion of genes encoding DUB submodule components *UBP8* or *SGF11* increased Sgf73 acetylation (Fig. 5*E*). In contrast, acetylation was not affected by the deletion of *BRE1* (Fig. 5*E*). Therefore, the SAGA DUB module may directly or indirectly regulate

the activity of Gcn5 or sirtuins toward Sgf73. Indeed, Sir2 was previously suggested to bind to SAGA (37), and it is tempting to speculate that the DUB module may regulate this interaction.

Immunoblotting also confirmed the sirtuin sensitivity of Esa1 interactor and NuA4 component Yng2 (38), and Bur1 acetylation marks (Fig. 6*A*–6*C*, supplemental Fig. S3*D*). As predicted by our SILAC experiments and by its association with Esa1 as a member of the NuA4 complex, Yng2 acetyla-
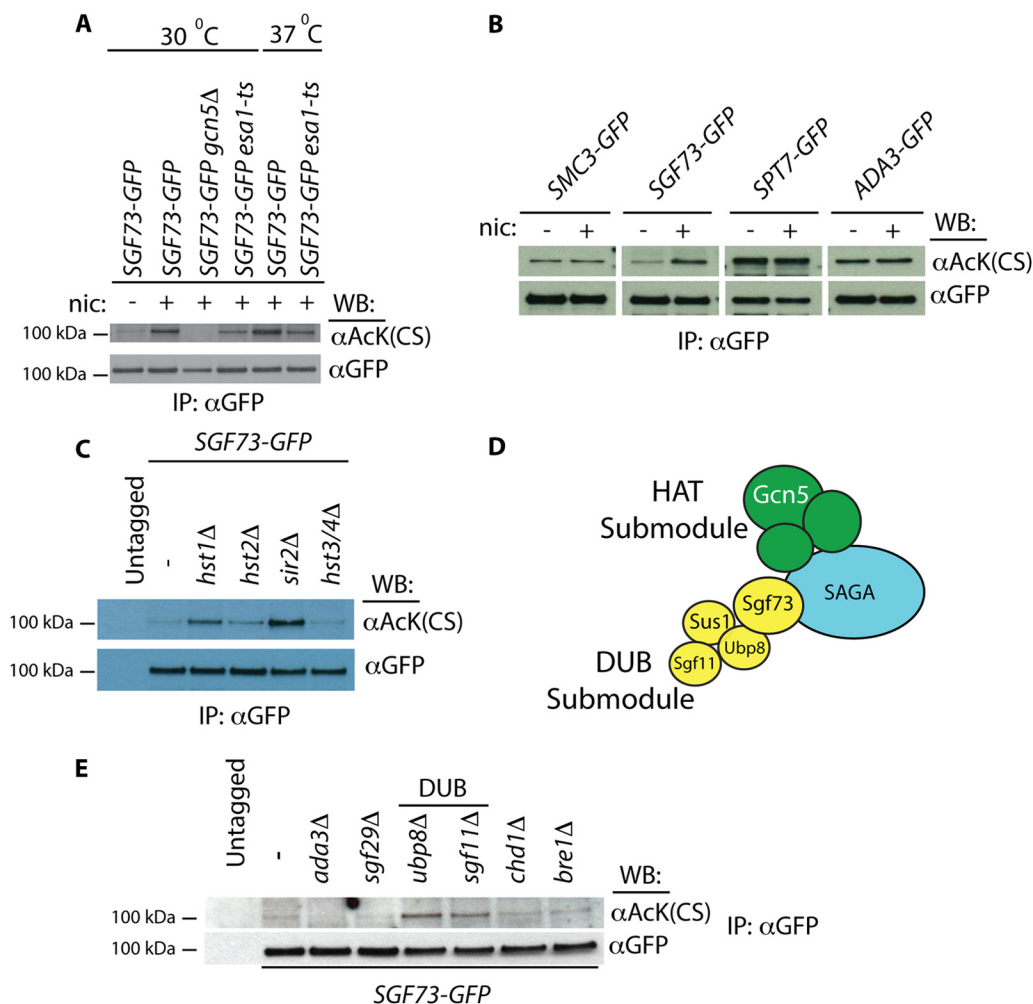
FIG. 5. **Regulation of Sgf73 acetylation.** *A*, Sgf73 acetylation depends on Gcn5 and is regulated by sirtuins. GFP-tagged proteins were immunoprecipitated from the indicated strains in the presence or absence of 20 mM nicotinamide. Where indicated, strains were incubated at 37 °C for 2 h. Acetylation status of the indicated proteins was measured using an antibody against acetylated lysine (from Cell Signaling (CS) as indicated). *B*, Sirtuins are selective in their action toward acetylated SAGA subunits. SAGA subunits were immunoprecipitated from the indicated strains and analyzed as in *A*. The Smc3 protein, a known acetylated protein unaffected by nicotinamide treatment, served as a control. *C*, Multiple sirtuins regulate Sgf73 acetylation. Immunoprecipitated Sgf73 from individual sirtuin mutant strains was analyzed as in *A*. *D*, Schematic representation of SAGA. Sgf73 binds the DUB module to SAGA. *E*, Sgf73 acetylation is increased in the absence of the SAGA DUB module. Sgf73 was immunoprecipitated from the indicated strains and acetylation was analyzed as described in *A*.

