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# Subunits of ADA-two-A-containing (ATAC) or Spt-Ada-Gcn5-acetyltrasferase (SAGA) Coactivator Complexes Enhance the Acetyltransferase Activity of GCN5*

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**Background:** Histone acetyltransferases (HATs) incorporated in large multiprotein complexes are involved in a wide variety of cellular processes, including transcription regulation.
**Results:** Subunits of HAT complexes enhance the enzymatic activity of their catalytic subunits.
**Conclusion:** The activity, but not the specificity of HAT complexes, is stimulated by the corresponding subunits.
**Significance:** We gained important insights into histone acetylation function and specificity of HAT complexes.

**Histone acetyl transferases (HATs) play a crucial role in eukaryotes by regulating chromatin architecture and locus specific transcription. GCN5 (KAT2A) is a member of the GNAT (Gcn5-related *N*-acetyltransferase) family of HATs. In metazoans this enzyme is found in two functionally distinct coactivator complexes, SAGA (Spt Ada Gcn5 acetyltransferase) and ATAC (Ada Two A-containing). These two multiprotein complexes comprise complex-specific and shared subunits, which are organized in functional modules. The HAT module of ATAC is composed of GCN5, ADA2a, ADA3, and SGF29, whereas in the SAGA HAT module ADA2b is present instead of ADA2a. To better understand how the activity of human (h) hGCN5 is regulated in the two related, but different, HAT complexes we carried out *in vitro* HAT assays. We compared the activity of hGCN5 alone with its activity when it was part of purified recombinant hATAC or hSAGA HAT modules or endogenous hATAC or hSAGA complexes using histone tail peptides and full-length histones as substrates. We demonstrated that the subunit environment of the HAT complexes into which GCN5 incorporates determines the enhancement of GCN5 activity. On histone peptides we show that all the tested GCN5-containing complexes acetylate mainly histone H3K14. Our results suggest a stronger influence of ADA2b as compared with ADA2a on the activity of GCN5. However, the lysine acetylation specificity of GCN5 on histone tails or full-length histones was not changed when incorporated in the HAT modules of ATAC or SAGA complexes. Our results thus demonstrate that the catalytic activity of GCN5 is stimulated by subunits of the ADA2a- or ADA2b-containing HAT modules and is further increased by incorporation of the distinct HAT modules in the ATAC or SAGA holo-complexes.**

Post-translational modifications of histones regulate transcription activation or repression. Nucleosomes composed of a $(H3\text{-}H4)_2$ tetramer flanked by two H2A-H2B dimers wrapped by 147 base pairs of DNA play a key role in chromatin compaction (1). To loosen chromatin structure and activate gene expression, transcription activators first bind to their cognate DNA binding sites and subsequently recruit multisubunit coactivator complexes. Around enhancers and promoter elements, coactivator complexes can then either reorganize the nucleosomes by using ATP-dependent chromatin remodeling factors or modify histones covalently. Chromatin-modifying coactivator complexes dynamically deposit and remove post-translational modifications on histones, thus creating or erasing docking surfaces for proteins that recognize histone modifications (2).

One of the first histone modifications discovered was acetylation of lysine residues. This modification is thought to activate gene expression by loosening the chromatin structure but also by creating docking surfaces for other proteins and protein complexes (2–5). GCN5 (also called KAT2A) was the first histone acetyltransferase (HAT)[5] or lysine (K) acetyltransferase (KAT) - enzyme to be identified and was classified to be part of the GNAT (Gcn5-related *N*-acetyltransferase) family of

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[5] The abbreviations used are: HAT, histone acetyltransferase; KAT, lysine acetyltransferase; GCN5, general control nonderepressible 5; y, yeast; h, human; SAGA, Spt Ada Gcn5 acetyltransferase; ATAC, Ada two A-containing; PCAF, p300/CREB-binding protein-associated factor; IP, immunoprecipitation; WB, Western blot.

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enzymes. Its activity as an acetyltransferase on histone substrates was first described in *Tetrahymena* (6). Later it was shown that this enzyme is homologous to the yeast Gcn5 protein, already known to be required for transcriptional activation (7–10). Gcn5 is evolutionary highly conserved from yeast to human (5, 11). Initial *in vitro* studies using microsequencing of labeled yeast histone H3 or H4 peptides and recombinant Gcn5 indicated that the enzyme displays a non-random specificity, mainly acetylating histone H3 at Lys-14 and to some extent H4 at Lys-8 position (12). A recent quantitative high throughput mass spectrometry-based assay, when using free recombinant histone H3 alone as substrate, demonstrated recombinant Gcn5-mediated acetylation at six lysines with the following efficiency: Lys-14 > Lys-9 ≈ Lys-23 > Lys-18 > Lys-27 ≈ Lys-36) (13).

Yeast (y) Gcn5 is part of two coactivator complexes, SAGA (Spt-Ada-Gcn5 acetyltransferase) and ADA (14). Mammalian GCN5 was also described to be part of two large HAT complex, called SAGA (15–17) or ATAC (Ref. 18 and references therein). The mammalian SAGA complexes all contain 18–19 evolutionary well conserved subunits (18). Interestingly, it was demonstrated that the subunits of SAGA complexes are organized in functionally distinct modules, including the HAT, the deubiquitination, and the structural and the activator interaction modules (18, 19). Originally, it was shown in yeast that yGcn5 interacts with yAda2 and yAda3 to form an Ada/Gcn5 module, which is important for acetylation of histones H3 and H4 (9, 20, 21). Also in human cells, hGCN5 interacts with hADA2b and hADA3 (22). More recently, Lee *et al.* (19) discovered that ySgf29 is also part of this HAT module. Thus, yGcn5 and hGCN5 are subunits of the HAT modules of the respective SAGA complexes, together with yAda2/hADA2b, yAda3/hADA3, and ySgf29/hSGF29.

A study combining *in vitro* acid-urea gel and quantitative mass spectrometry approach to measure the activity of yeast Gcn5 incorporated in the partial HAT module of the ySAGA and Ada complexes containing Gcn5-Ada2-Ada3 showed that Gcn5 acetylated free histone H3 with the following efficiency: H3K14 > H3K23 > H3K9 ≈ H3K18 > H3K27 > H3K36 (23). Purified yeast and human SAGA complexes were shown *in vitro* to acetylate H3 mainly at position Lys-14, but also Lys-9, Lys-18, and Lys-23 to some extent when using different pre-acetylated H3 tail peptides (24, 25).

Metazoan GCN5 was identified also as a subunit of a second coactivator HAT complex in *Drosophila* (26) and mammals, named ATAC (Ada two A-containing) (26, 27). Interestingly, the yAda2 protein has two paralogues in metazoans: ADA2a and ADA2b (28, 29). Although ADA2b is part of the HAT module of the SAGA complex, ADA2a was shown to be a subunit of the HAT module of the ATAC complex together with GCN5, ADA3, and SGF29 (18, 28, 29).

In addition to the diversity of GCN5-containing HAT complexes in metazoans, a second level of complexity exists in vertebrate organisms. In vertebrates, another HAT protein called PCAF (p300/CREB-binding protein (CBP)-associated factor, or KAT2B) is present that is 70% identical to GCN5 (30). Similarly to GCN5, PCAF can acetylate histones H3 and H4 (31). In mammalian cells no free GCN5 or PCAF is found. Both proteins are always integrated into either the ATAC or SAGA complexes. The presence of GCN5 or PCAF in SAGA or ATAC is mutually exclusive (27, 32).

Although eukaryotic SAGA complexes preferentially acetylate histone H3K9 and H3K14 lysine residues *in vivo* (24, 25, 33, 34), specificities of the metazoan ATAC complexes are less well understood (27, 35, 36). After knock down of ATAC subunits a decrease of acetylated H4K5 and acetylated H4K12, or H4K16 was observed in *Drosophila* (37–39). In contrast, analyses of changes in histone tail acetylation patterns upon knock-out or knockdown of specific ATAC subunits in mammalian cells suggested that ATAC preferentially acetylates histone H3 rather than H4 (27, 35, 36). A recent mass spectrometry-based strategy to precisely and accurately quantify histone mark modifications in *Drosophila* KC cells after RNAi ablation of GCN5 (present in ATAC and SAGA) demonstrated H3 acetylation marks decrease in the following order: H3K9+K14> H3K9 > H3K14 > H3K27 = H3K36 (34). Note that in this study no changes in H4 acetylation were reported after GCN5 knockdown, suggesting potentially redundant action of several other HATs on H4. Interestingly, this compensatory activity *in vivo* did not occur on H3. Furthermore, knock-out of yeast Gcn5 or knockdown of the ATAC and SAGA HAT module activity in mammalian cells resulted in a significant genome-wide reduction of H3K9ac mark at all active gene promoters (33). In agreement, deletion of GCN5 and PCAF in mammalian cells specifically and dramatically reduced acetylation on histone H3K9 (40).

