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Chaperone-mediated acetylation of histones by Rtt109 identified by quantitative proteomics

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Abstract

Rtt109 is a fungal-specific histone acetyltransferase (HAT) that associates with either Vps75 or Asf1 to acetylate histone H3. Recent biochemical and structural studies suggest that site-specific acetylation of H3 by Rtt109 is dictated by the binding chaperone where Rtt109-Asf1 acetylates K56, while Rtt109-Vps75 acetylates K9 and K27. To gain further insights into the roles of Vps75 and Asf1 in directing site-specific acetylation of H3, we used quantitative proteomics to profile the global and site-specific changes in H3 and H4 during *in vitro* acetylation assays with Rtt109 and its chaperones. Our analyses showed that Rtt109-Vps75 preferentially acetylates H3 K9 and K23, the former residue being the major acetylation site. At high enzyme to substrate ratio, Rtt109 also acetylated K14, K18, K27 and to a lower extent K56 of histone H3. Importantly, this study revealed that in contrast to Rtt109-Vps75, Rtt109-Asf1 displayed a far greater site-specificity, with K56 being the primary site of acetylation. For the first time, we also report the acetylation of histone H4 K12 by Rtt109-Vps75, whereas Rtt109-Asf1 showed no detectable activity toward H4.

Keywords

Histone; histone acetyltransferase; chaperone; acetylation; proteomics; mass spectrometry

1. Introduction

Eukaryotic DNA is assembled into a highly compact and dynamic nucleoprotein structure known as chromatin. The basic repeating subunit of chromatin is the nucleosome core particle (NCP) that comprises 147 base pairs of DNA wrapped nearly twice around a histone octamer [1]. The octamer is formed of an (H3-H4)₂ tetramer (two molecules each of both

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CONFLICT OF INTEREST

The authors declare no conflicting financial interests.

H3 and H4) paired with two H2A/H2B dimers. Neighbouring NCPs are joined by variable lengths of linker DNA (10–80 bp) [2] that binds to histone H1 family members to promote chromatin compaction into a 30-nm fibre [3]. The N-terminal tails of core histones protrude from the nucleosome, and contain multiple lysine residues that can be modified by post-translational modifications (PTMs) such as acetylation, methylation, ubiquitylation and sumoylation. These modifications can act individually or in a combinatorial fashion to mediate epigenetic regulation of chromatin structure [4, 5]. The acetylation of lysine residues represents one of the most extensively studied histone modifications thus far. This modification can directly affect the chromatin structure and/or favor the recruitment of other binding partners by providing a docking platform for histone modifying enzymes or ATP-dependent chromatin remodeling machineries [6–9]. The acetylation of specific lysine residues can also provide a chromatin mark for the regulation of gene expression [10] or to identify sites of DNA damage. For instance, recent studies have shown that acetylation of H3-K56 and H4-K16 is significantly elevated in mammalian cells in response to DNA damage [11–13].

Histone acetylation is regulated by two enzyme families known as histone acetyltransferases (HATs) and histone deacetylases (HDACs) [14, 15]. HATs catalyze the transfer of acetyl groups from acetyl-coenzyme A (CoA) to the ϵ -amino group of specific lysines on histone substrates while HDACs remove N-acetyl moieties on target lysine residues. These two enzyme families act antagonistically to modulate gene expression [16], and abnormal activity of some of these enzymes have been observed in different types of cancer [17]. For example, mutations in CBP (which encodes a HAT) and chromosomal translocation with MLL are hallmarks of different human diseases including specific types of leukemia [17–19].