tions were regulated by Esa1 HAT activity (Fig. 6*A*, left). Yng2 also copurified with an additional nicotinamide sensitive species. This was not an artifact of nicotinamide treatment, as the same species copurified with Yng2 recovered from a sirtuin triple-mutant strain (Fig. 6*B*). Therefore, sirtuins may regulate multiple members of the NuA4 complex. In line with this observation, Peng *et al.* previously described the SIRT1-regulated acetylation of the Esa1-like TIP60 and hMOF catalytic subunits in mammalian cells(39).

Bur1 is the homolog of human CDK9, whose active site acetylations are regulated by human sirtuin *SIRT2* (40). Bur1 regulates transcription elongation by phosphorylating the Spt5 elongation factor (23). As predicted by our data, mutation of neither *GCN5* nor *ESA1* were able to fully eliminate the acetylation signal observed on this protein (Fig. 6*C*). Ctk1,

another CDK involved in transcriptional elongation via CTD phosphorylation (41) was not sensitive to nicotinamide treatment but, like Bur1, its acetylation did increase in the sirtuin triple-mutant strain (Fig. 6*D* and supplemental Fig. S3*E*). Finally, Kin28, a third CTD kinase that was not found to be acetylated in our screens, showed little to no reactively with multiple anti-acetyllysine antibodies in immunoblot experiments (Fig. 6*D* and supplemental Fig. S2*C*). These data suggest a complex role for both sirtuins and for acetylation in general in the regulation of CDK-like enzymes.