In this study we set out to determine how the subunits of ATAC or SAGA HAT complexes regulate the activity of the GCN5 enzyme. To this end we compared the activities of the recombinant GCN5 enzyme in isolation, the GCN5-containing HAT modules of ATAC and SAGA, and the corresponding endogenous holo-complexes. *In vitro* acetylation assays performed on histone tail peptides, H3/H4 tetramers, and histone octamers as substrates show that GCN5 alone is less active when compared with its activity in the HAT modules or to the endogenous ATAC or SAGA complexes. Thus, the protein partners of GCN5 enhance its activity.

## Experimental Procedures

*DNA Constructs and Recombinant Protein Production*—The $His_6$-FLAG-hGCN5-expressing construct was described in Demény *et al.* (41). Recombinant ATAC and SAGA HAT modules were produced using the MultiBac system (42). Coding sequences for human GCN5, human ADA2a/b, mouse ADA3, and human SGF29 were inserted by sequence and ligation-independent cloning into the vectors pFL, pIDC, pIDK, and pIDS, respectively (43). The 5′ region of the GCN5 gene was engineered to code for a decahistidine tag followed by a tobacco etch virus protease cleavage site. Vectors were fused by Cre recombination and integrated into the EMBacY baculovirus genome via Tn7 transposition (44). Recombinant baculoviruses were generated as described (44) and used for protein complex production in *Sf*21 insect cell culture. Infected insect cells were harvested 72 h post cell arrest by centrifugation and stored at −80 °C until further use.

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*Immunoprecipitation and Western Blot Analysis*—Preparation of HeLa cell nuclear extract was described in Demény *et al.* (41). *Sf*9 cell lysates were made as described in Leurent *et al.* (45). Proteins from insect *Sf*9 cell lysates (from four 75-cm$^2$ Falcon flasks) or from 4 mg of HeLa cell nuclear extracts were immunoprecipitated with 100 $\mu$l of protein G-Sepharose (GE Healthcare) and ~20 $\mu$g of the different antibodies (as indicated in Fig. 1). His$_6$-FLAG-hGCN5 was purified using anti-FLAG M2-agarose beads (Sigma). Antibody-protein G-Sepharose- or M2-agarose-bound protein complexes were washed 3 times with immunoprecipitation (IP) buffer (25 mM Tris-HCl, pH 7.9, 10% (v/v) glycerol, 0.1% Nonidet P-40, 0.5 mM DTT, 5 mM $MgCl_2$) containing 0.5 M KCl and twice with IP buffer containing 100 mM KCl. After washing, proteins were eluted by a 1000× excess of the corresponding epitope peptide (for the anti-GCN5 5GC2A6 monoclonal antibody (mAb), MAEPSQAPTPAPAAQPRPLC; for anti-ADA3 pAb 2678, LEGKTGHGPGPGRPKSKN; for anti-ADA2a 2AD2A1 mAb, MDRLGSFSNDPSDKPPC; for anti-TAF10 23TA1H8 mAb, MSCSGSGADPEAAPASAASAC) (see also Fig. 1). Proteins were then visualized by staining the gels with Coomassie Brilliant Blue or silver stain or transferred to nitrocellulose membranes and probed with the indicated primary antibodies. Chemiluminescence detection was performed according to manufacturer's instructions (Amersham Biosciences). All antibodies raised against human ATAC or SAGA subunits used in this study were described previously (27). The anti-GCN5 mouse mAb 2GC was raised against the CKCNGWKNPKPPTAP epitope (amino acids 118–132 of hGCN5), and the anti-GCN5 mAb 4GC was raised against the IWESGFTMPPSEGTQLVPC epitope (amino acids 374–391 of hGCN5). Antibodies used for detection of histone modifications on Western blot were: H3K14 (46), H3K9Ac (#06-942; Millipore), and H4K5Ac (ab51997; Abcam).

*Peptide Acetylation Assay*—Histone tail peptides (1 $\mu$g; corresponding to the N-terminal tail of histone H3 or H4, as indicated in the figures) was added to the purified protein samples together with 1 $\mu$l of $H^3$-labeled acetyl-coenzyme A (0.05 $\mu$Ci per reaction) or $^{14}$C-labeled acetyl-coenzyme A (0.04 $\mu$Ci per reaction) in buffer R (50 mM Tris-HCl, pH 8, 20 mM KCl, 5 mM DTT, 4 mM EDTA) and incubated at 30 °C for 1 h. Samples were deposited on Whatman P81 nitrocellulose filters, washed 3 times for 10 min in ice-cold 50 mM $NaHCO_3$, pH 9, buffer, and dried. Filters were then dropped into 5 ml of ReadySafe liquid scintillation mixture (Beckman Coulter), and radioactivity was quantified by an LS6000SC Beckman counter.

*Histone Acetylation Assay*—Histones were purified from HeLa cells as described (47). Recombinant histone octamer purification was described in Tropberger *et al.* (48). Per reaction, 300 ng of histones were incubated with $^{14}$C-labeled acetyl-coA (0.04 $\mu$Ci per reaction) and 20–100 ng of the indicated GCN5-containing complexes in HAT buffer (50 mM Tris-HCl, pH 7.9, 10% glycerol, 0,1 mM EDTA, 50 mM KCl, 20 mM sodium butyrate, 1 mM DTT, and protease inhibitors). The reactions were incubated for 1 h at 30 °C, stopped by adding Laemmli buffer with 100 mM DTT, and boiled for 10 min. Proteins were then loaded on a 13% SDS-PAGE and analyzed by Coomassie Brilliant Blue staining. The gel was then incubated for 20 min in Amplify reagent (GE Healthcare; NAMP100) and dried. A blank phosphorimaging screen (Fuji) was placed on the gel, and the radioactive signal was analyzed and quantified with a Typhoon 8600 scanner.

*Mass Spectrometric Identification of Histone Acetylation*—For large scale histone acetylation assays, 1 $\mu$g of recombinant human histone octamers (reconstituted with H3.2 variant) were incubated with 100 ng of GCN5-containing complexes and 200 $\mu$M cold acetyl-coA. Reactions were incubated for 1 h at 37 °C in HAT buffer and frozen at −80°. Protein mixtures were then separated in two equal parts and TCA-precipitated overnight at 4 °C. Samples were centrifuged at 14,000 rpm for 30 min at 4 °C, and pellets were washed twice with 500 $\mu$l of cold acetone and urea-denatured with 8 M urea in 0.1 mM Tris-HCl, reduced with 5 mM tris(2-carboxyethyl)phosphine for 30 min, and then alkylated with 10 mM iodoacetamide for 30 min in the dark. Both reduction and alkylation were performed at room temperature and under agitation (850 rpm). One-half of the samples was double-digested with endoproteinase Lys-C (Wako) at a ratio 1/100 (enzyme/proteins) in 8 M urea for 6 h followed by an overnight-modified trypsin digestion (Promega) at a ratio of 1/100 (enzyme/proteins) in 2 M urea at 37 °C. The second half was digested with ArgC (Promega) at a ratio of 1/100 (enzyme/protein) in 4 M urea for 12 h at 37 °C. Peptide mixtures were then desalted on C18 spin-column and dried on a speed vacuum before LC-MS/MS analysis.

*LC-MS/MS Analysis*—Samples were analyzed using an Ultimate 3000 nano-RSLC (Thermo Scientific, San Jose, California) coupled in line with an linear trap Quadrupole (LTQ)-Orbitrap ELITE mass spectrometer via a nano-electrospray ionization source (Thermo Scientific). Peptide mixtures were loaded on a C18 Acclaim PepMap100 trap column (75 $\mu$m inner diameter × 2 cm, 3 $\mu$m, 100 Å; Thermo Fisher Scientific) for 3.5 min at 5 $\mu$l/min with 2% acetonitrile (ACN), 0.1% formic acid in $H_2O$ and then separated on a C18 Accucore nano-column (75 $\mu$m inner diameter × 50 cm, 2.6 $\mu$m, 150 Å; Thermo Fisher Scientific) with a 120-min linear gradient from 5% to 50% buffer B (A: 0.1% FA in $H_2O$; B: 80% ACN, 0.08% FA in $H_2O$) followed with 10 min at 99% B. The total duration was set to 150 min at a flow rate of 200 nl/min. The temperature was kept constant at 40 °C. Peptides were analyzed by Top 10-CID-HCD (collision induced dissociation and high energy collision dissociation) data-dependent mass spectrometry.

*Data Analysis*—Proteins were identified by database searching using SequestHT (Thermo Fisher Scientific) with Proteome Discoverer 1.4 software (Thermo Fisher Scientific), a human Swissprot database (release 2015_04, 20,169 entries). Precursor mass tolerance was set at 7 ppm, and fragment mass tolerances were set at 0.5 Da and 20 ppm for collision-induced dissociation and high energy collision dissociation fragmentation, respectively. Trypsin or ArgC was set as enzyme, and up to two missed cleavages were allowed. Oxidation (M) and acetyl (Lys) were set as variable modifications, and carbamidomethylation (C) was set as fixed modification. Peptides were filtered with a score *versus* charge state (1.5 z1, 2.5 z2, 3 z3, and 3.2 $z \geq 4$) and rank 1. Identified acetylated peptides resulting from the two digestions were combined.