The regulator of Ty1 transposition gene product 109 (Rtt109) is a fungal-specific HAT that acetylates newly synthesized histone H3 prior to its incorporation into chromatin. Rtt109 is involved in the response to genotoxic agents and the regulation of gene expression [20, 21]. Rtt109 has a conserved core region that is structurally related to other HAT enzymes though they share limited sequence homology. Recent structural and biochemical analyses of Rtt109 revealed significant homology to the metazoan p300/CBP HAT domain, but also highlighted important differences in their catalytic properties [22]. The enzyme activity of Rtt109 is greatly enhanced by its association with either Vps75 or Asf1 histone chaperones [23, 24]. These two chaperones are not structurally related but they both bind to new H3/H4 molecules [25, 26]. While Rtt109 interacts only weakly with Asf1, crystal structures revealed the formation of a tight and stable complex with the chaperone Vps75 [27–29]. The chaperones are required to present the substrate in a distinct orientation that confers target specificity to Rtt109. A recent review by D'Arcy *et al.* indicated that Rtt109-Vps75 sequentially acetylates histone H3 at K9 and K27, whereas the Rtt109-Asf1 complex primarily acetylates K56 [29]. However, the exact sites of acetylation targeted by Rtt109-Vps75 are not entirely clear as an early study by Berndsen *et al.* [30] reported that this enzyme acetylated H3K9 and H3K23 but not H3K27.

In the present study, we used quantitative proteomics to determine the site-specific acetylation of histones by Rtt109 associated with either the Vps75 or the Asf1 histone

chaperone. This is achieved using a two-pronged approach that we previously developed to profile changes in histone modifications between wild-type and HAT mutant yeast strains [31] and for human cells incubated with different HDAC inhibitors [32]. First, temporal changes in H3-H4 acetylation following incubation with Rtt109-Vps75 and Rtt109-Asf1 are determined from the LC-MS mass profiles of intact histones. Second, the acetylated histones are subjected to propionylation, tryptic digestion and LC-MS/MS analyses to identify acetylated lysine residues and their respective changes in stoichiometry. This strategy enabled precise and comprehensive determination of acetylated lysine stoichiometry to unambiguously identify chaperone-mediated acetylation of histones H3 and H4 by Rtt109.

2. Material and methods

2.1 Reagents

Capillary LC columns for nano LC-MS were packed in-house using Jupiter C₁₈ (3 µm particles Phenomenex), and fused silica tubing (Polymicro Technologies). Trapping columns were packed in-house using the same bulk material in Teflon tubing (Supelco). Water and acetonitrile (ACN) for chromatographic analysis were all HPLC grade (Fisher Scientific and in-house Milli-Q water). Trifluoroacetic acid (TFA), anhydrous methanol, ammonium bicarbonate, ammonium hydroxide, and propionic anhydride were all purchased from Sigma. Formic acid (FA) was obtained from EMD Science. Porcine modified trypsin (sequencing grade) was obtained from Promega.

2.2 Protein Purification

Recombinant (H3-H4)₂ tetramers or monomeric H3 were purified from *E. coli* as previously described [33]. The holoenzyme Rtt109-Vps75 was expressed and purified according to methods reported by Tang *et al.* [28]. Because Asf1 and Rtt109 do not form a stable complex, the two polypeptides were expressed and purified separately. The gene encoding full-length Rtt109 was cloned into a modified pET Duet vector (Novagen) with a TEV-cleavable GST tag, as previously described [22]. A gene encoding *S. cerevisiae* 6His-Asf1N(1–154) was constructed into a modified pET Duet vector (Novagen) with a thrombin/TEV-cleavable 6His tag. Both recombinant proteins were expressed in *Escherichia coli* BL21(DE3). After induction with 0.8 mM IPTG, cells were incubated overnight at 18°C and then harvested for protein purification. The GST-Rtt109 protein was purified using a standard GST-affinity procedure with on-resin TEV protease digestion. The cleaved Rtt109 protein was eluted off the resin using PBS buffer and then flash frozen using liquid nitrogen and stored at –80 °C for future use. The 6His-tagged Asf1 protein was purified through Ni-NTA affinity and ion exchange chromatography before being subjected to TEV protease cleavage in solution and size exclusion chromatography.