Sfp1, a factor that promotes transcription from ribosomal protein (RP) promoters, was found to be regulated by sirtuins and by Esa1 in our screens and these dependences were recapitulated in our IP-Western experiments (Fig. 6*E*). Like Ifh1, a target of the Gcn5 HAT, Sfp1 recruitment and/or ac-
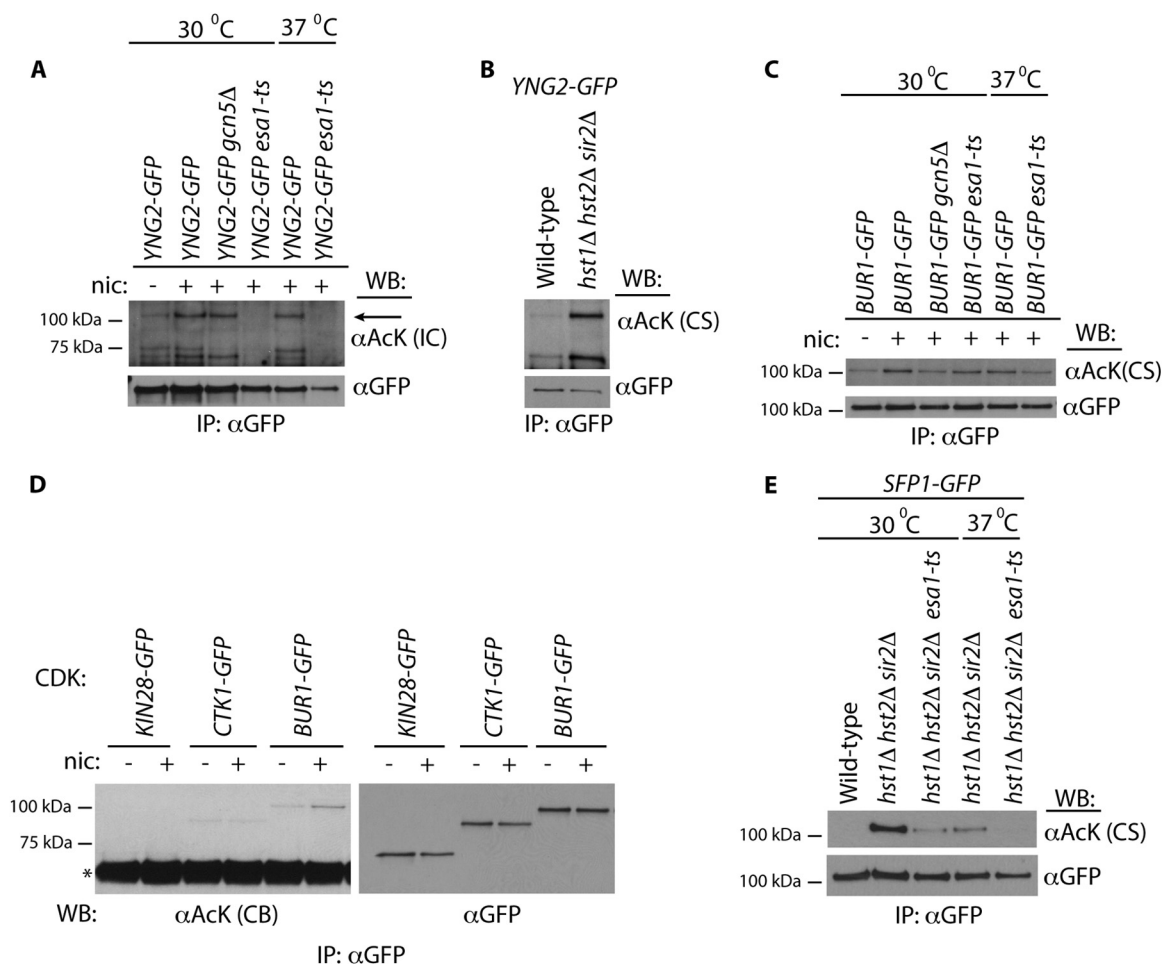
FIG. 6. **Exploring acetylation of non-histone substrates.** *A–E*, GFP-fusion proteins were purified from the indicated strains and acetylation was measured following SDS-PAGE and Western blotting, with an antibody against acetylated lysine. Anti-acetyllysine antibodies were from ImmuneChem (IC) or from Cell Signaling (CS) as indicated. Where indicated, nicotinamide was used at 20 mM. Treatment at 37 °C for *esa1-ts* analyses was for 2 h. Asterisk indicates nonspecific bands. Arrow indicates acetylated species copurifying with Yng2-GFP.

tivity at RP promoters is thought to be inhibited during stress, such as nutrient starvation or treatment with the TORC1 inhibitor rapamycin (42, 43). In contrast, both Gcn5 and Esa1 localize to RP promoters during periods of rapid growth (33, 44), suggesting that the activities of multiple HATS converge to regulate transcription under such circumstances by targeting unique substrates.

Role of Ada2 in Regulating Gcn5 Function—Previous work has shown that Ada2 serves as a cofactor for Gcn5-dependent acetylation of histone residues and that it is required for copurification of Gcn5 with SAGA and the related SALSA/SLIK complexes isolated via chromatography (27, 45–47). In agreement with these data, we found that Gcn5 does not co-immunoprecipitate with SAGA component Sgf73 in strains lacking *ADA2*, although the level of Gcn5 protein in total whole-cell lysates remained relatively unchanged (Fig. 7*A*). Moreover, in the absence of Ada2, immunoprecipitation of core SAGA component Spt7 failed to recover Gcn5 or Sgf73, suggesting a general destabilization of the complex (Fig. 7*B*).