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## Results

*Purification of GCN5- and GCN5-containing Complexes*—To test whether the incorporation of human GCN5 in either the ATAC or SAGA HAT modules or, alternatively, in the corresponding endogenous human ATAC and SAGA holocomplexes influence its activity and specificity, we set out to purify GCN5 and the respective complexes. Recombinant FLAG-tagged GCN5 was purified from baculovirus-infected *Sf*21 insect cells by anti-FLAG IP followed by FLAG peptide elution (Fig. 1*A*).

Subunits corresponding to either the HAT module of hATAC (GCN5, ADA2a, ADA3, and SGF29) or those of the HAT modules of hSAGA (GCN5, ADA2b, ADA3, and SGF29) were produced in insect cells using the MultiBac expression system (44, 49). Recombinant proteins were purified from extracts of MultiBac-infected *Sf*21 insect cells by double immunoprecipitation against hGCN5 and ADA3 (Fig. 1*B*). SDS-PAGE analysis of the purified fractions showed co-immunoprecipitation of the four subunits in apparently stoichiometric amounts, indicating that the recombinant proteins assemble in stable ATAC HAT module or SAGA HAT module (Fig. 1*C*).

Endogenous ATAC and SAGA complexes were purified by tandem IPs from human HeLa cell nuclear extracts (Fig. 1, *D* and *E*). To purify endogenous ATAC complexes, the first IP was performed by using an antibody against hADA2a, an ATAC-specific subunit, and eluted with the corresponding peptide (Fig. 1*D*). To obtain only GCN5 (KAT2A)-containing ATAC complexes (and to eliminate PCAF (KAT2B)-containing ATAC complexes), we carried out a second affinity purification using an anti-GCN5 antibody, which purified the endogenous GCN5-containing ATAC complex (Fig. 1, *D*, *F*, and *G*). Despite the fact that in human cells both GCN5- and PCAF-containing ATAC complexes exist, hereafter we will call the GCN5-containing ATAC complexes "ATAC," for simplicity. To purify endogenous SAGA, the first IP was performed by using an antibody against hTAF10 (a subunit of SAGA and TFIID). To exclusively purify SAGA complexes that contain GCN5, the second IP was performed using an antibody raised against hGCN5, and endogenous GCN5-containing SAGA complexes were eluted with the corresponding peptide (Fig. 1, *E*, *F*, and *G*). Despite the fact that in human cells both GCN5- and PCAF-containing SAGA complexes exist, hereafter we will call the GCN5-containing SAGA complexes "SAGA" for simplicity. The protein composition of the endogenous complexes was tested by Western blot (WB) assays using antibodies against either shared (ADA3 and SGF29) or specific subunits (ZZZ3 and ADA2b) (Fig. 1*F*) and by silver staining (Fig. 1*G*). To verify whether the immunopurified endogenous ATAC and SAGA complexes would contain contaminating HATs other than GCN5 or PCAF, the anti-ADA2a and the anti-TAF10 IPs (see Fig. 1, *D* and *E*) were subjected to mass spectrometry analyses. We did not detect any other HATs than GCN5 and PCAF in the analyzed IPs (supplemental Table 1).

*GCN5 Needs to Be Incorporated in Its Corresponding HAT Modules or Endogenous HAT Complexes to Exert Its Full Activity on Histone Tail Peptides*—To compare the specific HAT activity of GCN5 among the recombinant baculovirus over expressed samples and endogenous protein complex preparations, they were normalized for GCN5. First the recombinant GCN5 and ATAC and SAGA HAT module preparations were normalized either by Coomassie Blue staining or WB assays by using two different anti-GCN5 mouse mAbs (Fig. 2*A*). Next we verified whether the two different anti-GCN5 mAbs would detect the same way the recombinant and the endogenous human (h) GCN5s in the different preparations (Fig. 2*B*). As we did not observe any differences in their detection specificity, we used the 2GC mAb to normalize GCN5 among the recombinant baculovirus-overexpressed samples and endogenous protein complex preparations (Fig. 2*C*). Note that as the recombinant hGCN5 is FLAG- and His-tagged, it migrates slightly slower (Fig. 2*A*, *lanes 1*, *2*, and *4*) than the endogenous non-tagged hGCN5 in ATAC and SAGA complexes (Fig. 2, *B* and *C*, *lanes 3* and *5*).

Next, the enzymatic activities of recombinant GCN5 alone, incorporated into either the recombinant ATAC HAT or SAGA HAT modules, or the two endogenous ATAC or SAGA holocomplexes were measured by peptide acetylation assays using an N-terminal histone H3 peptide ranging from amino acids 5 to 20 (Fig. 2*D*). Interestingly, in this assay the same amount of GCN5 was 3–8 times more active when incorporated in either of the HAT modules or 12–14 times more active when part of the endogenous ATAC or SAGA complexes (Fig. 2, *D* and *F*). As ATAC has been described to acetylate also histone H4 (27, 35, 36), we tested the activity of GCN5 alone and the different purified complexes on a histone H4 tail peptide encompassing amino acids 1–19 (Fig. 2*E*). We observed that the same amount of GCN5 was only slightly more active (1.5-fold) on the H4 tail peptide when incorporated in the recombinant HAT modules but ~5 times more active in the endogenous ATAC or SAGA holo-complexes (Fig. 2, *E* and *F*). Generally, the proteins and complexes were ~10 times more active on the H3 tail peptide than on the H4 tail peptide (Fig. 2, *D* and *E*). Interestingly, we found that the acetylation activity of GCN5 is generally stimulated to a higher extent in the SAGA-type HAT module than in ATAC-type HAT module (Fig. 2, *D*, *E*, and *F*), suggesting a differential stimulatory role of ADA2a and ADA2b. Because the activity of GCN5 is further stimulated upon incorporation of the HAT modules into the corresponding endogenous HAT complexes, we conclude that distinct subunit environments in the HAT modules or in the endogenous complexes play a stimulatory role in the regulation of the activity of GCN5. Note, however, that post-translational modifications of GCN5 in the endogenous complexes may also participate in this stimulatory activity. Alternatively, the purification of rGCN5 alone, without its natural protein environment, may partially inhibit its activity.

*ADA2b Has a Stronger Stimulatory Activity on GCN5 Than ADA2a*—As the ATAC and SAGA HAT modules share the three subunits GCN5, ADA3, and SGF29, we postulate that the significant differences in acetylation activity of the ATAC HAT and the SAGA HAT modules on H3 peptides originate from sequence differences in the ADA2a and ADA2b subunits, which are 45% similar. To test this hypothesis, we produced and

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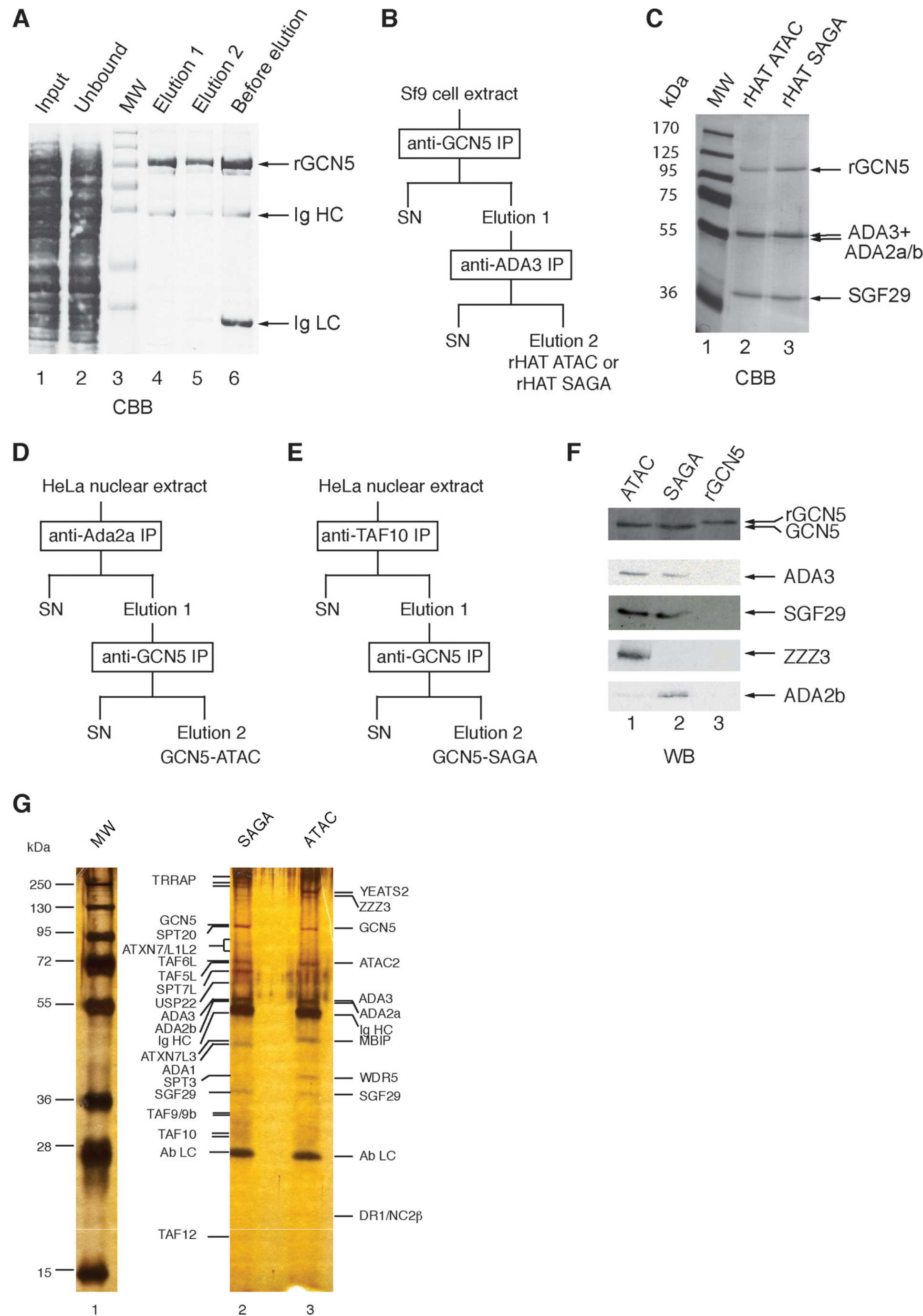