2.3 HAT assay

In vitro HAT assays were performed using recombinant (H3-H4)₂ tetramer (or H3 monomer), acetyl-CoA and Rtt109-Vps75/Asf1 enzyme-chaperone complexes. The assays were performed by incubating the substrates and enzyme/chaperone complexes for different time periods (0 to 90 min) at 30° C. Unless otherwise indicated, histone tetramers or monomers were incubated with Rtt109-Vps75/Asf1 enzyme-chaperone complexes at a 1:1

enzyme to substrate ratio. Acetyl-CoA was added to all samples to a final concentration of 100 μ M. The reaction buffer consisted of 1 mM DTT in 25 mM ammonium bicarbonate solution (pH 7.8). The enzymatic reactions were quenched with 5% TFA. A control sample (without Rtt109) was included to monitor assay conditions.

2.4 Sample preparation and protein digestion

Histone samples were desalted on a pre-equilibrated C18 ZipTip pipette tips (Millipore). Desalting was performed according to manufacturer's instructions. Each eluate was divided into two portions and dried in a speed-vac. The first portion of samples was resuspended in 0.2 % FA for analysis of intact histones by LC/MS. The remaining samples were subjected to propionylation, tryptic digestion and peptide sequencing by LC-MS/MS. The propionylation reaction is performed by adding 200 μ l of the propionylation reagent (de-ionized water: propionic anhydride, 2:1 (v/v)) to the histone samples and vortexing the mixture for 1 h at room temperature. Samples were then dried, resuspended in 0.1 M ammonium bicarbonate (pH 7.8), and digested overnight at 37°C using trypsin (1:50, enzyme:substrate ratio). Digests were resuspended in 0.2 % FA in water (v/v) prior to LC-MS/MS analyses [31].

2.5 LC/MS analysis of intact histones

Intact histone analysis was performed on an Agilent QTOF 6520 LC/MS system equipped with nano-ESI source. The mass spectrometer was operated in positive ion mode and scanned from m/z 400 to 1600. Prior to injection, histone samples were diluted to 200 ng/ μ l using 0.2% FA in water. An aliquot of 5 μ l was loaded on a custom C18 trap column (4 mm length, 360 μ m i.d.) for 5 min at 15 μ l/min using 0.2% FA. Histones were then eluted onto a C18 analytical column (10 cm length, 150 μ m i.d.) using a gradient of 5 to 60% aqueous ACN (0.2% FA) in 60 min at 600 nl/min. The Agilent MassHunter Workstation Software (version B.01.04) was used to deconvolute the multiply-charged protein ions over a discrete time-segment of the LC-MS run.

2.6 LC-MS/MS analysis of tryptic peptides

Tryptic digests of propionylated histone samples were analyzed on LTQ-Orbitrap XL mass spectrometer (Thermo Fisher scientific) coupled to an Eksigent nano-LC system. The same column and solvent systems were used as described above for the LC/MS analysis of intact histones. The mass spectrometer was operated in a data-dependent acquisition mode with full scan (m/z 300 – 2000) resolution set to 60 000, with a target value of 1.0×10^6 . The six most abundant precursor ions were selected for fragmentation in the LTQ by CID at a normalized CE setting of 35. Fragment ions were analysed in the LTQ ion trap over the mass range of m/z 200 – 2000 with a target value of 1.0×10^4 .

2.7 Database searching and peptide clustering

MS data were analyzed using the Xcalibur software (version 2.0.7). Peak lists were generated using the Mascot distiller software (version 2.3.2.0, Matrix science) where MS processing was achieved using the LCQ_plus_zoom script. Database searches were performed using the search engine Mascot (version 2.2.0, Matrix Science, London, UK)