Intriguingly, however, Ada2 is not required for Gcn5-dependent acetylation of Rsc4 (48), suggesting that Gcn5 may target some substrates independently of its known interaction partners. In support of this idea, strains lacking *ADA2* grew better than those lacking *GCN5*, relative to control strains, especially on synthetic media (supplemental Fig. S3). Thus, we used acetylome profiling to determine whether Ada2 is required for Gcn5-dependent regulation of non-histone substrates on a global scale (Fig. 7*C*). We first carried out SILAC experiments comparing the acetylome of *ada2Δ gcn5Δ* double mutants to that of *ada2Δ* single mutant control strains, focusing on high and medium class Gcn5-regulated sites as defined in supplemental Table S1 (Fig. 7*D*). If Ada2 is required for a given substrate's acetylation(s), acetylated peptides containing the target site will be recovered from each strain at the same low level, and the ratio of measured intensities be equal to ~one (*i.e. ada2Δ* and *gcn5Δ* mutants are epistatic). In contrast, if Ada2 (and an intact SAGA complex) is entirely unnecessary for the regulation of a protein's acetylation by
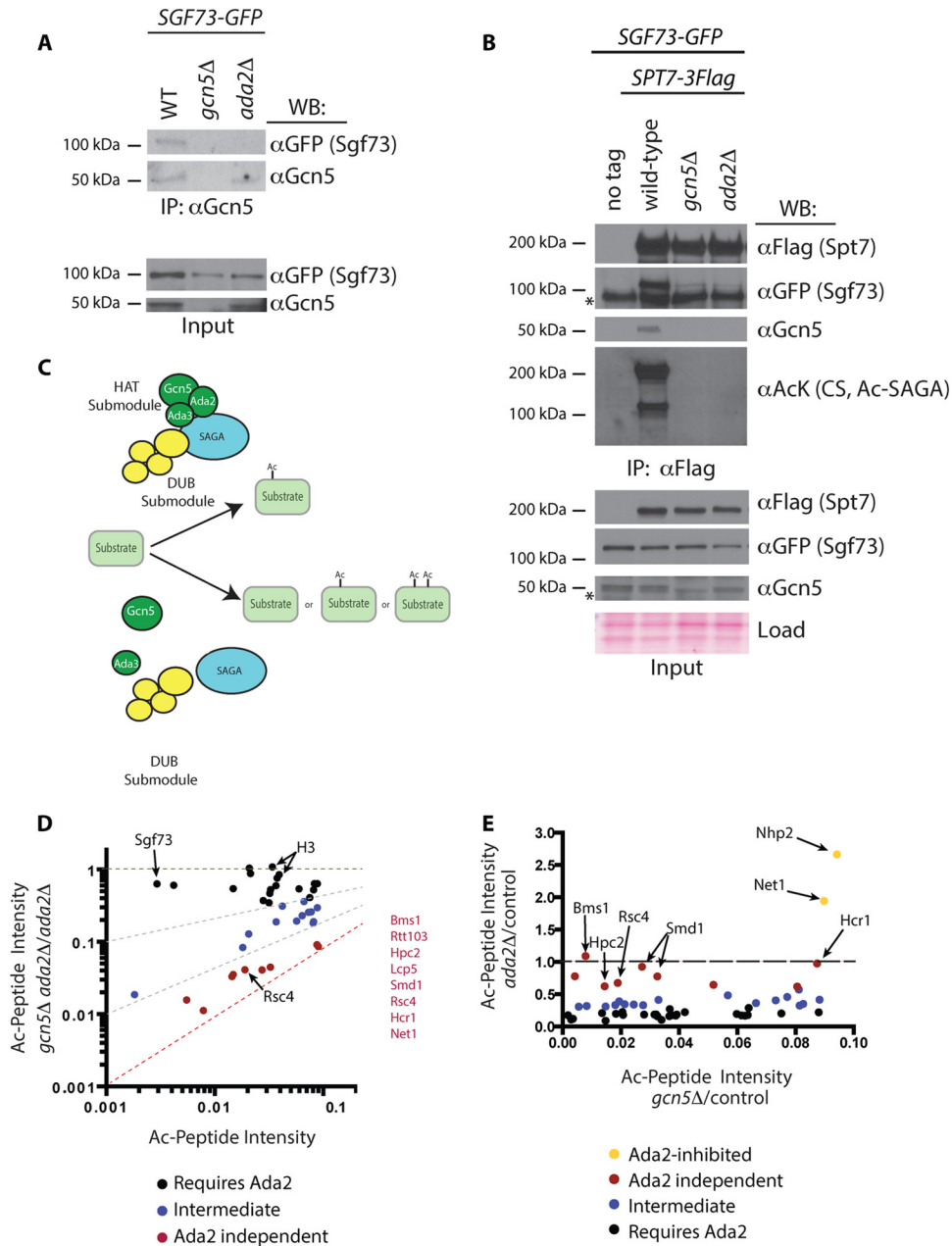
FIG. 7. **Ada2 selectively regulates Gcn5-dependent acetylations.** *A*, Deletion of *ADA2* prevents Gcn5 association with SAGA. Gcn5 was immunoprecipitated from the indicated strains and the presence of SAGA was tested via immunoblotting with an antibody against GFP, which was expressed as a fusion with SAGA component Sgf73. *B*, Flag-tagged SAGA component Spt7 was immunoprecipitated with an antibody against the Flag epitope and levels of associated SAGA components were measured with the indicated antibodies. *C*, Rationale - three possible outcomes for acetylation status of Gcn5 substrates in the absence of the Ada2 protein. *D*, Ada2 has differential effects on Gcn5-dependent acetylations. Average ratio of acetylated peptides in *gcn5Δ ada2Δ versus ada2Δ* cells is plotted on the Y axis for previously classified high and medium confidence Gcn5 substrates. *gcn5Δ versus* control data (supplemental Table S1) is plotted on the X axis. The dotted red line represents the point at which each substrate's acetylations are completely unaffected by loss of *ADA2*, and disruption of the SAGA complex. The dotted black line represents the point at which each substrate's acetylations are completely dependent on *ADA2*. *E*, Average ratio of acetylated peptides in *ada2Δ versus* wild-type is plotted on the *y* axis. The ratio of acetylated peptides in *gcn5Δ versus* control is plotted on the *x* axis. Ada2 (and hence SAGA complex) independent acetylations should approach the black line. For both D) and E) all experiments were carried out in the *hst1Δ hst2Δ sir2Δ* triple mutant background. Gcn5 candidate substrates examined were medium and high confidence regulated peptides from supplemental Table S1 and are, by definition, under-represented by at least tenfold in *gcn5Δ* mutant cells. Raw data used is listed in supplemental Table S4.