FIGURE 1. **Purification of GCN5, recombinant HAT modules, and endogenous ATAC and SAGA complexes.** *A*, purification of recombinant GCN5. SDS-PAGE analysis of the GCN5 purification procedure showed input and unbound samples (*lanes 1* and *2*, respectively), the peptide elutions (*lanes 4* and *5*), and GCN5-bound antibody beads before elution (*lane 6*). Proteins were visualized by Coomassie Brilliant Blue (*CBB*) stain. The antibody heavy chains (*Ig HC*) and light chains (*Ig LC*) are indicated. *MW*, molecular mass marker (170, 125, 95, 75, 55, 36, and 24 kDa). *B*, purification scheme of the recombinant ATAC and SAGA HAT modules (rHAT ATAC and rHAT SAGA). *SN*, supernatant. *Elution*, peptide elution with a 1000× excess of peptide against which the indicated antibodies were raised. *C*, purification of recombinant HAT modules of ATAC and SAGA. SDS-PAGE analysis of the purified complexes following the procedure depicted in *B*. Proteins were visualized by Coomassie Brilliant Blue stain. Note that the ATAC HAT module contains ADA2a, whereas the SAGA HAT module contains ADA2b. *MW*, molecular mass marker (as in *panel A*), and protein identities are indicated on the *left* and *right of the gel*, respectively. *D* and *E*, schematic representation of the purification of endogenous GCN5-containing ATAC and SAGA complexes from HeLa cell nuclear extracts. *SN*, supernatant containing the unbound proteins. *Elution*, peptide elution with a 1000× excess of peptide against which the indicated antibodies were raised. *GCN5-ATAC*, GCN5-containing ATAC complex; *GCN5-SAGA*, GCN5-containing SAGA complex. *F*, Western blot analyses of endogenous ATAC (*lane 1*) and SAGA (*lane 2*) complexes following the purification procedures depicted in *D* and *E*, respectively. Recombinant GCN5 (*lane 3*) was also analyzed as the control. Note that the recombinant hGCN5 is FLAG tagged, and thus it migrates slightly slower than the endogenous hGCN5 in ATAC and SAGA. Primary antibodies are indicated on the *right*. GCN5, ADA3, and SGF29 are common subunits of ATAC and SAGA, whereas ZZZ3 and ADA2b are ATAC- and SAGA-specific subunits, respectively. The GCN5 content of the endogenous complexes was normalized to the amount of recombinant GCN5. *G*, the subunits of endogenous ATAC (*lane 2*) and SAGA (*lane 3*) complexes purified as depicted in *D* and *E*, respectively, were visualized by silver nitrate staining. The subunits of the respective complexes are indicated. Molecular mass markers (*MW*; *lane 1*) are indicated in kDa. The antibody heavy chains (*Ig HC*) and light chains (*Ig LC*) are indicated.

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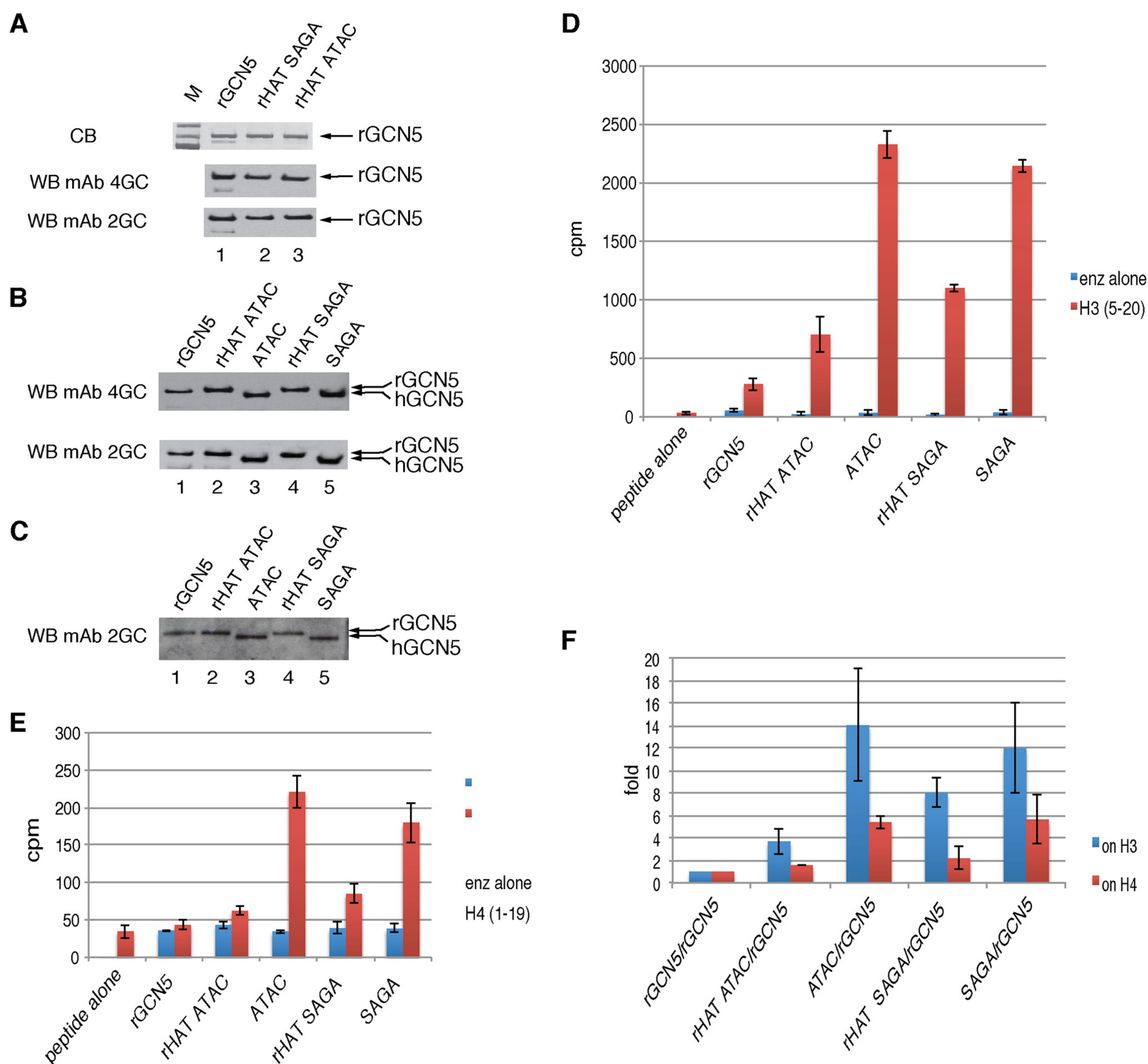


FIGURE 2. **Acetylation activity of the GCN5 enzyme depended on its protein environment.** *A*, normalization of recombinant GCN5 and GCN5-containing HAT complexes. Amounts of purified recombinant ATAC HAT and SAGA HAT modules were normalized to recombinant GCN5 and analyzed by either Coomassie Blue (*CB*) staining or by WB with two different anti-hGCN5 mouse mAbs 2GC and 4GC. *B*, purified recombinant GCN5, ATAC HAT, and SAGA HAT modules and endogenous ATAC and SAGA complexes were tested by WB with two different anti-hGCN5 mouse mAbs 2GC and 4GC. *C*, amounts of purified recombinant GCN5, ATAC HAT, and SAGA HAT modules and endogenous ATAC and SAGA complexes were normalized by WB with the anti-hGCN5 mouse mAbs 2GC. Note that in *panels B* and *C* the recombinant hGCN5 is FLAG- and His-tagged alone, or incorporated in the HAT modules (*lanes 1*, *2*, and *4*). Thus, it migrates slightly higher than the endogenous non-tagged hGCN5 (*lanes 3* and *5*). *D*, acetylation of an H3 tail peptide revealed by a filter-binding assay. H3 tail peptide covering amino acids 5–20 was incubated with radioactive acetyl-coenzyme A and the indicated protein or protein complex preparation. Reactions were carried out in quadruplicate in the presence (H3 amino acids 5–20) or absence of the H3 tail peptide, and acetylation was measured in cpm. *Error bars* indicate S.D. *E*, acetylation assay as in *D* but with an H4 tail peptide encompassing residues 1–19. *F*, -fold activation of GCN5 activity of the HAT modules or the endogenous ATAC and SAGA complexes compared with the activity of recombinant GCN5 on the H3 and H4 tail peptides used in *D* and *E*. Activation was calculated from three biological replicates. *Error bars* represent S.D.