with the concatenated forward and reverse *Saccharomyces* Genome Database (SGD, version 64.1). The tolerance window for experimental precursor mass values and fragment ion mass values were set to ± 15 ppm and 0.5 Da, respectively. The number of allowed missed cleavage sites for trypsin was set to 5 and acetylation (K), propionylation (K), oxidation (M), and deamidation (NQ) were all selected as variable modifications. No fixed modification was included in the search. All peptide identifications were transferred to ProteoConnections [34], an in-house developed bioinformatics tool, to generate a .csv (comma separated value) file that can be opened and modified in Excel. The .csv output file contained only distinct peptides (peptide with maximum score is kept) with assigned Mascot score of greater than or equal to 20. Manual MS/MS spectra verification was performed on each modified peptide to confirm the sequence assignment. The clustering of peptide abundances across different experimental conditions was performed using in-house tools as described previously [32]. Briefly, raw data files (.raw) from the Xcalibur software were converted into peptide map files representing all ions according to their corresponding m/z values, retention time, intensity, and charge state. An intensity threshold of 5000 counts was set as a cut-off for peptide detection. Peptide abundances were determined using the peak top intensity values. Clustering of peptide maps across different sample sets were performed on peptides associated to a Mascot entry using hierarchical clustering with tolerances of ± 15 ppm and ± 1 min for peptide mass and retention time, respectively. Normalization of retention time is performed on the initial peptide cluster list using a dynamic and nonlinear correction that confines the retention time distribution to less than 0.1 min ($<0.3\%$ relative standard deviation, RSD) on average. Global intensities were normalized based on the average intensity of all peptides signals. Peptide clusters showing reproducible changes in abundance across conditions were manually inspected to validate identification and changes in abundance.

3. Results

3.1 Nano-LC/MS analysis of histones

Our MS analyses of histones involve a two-pronged strategy to profile the distribution of molecular mass and identify sites of modification [31]. This approach utilizes both intact histones and tryptic peptides to profile global and site-specific changes in histone modifications. First, intact histones are analyzed to determine changes in their mass profiles that can be attributed to specific modifications. Second, peptide maps generated from nano LC-MS/MS analysis of the corresponding tryptic digests are compared to identify peptides showing statistically meaningful changes in abundance across conditions. In the present study, we extended the application of this approach to determine the kinetics and site-specificity of Rtt109 toward histones H3 and H4. The experimental workflow is illustrated in Figure 1. Prior to MS analysis, recombinant histones H3 and H4 (monomeric or tetrameric forms) were incubated with either Rtt109-Vps75 or Rtt109-Asf1 HAT complex for different time periods (0 to 90 min). A portion of each intact histone sample was analyzed by LC/MS on a Q-TOF instrument, while the remaining sample was propionylated and digested by trypsin prior to peptide sequencing by LC-MS/MS on a LTQ-Orbitrap XL mass spectrometer.

3.2 Extent of histone acetylation by Rtt109-Vps75 and Rtt109-Asf1 complexes

To profile the progressive changes in acetylation of the H3:H4 tetramers upon incubation with either Rtt109-Vps75 or Rtt109-Asf1 complexes, we first analyzed the enzymatic products using LC-MS. An enzyme to substrate ratio of 1:1 was maintained in all cases. H3 and H4 histones along with Rtt109 and its chaperones were chromatographically separated and displayed abundant multiply-charged ion spectra from which the molecular mass could be determined. Under the present chromatographic conditions, we identified H4 (M_{obs} : 11237 Da), and H3 (M_{obs} : 15225 Da), at 43.6 and 47.1 min respectively (Supplementary Figure 1). The observed molecular mass was within ± 1 Da of the value calculated from the protein sequence. To account for changes in histone acetylation over different incubation periods, we normalized the intensity of individual peak to that of histones from the control sample. The relative proportion of histones displaying different degrees of acetylation was determined from the abundance ratio of a specific acetylated form to the sum of all forms of identified histones.