Gcn5, the measured ratio will be identical to that observed in the *gcn5Δ versus* (wild-type) control experiment. By definition, all Gcn5-regulated sites examined were down-regulated ~10–1000-fold in strains lacking *GCN5* (supplemental Table S1). We found that Ada2's contribution to Gcn5 activity varied significantly depending on the protein and site in question (Fig. 7D). Ada2 was required for Gcn5-dependent acetylation of histones and of SAGA component Sgf73. In contrast, Rsc4, as predicted (48), was one of ~eight proteins whose acetylation showed only a minor requirement for functional Ada2 (Fig. 7D).

We also directly examined the effect of an *ada2Δ* mutation alone on the acetylation status of high and medium confidence Gcn5-dependent sites, relative to a control strain (Fig. 7E). On average, the deletion of *ADA2* resulted in a more mild effect on these same sites relative to the deletion of *GCN5*, and there was no apparent correlation between the strength of defects observed in the absence of each individual gene (Fig. 7E). Here again, we observed that the contribution of Ada2 to each acetylation mark varied greatly (Fig. 7E). Also, acetylation marks on Net1 and Nhp2 were up-regulated in *ada2Δ* mutants, suggesting that Ada2 may actually slightly inhibit Gcn5-dependent acetylation of these targets. Importantly, the great majority of substrates found to be Ada2-independent in each individual analysis (Figs. 7D and 7E) overlapped significantly.

DISCUSSION

We provide here a resource that associates acetylation and deacetylation machineries with specific acetylation marks. Our data set underscores the scope of acetylation as a regulatory mechanism, provides valuable insight into the variety of pathways regulated by unique enzymes, and suggests that acetylation and deacetylation enzymes cooperate to regulate multiple pathways important for cellular growth and division. The large number of potential substrates uncovered for sirtuins, Esa1 and Gcn5 allowed for the first generation of *in vivo*-derived consensus sequences for these enzymes. Intriguingly, the consensus sequence for Gcn5 was very similar to that of sirtuin enzymes. This observation is in line with the high-degree of overlap observed between Gcn5 and sirtuin-regulated acetylation sites. Our ability to define consensus sequences for these enzymes underscores the quality of our data sets and suggests that many of the regulated sites are direct targets, although we cannot completely rule out the effect of cross-enzyme regulation on the observation of site preference. SAGA subunit Sgf73, for example, is regulated by multiple sirtuin enzymes. Our Gcn5 consensus of S/A-X-K-K/R-P differs from the consensus suggested by Rojas *et al.*, G-K-X-P, derived from a costructure of Gcn5 with an H3 peptide. We suggest that our consensus sequences, which are based on a large number of potential substrates, will be better suited to facilitate the study of HAT and HDAC function on a global scale.