purified recombinant heterotrimeric (GCN5/ADA3/ADA2a or ADA2b) and heterotetrameric (GCN5/ADA3/SGF29/ADA2a or ADA2b) HAT modules. Because GCN5 does not form stable dimeric complexes with either ADA2a or ADA2b alone, we did not test the influence of these single subunits on acetylation activity of GCN5 (22, 50).[6] Subunit compositions of the multimeric recombinant complexes were verified by Western blot (Fig. 3*A*). When we tested the tri- and tetrameric complexes in our peptide acetylation assays on H3 tail peptides, we found that the ADA2a-containing HAT complexes were about two times less active than the ADA2b-containing HAT complexes (Fig. 3*B*). These results show that ADA2b has a stronger stimulatory effect on the HAT activity of GCN5 than ADA2a. Interestingly, the incorporation of SGF29 in the respective HAT modules, either ATAC type or SAGA type, did not further stimulate the activity of GCN5.

[6] A. Riss, E. Scheer, M. Joint, S. Trowitzsch, I. Berger, and L. Tora, unpublished observations.



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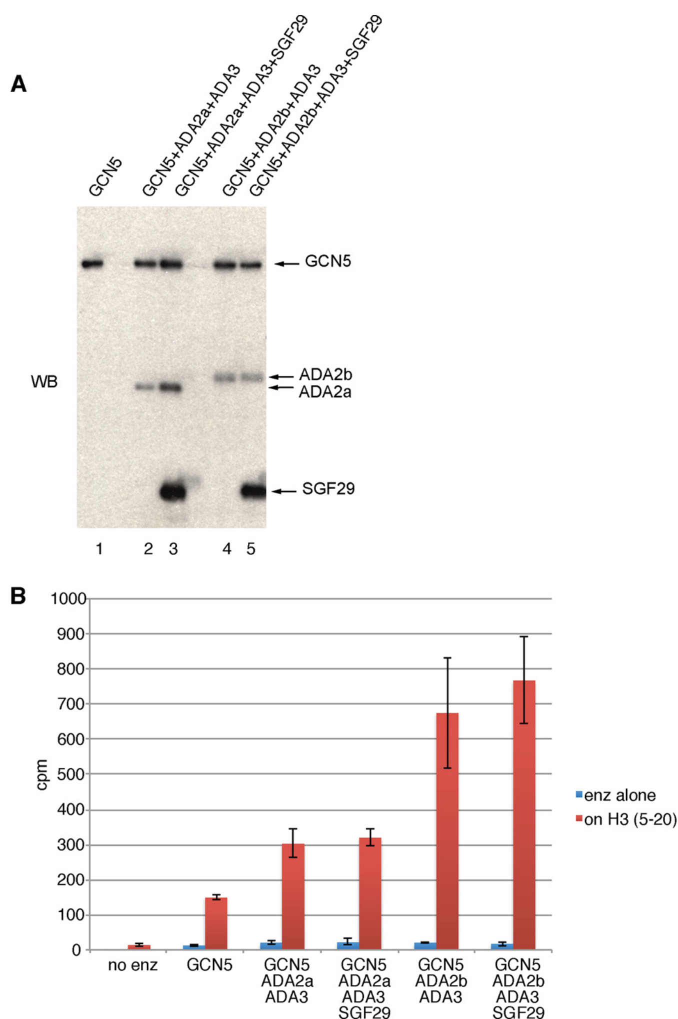
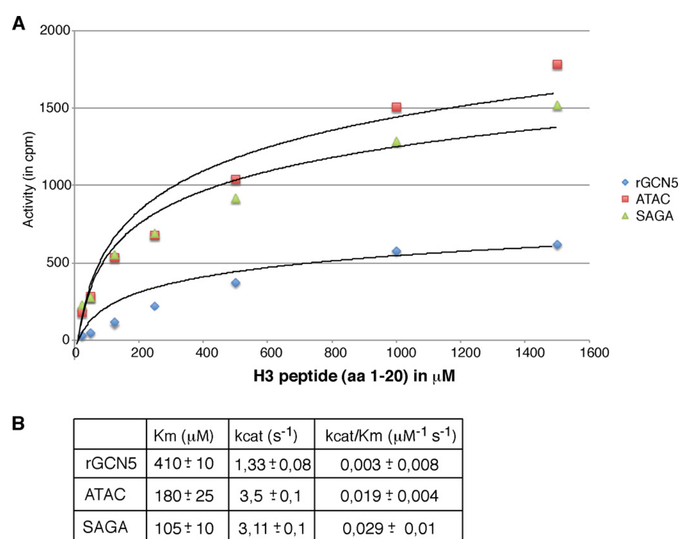



FIGURE 3. **Acetylation activity of GCN5 was differentially regulated by the subunits ADA2a or ADA2b within recombinant HAT modules.** *A*, Western blot analysis of purified recombinant GCN5 and heterotrimeric and heterotetrameric recombinant HAT modules of ATAC and SAGA. Primary antibodies used to identify subunits are indicated on the *right*. Amounts of GCN5 within the HAT modules were normalized to recombinant GCN5. *B*, acetylation of an H3 tail peptide by recombinant GCN5 and heterotrimeric and heterotetrameric HAT modules of ATAC and SAGA. Experimental conditions were as in Fig. 2*B*. Subunit compositions of the HAT modules tested are indicated.

*Subunits of ATAC and SAGA Complexes Enhance Both the Substrate Binding and the Turnover Rate of GCN5-Substrate Complexes*—To obtain more insights into the specific role of the GCN5-associated proteins, we set out to determine the kinetics of acetylation by GCN5 alone or incorporated into endogenous HAT complexes. To this end we measured acetylation rates by filter binding assays at increasing concentrations of H3 peptide (amino acids 1–20) at constant acetyl-CoA concentrations (Fig. 4*A*). The obtained data were fitted to the Michaelis-Menten equation. Compared with GCN5 alone, the endogenous ATAC and SAGA complexes decrease the $K_m$ of GCN5 alone from ~410 $\mu$M to ~180 $\mu$M (for ATAC) and 105 $\mu$M (in the case of SAGA) (Fig. 4, *A* and *B*). These results suggest that subunits of ATAC and SAGA complexes enhance H3 peptide binding. Next the turnover rate of GCN5 alone-, ATAC-, and SAGA-substrate complex was determined by calculating $k_{cat}$. Our measurements indicate that ATAC and SAGA subunits also increase the turnover rate of GCN5 alone from ~$k_{cat}$ 1.33 $s^{-1}$ to ~3.5 $s^{-1}$ (for ATAC) or 3.1 $s^{-1}$ (for SAGA) (Fig. 4*B*). The $k_{cat}/K_m$ calculations show that the incorporation of GCN5 in endogenous HAT complexes increases the catalytic efficiency of GCN5 by ~6 (in ATAC) to 10-fold (in SAGA) (Fig. 4*B*). Thus our results demonstrate that the distinct subunits of ATAC and SAGA complexes can enhance both the substrate binding and the turnover rate of GCN5-substrate complexes.




B

| | Km (µM) | kcat ($s^{-1}$) | kcat/Km ($\mu M^{-1}$ $s^{-1}$) |
|---|---|---|---|
| rGCN5 | 410 ± 10 | 1,33 ± 0,08 | 0,003 ± 0,008 |
| ATAC | 180 ± 25 | 3,5 ± 0,1 | 0,019 ± 0,004 |
| SAGA | 105 ± 10 | 3,11 ± 0,1 | 0,029 ± 0,01 |

FIGURE 4. **Subunits of ATAC and SAGA complexes enhanced both the substrate binding and the turnover rate of GCN5-substrate complexes.** *A*, kinetic measurements comparing the activity of GCN5 alone with endogenous purified ATAC and SAGA complexes on different concentration of H3 tail peptide (from amino acid 1–20) using [$^{14}$C]acetyl CoA in a filter binding assay. Peptide bound radioactivity was quantified in two biological replicates. The activities of the different GCN5-containing preparations are represented in cpm. *B*, summary of kinetic parameter calculations.