Figure 2 show the intact mass profiles of acetylated histone H3 (M_{theo} 15225 Da) following the *in vitro* HAT assay with Rtt109-Vps75 and Rtt109-Asf1 complexes. As expected, a progressive increase in histone H3 acetylation is observed over the first 90 min of incubation. Interestingly, a broader distribution of acetylated products is observed for Rtt109-Vps75 compared to Rtt109-Asf1 although identical enzyme:substrate ratios were used in all cases. Acetylation of H3 by Rtt109-Vps75 proceeded rapidly, and up to three acetyl groups were detected within the first minute of incubation (Figure 2A). In contrast, acetylation of H3 by Rtt109-Asf1 progressed more slowly, and the first acetylated form of H3 was significantly detected only after 5 min of incubation (Figure 2B). We observed that incubation of H3 with Rtt109-Vps75 yielded up to 5 acetylation sites over the first 90 min of the *in vitro* HAT assay. The relative proportion of each acetylated form of H3 is shown in Figure 2C. As indicated, H3 is rapidly acetylated by Rtt109-Vps75 within the first 15 min. The successive acetylation resulted in mono to penta acetylated H3 with the tetra acetylated form being predominant beyond 30 min of incubation. In contrast, Rtt109-Asf1 showed a narrower distribution of H3 acetylation, and no more than three sites were detected over the same incubation period (Figure 2D). The relative proportion of unmodified H3 decreased very slowly, and the mono-acetylated H3 remained the most abundant form detected after 90 min of incubation.

Although Vps75 and Asf1 were previously reported to affect the acetylation status of histone H3, we also examined the activity of these chaperones on the acetylation of H4 by Rtt109. Similarly to that described before, we determined the extent of acetylation of histone H4 following incubation of the H3:H4 tetramers with Rtt109-Vps75 and Rtt109-Asf1 (Figure 3). Upon incubation with Rtt109-Vps75, no significant acetylation of histone H4 (M_{obs} : 11237 Da) was observed until 5 min of incubation (Figure 3A). Longer incubation periods led to increased abundance of the mono-, di-, and tri-acetylated acetylated forms of H4 at 11279, 11321 and 11363 Da, respectively. However, the monoacetylated form of H4 was the predominant reaction product of Rtt109-vps75, while the original H4 substrate remained present over the entire incubation period (Figure 3B). In contrast, Rtt109-Asf1 did not show any activity towards histone H4 acetylation in spite of the high enzyme to substrate ratio

used (Figure 3C). To our knowledge, no previous study has reported the activity of Rtt109 on the acetylation of histone H4.

To address whether acetylation of histones H3 and H4 was dependent on the oligomeric state of the substrates, we performed *in vitro* HAT assays using tetrameric histones (H3-H4)₂ or individual H3 or H4 histones. These experiments indicated that the chaperone-dependent activity of Rtt109 is not significantly affected by the oligomeric state of the histone substrates, and trends in acetylation observed for histone monomers were similar to those of tetramers (Supplementary Figure 2).

3.3 Effect of enzyme to substrate ratio on histone acetylation

Next, we examined the activity of Rtt109-Vps75 at different enzyme to substrate ratios. We performed *in vitro* HAT assays at enzyme to substrate molar ratios of 1:200, 1:100, 1:50 and 1:10 for a fix incubation period of 15 min. The relative proportion of all detected acetylated histones at different enzyme:substrate ratios is shown in Figure 4. At a molar ratio of 1:200 we observed two acetylated forms of H3, though their relative abundances were generally low (Figure 4A). Further analyses of these tryptic digests using LC-MS/MS identified two acetylation sites at position K9 and K23 of H3, with K9 being the most abundant (see section 3.4). The relative proportion of the mono acetylated form increased by 10-fold for an enzyme to substrate ratio of 1:100 and further increase in the mono and multiply acetylated forms of H3 were observed for higher enzyme:substrate ratios. We also examined the acetylation profiles of H4 under the same incubation conditions (Figure 4B). These analyses revealed that a mono acetylated form of H4 is typically observed irrespective of the enzyme to substrate ratio used. These observations are consistent with those noted previously for an enzyme to substrate ratio of 1:1. LC-MS/MS analyses of the corresponding tryptic digests indicated that the primary site of H4 acetylation by Rtt109-Vps75 is K12 (see section 3.4). It is noteworthy that only the monoacetylated histone H3 was detected for incubation periods of 15 min and 60 min when experiments were performed using Rtt109 alone (Figure 4C). Consistent with previous reports, we identified K9 as the monoacetylated residue of H3 using LC-MS/MS of the corresponding tryptic digest [30].