We define 52 high-confidence proteins containing acetylation marks that were up-regulated in a strain mutated for *HST1*, *HST2*, and *SIR2.* We find that all three of these sirtuins may play a role in the deacetylation of Sgf73. Together with our previous finding that Ifh1 acetylation is regulated by both Hst1 and Sir2, these data suggest that multiple sirtuins may cooperate to regulate substrates' acetylations. Sirtuins may target the same sites redundantly within the same molecule. Alternatively, unique populations of acetylated substrates may be targeted by different sirtuins at different times or in unique cellular compartments. Sir2, for example, is recruited to the nucleolus as a component of the RENT complex (49, 50), which functions in rDNA silencing and in regulating the localization of the Cdc14 phosphatase (49, 51). Our sirtuin data sets were enriched for proteins involved in ribosome biogenesis, which occurs within the nucleolus (43). It is tempting to speculate that it is Sir2 that is directly responsible for deacetylating these proteins and that their deacetylations are required for the maintenance of nucleolar function. Hst1, being more evenly distributed throughout the nucleus (52), may function to restrict acetylation of these and other proteins during their transit through the nucleoplasm or following their localization to chromatin. Hst2 is the only yeast sirtuin that localizes exclusively to the cytoplasm (52–54), and may play a significant role in regulating the landscape of protein acetylation therein. Hst2 may also regulate nuclear-cytoplasmic shuttling of proteins that function in multiple cellular compartments.

Acetylome profiling revealed that the HATs Esa1 and Gcn5 might cooperate with sirtuin enzymes to regulate important biological pathways (such as ribosome biogenesis). We identified 23 proteins containing high-confidence acetylation sites regulated by Esa1 and 34 such candidates regulated by Gcn5. Using a proteome-array, Lin *et al.* (18) identified 91 proteins as potential targets of Esa1 *in vitro*. Although we identified acetylation sites on 27 of these proteins in experiments employing the *esa1-ts* mutation, none of these sites were defined as Esa1-regulated using our stringent criteria (supplemental Tables S1 and S2). A separate study by Mitchell *et al.* (13), identified 38 proteins acetylated by the Esa1-containing complex NuA4 *in vitro*. Our analysis identified four of these proteins (Esa1, Eaf3 Epl1, and Yng2 – all members of the NuA4 complex) as having high or medium confidence Esa1-regulated sites. Esa1 may regulate other potential substrates identified by these studies only under specific conditions. In contrast to experiments with *gcn5* and *esa1* mutants, strains carrying a deletion of *HAT1* showed down-regulation of only a single acetylation site. Hat1, therefore, may have a more limited pool of non-histone substrates relative to Gcn5 and Esa1. Alternatively, Hat1 may function redundantly with other HATS, or, as suggested above for Esa1, may target substrates only under specific circumstances.

In our sirtuin Δ, *gcn5Δ*, *hat1Δ*, and *esa1*-ts data sets, we mapped a combined total of 3686 unique acetylation sites on 1494 proteins. An analysis of these sites suggests that high-

confidence sirtuin, Esa1, or Gcn5-regulated acetylation sites are more likely than total acetylation sites to be located in disordered regions (76% *versus* 47%; supplemental Table S3). Regulated acetylation sites were also more likely to be conserved in each species between yeast and human than total acetylated sites or lysines in general (22% *versus* 14 and 8%, respectively), and to be located within close proximity to other previously characterized post-translational modifications (25% for regulated sites, 10% for all acetylation sites, and 5% for lysines). In order to facilitate the use of our data as a resource, supplemental Table S3 also includes other metrics for each acetylation site mapped in our analyses, including proximity to known regulatory motifs and to amino acid substitutions giving rise to previously documented phenotypes.