*GCN5 HAT Activity on Histone H3/H4 Tetramers Is Stimulated by Interactions with ATAC and SAGA Subunits*—To assess the influence of the globular domains of histones on the acetylation activity of GCN5 and GCN5-containing complexes, we compared the activities of normalized amounts of GCN5-containing complexes on H3/H4 tetramers and H2A/H2B dimers purified from HeLa cells (Fig. 5*A*). In good agreement with previous observations (Ref. 22 and references therein), neither GCN5 nor the GCN5-containing complexes acetylated purified human H2A/H2B dimers (Fig. 5*B*). Interestingly, we hardly detected any acetylation activity of GCN5 when we used H3/H4 tetramers in the HAT assay (Fig. 5*C*, *lane 2*). In contrast, acetylation on histone H3 was detected when using either the HAT modules of ATAC or SAGA (Fig. 5*C*, *lanes 3* and *5*, respectively) or the two endogenous HAT complexes (Fig. 5*C*, *lanes 4* and *6*, respectively). Quantification of the HAT assays showed a gradually increasing activity of GCN5 on histone H3 from the recombinant HAT modules to the endogenous complexes (Fig. 5*D*). Under the conditions used, the recombinant HAT complexes preferentially acetylated histone H3, whereas the endogenous ATAC and SAGA complexes showed also weak activity on H4 (Fig. 5*D*). Importantly, these results on full-length histones reflect the acetylation assays with histone tail peptides and corroborate that the interaction partners of GCN5 within the distinct complexes play an important role in regulating GCN5 enzymatic activity.

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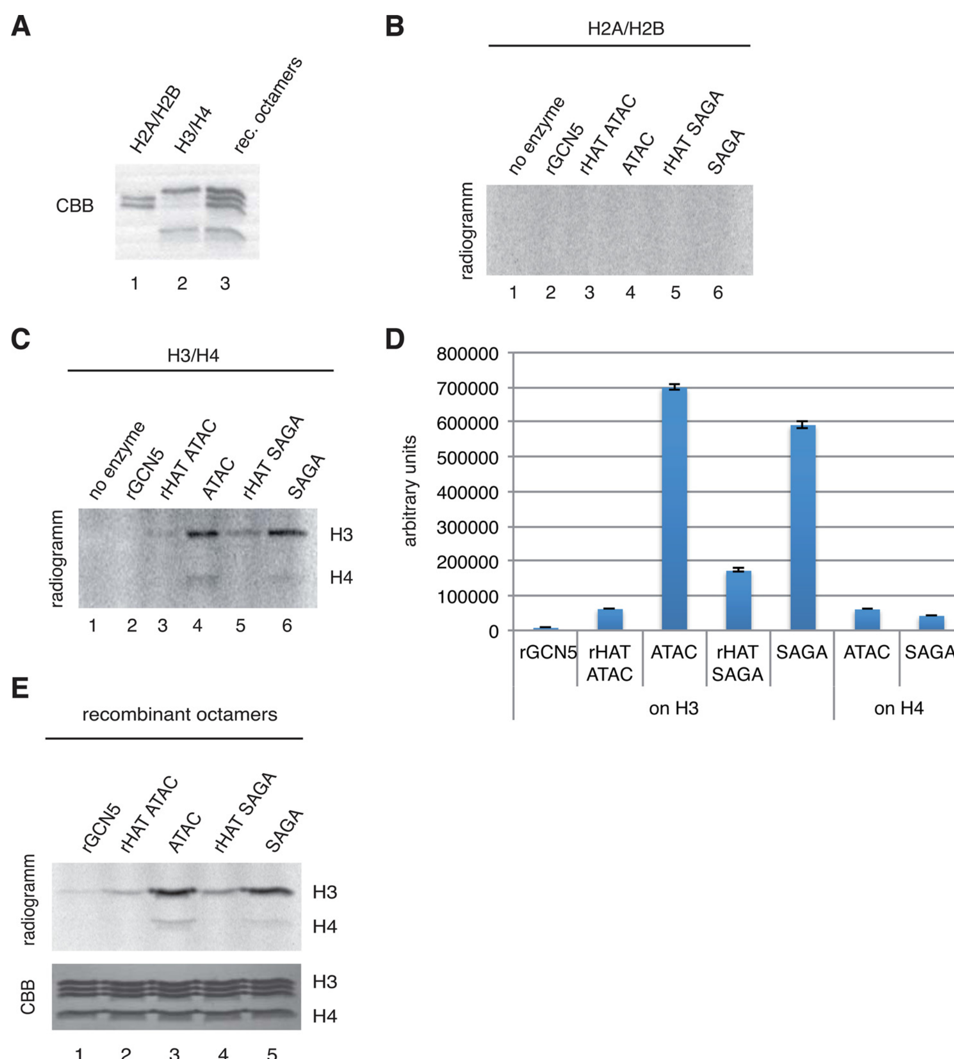


FIGURE 5. **The acetylation activity of GCN5 on recombinant histone assemblies gradually increased with subunit complexity of the HAT modules.** *A*, purification of human H2A/H2B dimers, H3/H4 tetramers, and histone octamers. SDS-PAGE analysis of H2A/H2B dimers and H3/H4 tetramers purified from human HeLa cells and recombinant histone octamers produced in *Escherichia coli*. Proteins were visualized by Coomassie brilliant blue (*CBB*) stain. Protein identities are indicated. *B*, acetylation tests on purified HeLa H2A/H2B dimers with recombinant GCN5, the HAT modules of ATAC and SAGA, and the endogenous ATAC and SAGA complexes. H2A/H2B dimers were incubated with radioactive acetyl-coenzyme A and normalized amounts of GCN5- and GCN5-containing complexes. Histones were resolved by 15% SDS-PAGE and dried, and radioactive signals were detected by radiography using an image intensifier screen. *C*, acetylation tested were as in *B* but purified HeLa H3/H4 tetramers were used. *D*, quantification of acetylation of histones H3 and H4 by GCN5-containing complexes. Quantified signals from two experiments are displayed in arbitrary units with signal deviations indicated as error bars. *E*, acetylation tests as in *B* but performed on recombinant histone octamers.

Next we performed HAT assays using recombinant histone octamers as substrates (Fig. 5, *A*, *lane 3*, and *E*). Using the same enzyme preparations on either recombinant octamers or tetramers purified from HeLa cells, we observed very similar enhancement of GCN5 activity by its incorporation in the different complexes (compare Fig. 5, *C*, *D*, and *E*). These results further demonstrate that the proteins interacting with GCN5 in the distinct complexes stimulate the GCN5 catalytic activity to a similar extent on purified endogenous H3/H4 tetramers and recombinant histone octamers. As histone octamers may have a tendency to dissociate into dimers or tetramers under certain *in vitro* reaction conditions, the octamers used here and later in our study may also be considered simply as H2A/H2B dimers and H3/H4 tetramers, which may not be physiological substrates of these complexes *in vivo*.

*The Lysine Acetylation Specificity of GCN5 on Histone Tails or Full-length Histones Is Not Changed When Incorporated in the HAT Modules of ATAC or SAGA Complexes*—Next we asked whether GCN5 alone or incorporated in the endogenous HAT complexes has different acetylation specificity. To this end, we carried out HAT assays using distinct histone H3 peptides that covered the H3 tail from amino acids 1 to 40 (Fig. 6*A*). In this assay the only two peptides that were acetylated by either GCN5 or the endogenous ATAC and SAGA complexes were those that contained the H3 tail sequences encompassing amino acids 5–20 (Fig. 6*A*).

To identify acetylation sites with single residue resolution, we used histone H3 tail peptides encompassing amino acids 5–20, where the three potential acetylation sites Lys-9, Lys-14, and Lys-18 were individually mutated to arginine (Fig. 6*B*). We again compared specificity of GCN5 alone with the specificity of GCN5 in endogenous complexes. Interestingly, only the H3K14R mutation had a drastic inhibitory effect on the HAT activity of both GCN5 and the endogenous complexes, suggest-