3.4 Identification of modified residues and determination of acetylation stoichiometry

To determine the extent and location of acetylation sites following *in vitro* HAT assay, all samples were subjected to propionylation, tryptic digestion and LC-MS/MS analyses. Propionylation of free Lys residues of histones was required to prevent the formation of small hydrophilic tryptic peptides that would be either too short for MS detection or could not be retained efficiently by reversed phase LC [31]. The LC-MS/MS analyses of the corresponding tryptic digests enabled the identification of at least 18 distinct peptides comprising 20 Lys residues, and these peptides typically contained one or two modified sites (see supplementary tables 3–4 and supplementary figure 3). The extent of acetylation of a specific Lys residue was determined from the intensity ratio of the acetylated peptide to the sum of all forms of the corresponding tryptic peptide (acetylated and propionylated). For example, Figure 5A shows representative extracted ion chromatograms of the doubly-protonated peptide ions corresponding to the mono-acetylated (K9ac, K14pr) and di-propionylated (K9pr, K14pr) forms of the tryptic peptide ⁹KSTGGKAPR¹⁷ at an incubation

time of 1 min. The intensity ratio of the $^9\text{KacSTGGKprAPR}^{17}$ peptide to that of all forms of this peptide corresponded to 0.2 or an acetylation stoichiometry of 20%. The MS/MS spectrum of m/z 500.28²⁺ assigned to the monoacetylated tryptic peptide $^9\text{K(ac)STGGK(pr)APR}^{17}$ is shown in Figure 5B. The incremental 42 Da mass shift of the b_2 – b_5 fragment ions confirmed the acetylation of K9 residue while an additional mass shift of 56 Da on b_6 – b_8 fragment ions was consistent with a propionylated K14 residue. The doubly-propionylated form of the same tryptic peptide displayed similar fragmentation patterns except that 56 Da shifts were observed for both K9 and K14 residues. Manual validation was performed on each MS/MS spectrum to confirm the identity of the modified peptides and to correlate the abundance of all peptides across the entire incubation period. Figure 5C shows the site occupancy of Lys acetylation following *in vitro* incubation with Rtt109-Vps75. This graph indicates that Rtt109-Vps75 preferentially acetylates H3 at K9 and K23 residues with the former site displaying the most significant changes in acetylation during this period. Additional acetylation sites at K14, K18, K27 and K56 were also identified beyond 15 min suggesting that Rtt109-Vps75 can promiscuously catalyze reactions on other sites when long incubation periods or high enzyme to substrate ratios are used.

In contrast, *in vitro* reactions performed with histone H3 incubated with Rtt109-Asf1 showed strikingly different patterns with a limited number of acetylated residues (Figure 5D). We noted that Rtt109-Asf1 displayed greater substrate specificity towards K56 acetylation, a residue that is located in the histone fold domain unlike the N-terminal Lys residues (e.g. K9, K23) modified by Rtt109-Vps75 (Figure 5E). Extended incubation periods with Rtt109-Asf1 led to the acetylation of K14 and K23 residues although these sites could arise from promiscuous enzymatic reactions.