An earlier study examining global acetylation in yeast identified a similar number of sites (4208) (12). Surprisingly, only 21% of the total acetylation sites identified in these two studies were found in both data sets (supplemental Fig. S4). The variations in acetylated sites detected may result from the use of different anti-acetyllysine antibodies and purification strategies. Regardless, these numbers illustrate the importance of varied applications of acetylome profiling and suggest that continued application of these techniques is likely to uncover still other novel acetylation sites on new target proteins.

Most of the 3686 acetylation sites that we identified in our study were not significantly up-regulated or down-regulated in any of the deletion mutants analyzed (supplemental Table S2). *S. cerevisiae* contains at least nine other proteins suggested to have lysine acetyltransferase activity and seven additional proteins with deacetylase activity (www.yeastgenome.org), which may contribute to the regulation of such sites. Of these enzymes, the type I HDAC Rpd3 has received significant interest. Henriksen *et al.* reported sites on 44 proteins to be up-regulated in strains lacking *RPD3* (12). Five of these proteins were also included in our high-confidence sirtuin data set, with only three proteins (Duo1, H3, and Sgf73) having one or more sites regulated by both Rpd3 and sirtuins. These data suggest different classes of HDAC usually have unique target proteins. The role of Rpd3 in regulating non-histone substrates has also been approached genetically. Using a Synthetic Dosage Lethality (SDL) strategy (55), Kaluarachchi-Duffy *et al.* identified 244 proteins whose overexpression exacerbated the growth defect imparted by an *rpd3Δ* strain (14). A similar strategy may be useful in prioritizing candidate sirtuin substrates identified in our work for follow-up analyses. Finally, a number of recent studies suggest that nonenzymatic acetylation of substrates can also occur, particularly within the mitochondria (56, 57). Although some such acetylations may occur to significant levels and even be regulated via deacetylation, others may contribute to a low level of nonfunctional "background" acetylation. Such findings underscore the importance of associating acetylation marks with specific enzymes.

HATs and HDACs often exist in multi-subunit complexes, and significant efforts have been focused on deciphering the roles of auxiliary subunits in regulating histone acetylation during transcription. The role of these proteins in targeting non-histone substrates, however, has been largely ignored. Using acetylome profiling, we were able to examine the contribution of Ada2, which is required for Gcn5 incorporation into SAGA, to protein acetylation on a proteome-wide scale. Surprisingly, our results suggest that Ada2 is not required for all Gcn5-dependent acetylation events.

How is Gcn5 locating its substrates in the absence of an Ada2-dependent connection to SAGA? "Free" Gcn5 may target these substrates in the nucleoplasm. Gcn5 could also interact with acetylated histones directly via its bromodomain to target HAT activity to chromatin bound substrates. Intriguingly, human *GCN5* is overexpressed in lung cancer, with its expression level correlating with tumor size (58). Although it is unclear what percentage of this protein is incorporated into complexes, it is conceivable that some effects of h*GCN5* overexpression may stem from aberrant acetylation of select non-histone substrates that hGCN5 can target independently of its normal interaction partners.

In addition to its role in recruiting Gcn5 to HAT complexes, Ada2 promotes Gcn5 acetylation of histone substrates *in vitro*, either by facilitating substrate binding or by promoting catalytic activity (45, 46). Ada2 is likely to play a similar role in the selection of non-histone substrates, although analysis of alleles that disrupt the functional Gcn5-Ada2 interaction, while still allowing for robust incorporation into the SAGA complex, are necessary to test this hypothesis. Intriguingly, the absence of Ada2 caused an increase in acetylation of several Gcn5 substrates identified in our acetylome profiles (Fig. 7*E*). Hence, Ada2, and possibly other SAGA components, may also restrict acetylation of some substrates. Finally, hGCN5 can bind either to hADA2a or hADA2b, which specify incorporation into SAGA-like complexes ATAC and STAGA, respectively (59). It is tempting to speculate that these related adaptors dictate unique sets of non-histone substrates for hGCN5 following specific stimuli or in different tissues. Importantly, other HATs also exist in large multisubunit complexes including Sas3 (a component of NuA3), and Esa1 (a component of NuA4 that was also profiled here). Therefore, it will also be informative to decipher the roles of the auxiliary subunits of these complexes in substrate targeting.

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S This article contains supplemental Figs. S1 to S4 and Tables S1 to S6.

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