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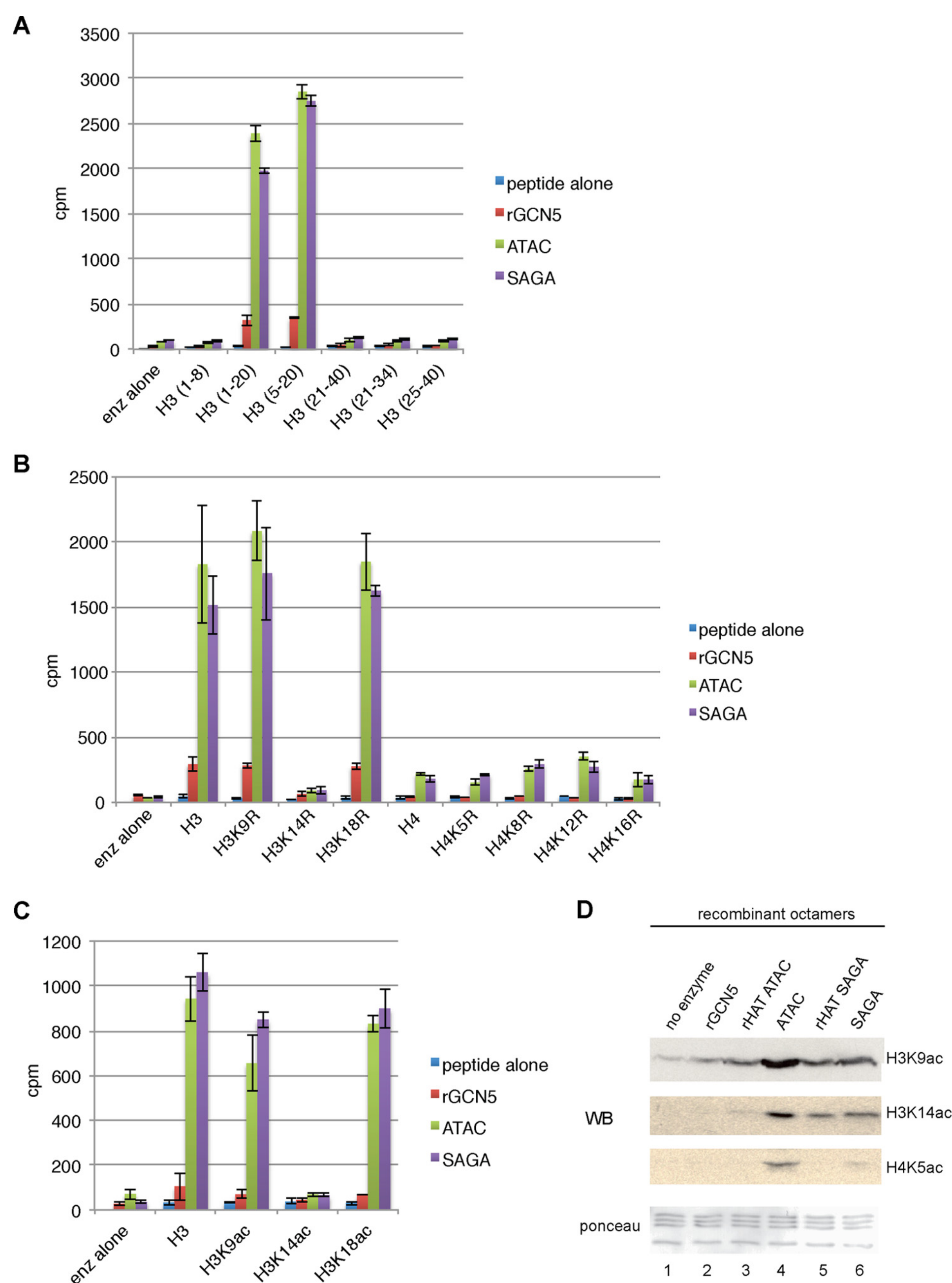


FIGURE 6. **In peptide tail HAT assays the specific acetylation site of GCN5 and the endogenous HAT complexes was H3K14, whereas on full-length histones the acetylation specificity was broader.** *A*, acetylation assays on H3 tail peptides. Histone H3 tail peptides encompassing different regions of the H3 tail (as indicated) were assayed for acetylation by recombinant GCN5 and the purified endogenous ATAC and SAGA complexes as in Fig. 2*B*. Reactions were carried out in quadruplicate, and acetylation was measured in cpm. *B*, acetylation assays as in *A* but using H3 (amino acids 5–20) peptides in which lysine residues Lys-9, Lys-14, and Lys-18 were consecutively substituted with arginines. *C*, acetylation assays as in *A* but using the H3 (amino acids 5–20) peptide in which lysine residues Lys-9, Lys-14, and Lys-18 were consecutively substituted with acetylated lysines. *D*, acetylation tests on recombinant histone octamers with normalized amounts of recombinant GCN5, the HAT modules, and the endogenous complexes (as indicated). Protein preparations were assayed for acetylation activity on recombinant octamers as in Fig. 4 but with cold acetyl-CoA. Histone octamers were resolved by SDS-PAGE and probed for acetylation by Western blot using the indicated antibodies. Successful transfer of histones to the membrane was verified by Ponceau S stain.

ing that H3K14 is the main acetylation site for GCN5 in isolation and associated with the endogenous complexes.

To test whether pre-acetylation of H3 lysine residues could have an influence on the GCN5-mediated acetylation of adjacent lysine residues, we generated H3 tail peptides (amino acids 5–20), in which each of the three lysine residues were synthetically pre-acetylated at the same time (Fig. 6*C*). Interestingly, pre-acetylation of H3K14 abolished acetylation of all the other sites in the peptide. These results corroborate that in our H3 peptide acetylation assay, the main acetylation site for GCN5 is H3K14, independent of the subunit environment of GCN5. Taken together, we show here that GCN5 specifically acetylates lysine H3K14 in isolation and when incorporated in the endogenous complexes and that the activity, but not the specificity, of the enzyme is altered when incorporated in the endogenous complexes.

We had observed a weak histone H4 acetylation activity of GCN5 when incorporated in ATAC and SAGA complexes (Fig.

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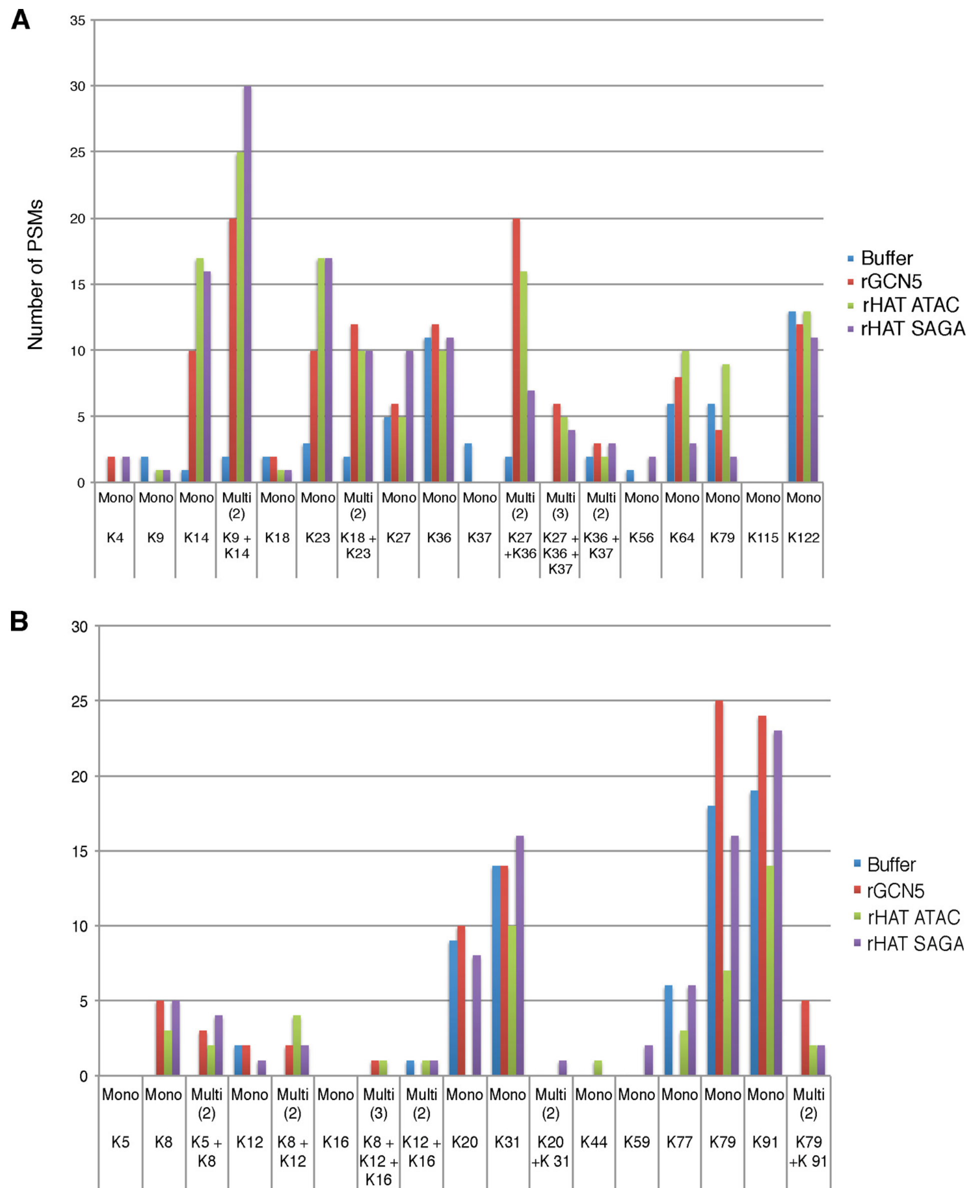


FIGURE 7. **GCN5 and the HAT modules of ATAC or SAGA acetylated the same lysine residues on histone octamers.** *A* and *B*, acetylation reactions were set up with GCN5 and the HAT modules of ATAC or SAGA using recombinant histone octamers. Acetylated lysine residues were identified on histone H3 (*A*) or H4 (*B*) by LC-MS/MS analysis using Orbitrap. The numbers of specific acetylated peptide-spectrum matches (*PSM*) containing the indicated different individual lysine (*K*) residues or their identified combination (as indicated) on H3 or H4 are represented. *Buffer*, the reaction in which no enzyme was included (control).

2*E*). Therefore, we next tested whether the specificity of GCN5 would change when assayed on a 19-mer histone H4 tail peptide in which the four potential lysine residues were individually mutated to arginine (Fig. 6*B*). Neither of the Lys-to-Arg mutations significantly abolished the weak HAT activity of the tested ATAC or SAGA complexes, suggesting that there is no specific acetylation site on these H4 peptides (Fig. 6*B*).