We also examined the enzymatic activity of Rtt109-Vps75 and Rtt109-Asf1 on the acetylation of H4 also present in the histone tetramer. Figure 6 shows the sites and extent of acetylation of histone H4 following *in vitro* incubation with Rtt109-Vps75. We identified a total of 3 acetylation sites on H4, consistent with that observed from intact mass measurements (Figure 3). The LC-MS/MS analysis for the tryptic digest of histone tetramers after 15 min incubation with Rtt109-Vps75 is shown in Figure 6A for the mono acetylated (K12ac, K5pr, K8pr, K16pr) and the fully propionylated tryptic peptide $^4\text{GKGGKGLGKGGAKR}^{17}$. The MS/MS spectrum of m/z 740.94²⁺ corresponding to the monoacetylated tryptic peptide $^4\text{GKGGKGLGKacGGAKR}^{17}$ is shown in Figure 6B. An incremental shift of 56 Da is observed for fragment ions y_{2-5} and y_{10-12} consistent with propionylation at K16 and K8 residues. The location of the acetylated K12 residue was confirmed by the observation of a mass shift of 42 Da for y_{6-9} fragment ions while b_2 (m/z 242.0) and b_3 (m/z 299.3) displayed a 56 Da shift, in agreement with a propionylated K5 residue. The acetylation profiles of K8, K12 and K16 residues are shown in Figure 6C for the 90 min incubation period with Rtt109-Vps75. The acetylation of H4K12 proceeded more rapidly than that of K8 and K16 residues (Figure 6C). An Rtt109-Vps75-dependent acetylation of histone H4 has not been reported in a literature, thus far. Interestingly, no acetylation of H4 was observed when the same reaction was performed using Rtt109-Asf1

suggesting again that this chaperone exert more specific catalytic activity toward its histone substrate.

DISCUSSION

This study describes a comprehensive proteomics approach to determine the chaperone-dependent specificity of the Rtt109 enzyme. This approach is based on a two-pronged strategy used previously to profile global and site-specific changes in histone acetylation upon HDACi treatment [31]. Our global analyses revealed that the sites acetylated in histones H3 and H4 vary significantly depending on the type of chaperone associated with the Rtt109 HAT. While this enzyme can acetylate H3K9 on its own, its association with either Vps75 or Asf1 chaperones not only increases its catalytic activity but also confers additional substrate selectivity. *In vitro*, Rtt109-Vps75 catalyzed the acetylation of histone H3 and exhibited a broader substrate activity as evidenced by the number of acetylated forms detected (Figures 2A and 3B). Our peptide sequencing analysis identified two preferential acetylation sites on H3 (K9 and K23) and only one on H4 (K12). It is known that H4K12 is acetylated *in vivo*. However, we cannot exclude the possibility that promiscuous acetylation of H4K12 by the Rtt109 - Vps75 enzyme could take place *in vivo* as observed here for the *in vitro* HAT assay. The significance of this finding would clearly require further functional analyses given recent evidences indicating that acetylation of H4 K12 was shown to regulate dynamic telomere properties in *Saccharomyces cerevisiae* [35].

The identification of H3K23 acetylation using quantitative proteomics also provided clear information on the site-specific activity of Rtt109-Vps75. Although several studies agree on K9 as a primary H3 acetylation site, conflicting results were reported regarding a second acetylation site at position K23 or K27 [24, 30, 36]. These sites were previously characterized using western blotting, and ambiguity on site assignment could possibly arise from cross reactivity of antibodies against H3 N-terminal tail acetylation sites that harbour similar sequences flanking acetylated residues [32, 37]. By using quantitative proteomics we unambiguously identified H3K23 as a second acetylation site targeted by Rtt109-Vps75. It is noteworthy that additional acetylation sites (e.g. K14, K18, K27 and K56) were observed after extended incubation periods (or at high enzyme-to-substrate ratio) showing that, at least *in vitro*, Rtt109-Vps75 can promiscuously acetylate sites other than K9 and K23. The physiological significance of the acetylation site preference exhibited by Rtt109-Vps75 is still unknown. Additional biochemical, structural and *in vivo* studies will be necessary to address the functional significance of this finding.