Next we analyzed the acetylation specificity of the different GCN5-containing complexes on recombinant octamers using a "cold" HAT assay and by revealing the acetylation sites with specific antibodies raised either against H3K9ac, H3K14ac, or H4K5ac (Fig. 6*D*). In this acetylation test all the GCN5-containing preparations acetylated H3K9 and H3K14; however, with gradually increasing activity, as described above (Fig. 6*D*, see also Fig. 5). This experiment suggests that on histone octamer (or H2A/H2B dimers and H3/H4 tetramers), the acetylation specificity of GCN5 is broader than on H3 tail peptides, but the specificity of the GCN5 does not change when incorporated in the HAT modules or in the endogenous complexes.

*GCN5 Alone or Incorporated in the HAT Module of ATAC or SAGA Can Acetylate Histone H3 and H4 at Very Similar Lysine Positions in Octamers*—To determine all the acetylation sites of GCN5 alone or in the HAT modules of ATAC or SAGA and to identify their preferred sites of acetylation on recombinant histone octamers (or H2A/H2B dimers and H3/H4 tetramers), we established a mass spectrometry-based approach in conjunction with large scale HAT assays (Fig. 7). HAT reactions were carried out under saturating conditions with cold acetyl-CoA. Histones were then digested by trypsin and ArgC, and the obtained peptides were subjected to LC-MS/MS analysis using an Orbitrap. From their mass spectra peptide-spectrum, matches were determined containing no, one, two, or three

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acetylated lysine positions. The number of peptide-spectrum matches obtained for a given acetylated lysine residue either on histone H3 (Fig. 7*A*) or H4 (Fig. 7*B*) in the three GCN5-containing fractions is represented including the negative control sample. These data show that under the used *in vitro* conditions on histone octamers, GCN5 alone or incorporated in the HAT module of ATAC or SAGA can acetylate histone H3 and H4 at very similar lysine positions (Fig. 7, *A* and B). The preferred acetylation sites of GCN5 on the recombinant histone octamers (or H2A/H2B dimers and H3/H4 tetramers) are the following: H3K9+K14, H3K14, H3K23, H3K18+K23, H3K27+K36, H3K27+K36+K37, and H4K5+K8, H4K8, H4K8+K12, H4K79+K91 (Fig. 7*B*). In this assay no significant acetylation was detected on positions H3K4, H3K9 (alone), H3K18 (alone), H3K36 (alone), H3K37 (alone), H3K56, H3K64, H3K79 (alone), H3K115, and H3K122, and H4K5 (alone), H4K12, H4K16, H4K20, H4K31, H4K44, H4K59 and H4K77, H4K79, and H4K91. These data, taken together, further show that the acetylation specificity did not change when GCN5 acted alone or incorporated in the HAT modules of either ATAC, or SAGA on recombinant histone octamers (or H2A/H2B dimers and H3/H4 tetramers).

## Discussion

Nuclear acetyltransferases are involved in a wide variety of cellular processes, including transcription regulation, chromatin structure, DNA repair, cell cycle control, and many others. However, it remains unclear how acetyltransferase enzymes are regulated and how such regulation controls the acetylation of specific targets at given lysine residues. A huge variety of *in vitro* and *in vivo* methods was employed to characterize the function of GCN5, but none systematically tested its activity and specificity alone or associated with its structural modules or complexes. We set out to understand GCN5 acetylation in a systematic way, starting from the isolated enzyme and by building up the corresponding HAT complexes.

Our study demonstrates that the subunits of the HAT modules and the incorporation of the HAT modules in to the endogenous complexes enhance the enzymatic activity of GCN5. This is important, as in cells GCN5 is always incorporated into endogenous complexes, such as SAGA and ADA in yeast, or ATAC and SAGA in metazoan cells (18, 27, 51). Moreover, in our assays using normalized GCN5 amount-containing complexes we found that the ADA2b-containing SAGA HAT module stimulated the HAT activity of GCN5 better than that of the ADA2a-containing ATAC HAT module. It has been described that ADA2b, but not ADA2a, increases the acetylation of nucleosomes by GCN5 (22). Both ADA2a and ADA2b have SANT and SWIRM domains that are found in several proteins implicated in chromatin function. Analyses of the role of the SANT domain in yeast Ada2p revealed that the domain was required for full HAT activity of the associated Gcn5p (52, 53) and suggested that the SANT domain of yAda2p is a histone tail binding or presentation module (52). Thus, it is possible that the ADA2b SANT domain plays a role in the recognition of histones and that the related ADA2a SANT domain has a weaker histone tail binding or presentation capability. To further dissect the roles of SANT and/or SWIRM domains in the enhanced regulation of GCN5 and to provide insight into the mechanism of histone acetylation by the ADA2a- or ADA2b-containing HAT modules, chimeric ADA2a and ADA2b proteins have to be created for further functional tests. Importantly, when the ATAC- or SAGA-type HAT modules are associated with the corresponding endogenous complexes, the HAT activity of GCN5 is further stimulated, suggesting that the HAT modules' interactions with the respective other functional modules in the ATAC or SAGA complexes would further accommodate the HAT modules structures for a full activity. Nevertheless, the full activities of the two endogenous complexes (normalized for GCN5) are rather comparable under all our assay conditions.

Recent structural and functional studies of the second enzymatic module of SAGA, the deubiquitination (DUB) module, also demonstrated an allosteric regulation of DUB enzymes (yUbp8 or hUSP22) through multiple interactions with other subunits of the DUB module of SAGA (54–56). These observations together with the above-described regulation of GCN5 suggest a common principle for the regulation of the enzymatic activities of ATAC and SAGA and may be even for all chromatin-modifying complexes with enzymatic activities.

In contrast to the enhanced activity of GCN5 by the corresponding subunits, the incorporation of GCN5 in different complexes did not significantly change the specificity of the different GCN5-containing enzyme preparations using H3 and H4 tail peptides, full-length H3/H4 tetramers, or recombinant histone octamers as substrates in our *in vitro* assays. Interestingly, however, we observed a broadening of the lysine acetylation specificity of GCN5 and the corresponding complexes when we used either full-length endogenous histone H3/H4 tetramers or recombinant histone octamers. In peptide tail assays all the activities acetylated strongly and specifically H3K14 (and ~10 times less H4), whereas in the assays using full-length histones or recombinant histone octamers the specificity broadened. On histone octamers (or H2A/H2B dimers and H3/H4 tetramers) GCN5 and the HAT modules were able to specifically acetylate H3 and/or H4 tail residues and/or their well defined combinations (H3K9+K14, H3K14, H3K23, H3K18+K23, H3K27+K36, H3K27+K36+K37, and H4K5+K8, H4K8, H4K8+K12, H4K79+K91; Fig. 7). These data together suggest that the main site targeted by GCN5 on H3 tail is H3K14 and that either GCN5 or the other HAT module subunits are able to bind H3/H4 tetramers or histone octamers, which would then target GCN5 to additional sites, such as H3K9, H3K23, H3K27, H3K36, H4K5, and H4K8. It is thus conceivable that histone fold recognition/binding via SANT domains of ADA2a or ADA2b could bring the histone tails in close proximity to the catalytic site of GCN5, therefore, broadening the specificity due to local enrichment. In contrast, when using tail peptides local enrichment would not be possible and only "perfect fit" peptides would have high enough affinities to be acetylated. Note, however, that H3/H4 tetramers and/or histone octamers may not represent the physiological targets of ATAC and SAGA *in vivo*. In future experiments it would be interesting to test whether pre-methylated and/or pre-acetylated histone octamers or nucleosomes would change the specificity of the HAT and/or the endogenous complexes

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toward certain lysine residues. Nevertheless, in our assays the GCN5-dependent acetylation sites are specific, as we did not obtain significant acetylation on several lysine residues present either on tails or globular domains of H3 or H4 (Fig. 7).

The enhanced substrate recognition and turnover of GCN5 by its associated subunits in ATAC or SAGA could play an important role in the function of these complexes. GCN5 within the SAGA complex is recruited at specific loci on the genome, where it will act on its specific histone substrates. The targeting of GCN5 to the chromatin is suggested to be carried out by several "reading domains" of SAGA and ATAC complexes, such as the bromodomain of GCN5 itself binding to acetylated chromatin marks and/or the SGF29 double Tudor domain known to bind H3K4 trimethylation (57–59). Thus, future structural and further enzymatic studies will be required to better understand the acetylation function, specificity, and targeting the GCN5-containing complexes to recombinant nucleosomes, premodified nucleosomes, and different chromatin templates in either the context of the ATAC or the SAGA HAT complexes.

*Author Contributions*—A. R. designed, performed, and analyzed the experiments and wrote the first draft of the paper. E. S. performed experiments. L. T. designed and performed the experiments shown in Fig. 7, and M. J. analyzed the experiments shown in Fig. 7. S. T. and I. B. constructed and provided the MultiBac expression vectors and participated in writing the paper. All authors reviewed the results and approved the final version of the manuscript. L. T. conceived and coordinated the study and wrote the paper.

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