In contrast to Rtt109-Vps75, Rtt109-Asf1 catalyzed the acetylation of histone H3 but not H4 (Figures 2D and 3C). This complex showed a striking specificity toward H3K56 acetylation, even at very high enzyme to substrate ratios. H3K56 is located at the C-terminal edge of an α -helix that is unique to histone H3 and is not part of the histone-fold domain. In addition, H3K56 cannot be acetylated by Rtt109 when H3 is incorporated into nucleosomes [38]. Rtt109 acetylation is mostly associated with non-nucleosomal, nascent histone H3 in yeast [39, 40]. Rtt109 acetylation of newly synthesized H3 is consistent with recent evidences on its activity in the cytoplasm [41]. It is noteworthy that the orderly sequence by which H3 is acetylated or the events leading to the specific recruitment of Vps75 and Asf1 chaperones to

Rtt109 are presently unknown. However, the possibility of determining enzyme-specificity *in vitro* or *in vivo* using quantitative proteomics opens up new perspectives to decipher the roles of chaperones in regulating HAT activity and substrate selectivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FA	formic acid
HAT	histone acetyl transferase
Rtt109	regulator of Ty1 transposition gene product 109
MS	mass spectrometry
Asf1	Anti-silencing function 1
Vps75	Vacuolar protein sorting 75
HDAC	histone deacetylase
ac	acetylation
pr	propionylation
RT	room temperature

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Figure 1.
Overview of the analytical scheme for profiling histone modifications.

Figure 2. LC/MS analyses of intact histone H3 obtained from *in vitro* HAT assay
 (A) Intact mass profiles of histone H3 obtained from *in vitro* HAT assay with Rtt109-Vps75 and (B) with Rtt109-Asf1. Incubation times (from top to bottom) are 0, 1, 5, 15, 30, 60 and 90 min. (C) Bar graph represent the extent of H3 acetylation after HAT assay with Rtt109-Vps75 and (D) with Rtt109-Asf1.

Figure 3. LC/MS analyses of intact histone H4 obtained from *in vitro* HAT assay

(A) Intact mass profiles of histone H4 obtained from *in vitro* HAT assay with Rtt109-Vps75.

(B) Bar graph showing the extent of H4 acetylation after assay with Rtt109-Vps75. (C)

Intact mass profiles of histone H4 obtained from *in vitro* HAT assay with Rtt109-Asf1.

Incubation times (top to bottom) are 0, 1, 5, 15, 30, 60, 90 min.

Figure 4. Relative proportion of histone acetylation by Rtt109-Vps75 for different enzyme to substrate ratios

Distribution of acetylated histone for (A) H3 and (B) H4. (C) *in vitro* acetylation of histone H3 acetylation by Rtt109 alone (without chaperone) after an incubation period of 15 min (upper panel) and 60 min (lower panel).

Figure 5. Identification of modified residues and determination of acetylation stoichiometry of H3 incubated with Rtt109-Vps75/Asf1

(A) Extracted ion chromatograms of the doubly charged tryptic peptides corresponding to the acetylated and propionylated forms of ⁹KSTGGKAPR¹⁷. (B) MS/MS spectrum of the acetylated tryptic peptide ⁹KacSTGGKAPR¹⁷. Acetylation stoichiometry of H3 lysine residues following incubation with (C) Rtt109-Vps75 and (D) Rtt109-Asf1. (E) Primary acetylation sites of Rtt109-Vps75 (K9 and K23 located on the N-terminus tail of H3) and Rtt109-Asf1 (K56 located on the H3 fold domain). The cartoon representation of yeast histone H3 (red) and H4 (blue) are taken from nucleosome PDB 1ID3 (prepared using the

Pymol Molecular Graphics system, version 1.2r1). (F) Amino acid sequence of yeast histone H3.

Figure 6. Identification of modified residues and determination of acetylation stoichiometry of H4 incubated with Rtt109-Vps75

(A) Extracted ion chromatograms of the doubly charged tryptic peptides corresponding to the acetylated and propionylated forms of $^4\text{GKGGKGLGKGGAKR}^{17}$. (B) MS/MS Spectrum of the acetylated tryptic peptide $^4\text{GKGGKGLGKacGGAKR}^{17}$. (C) Acetylation stoichiometry of H4 lysine residues (K5, K8, K12) following incubation with Rtt109-Vps75. (D) Amino acid sequence of yeast histone H4.