Published in final edited form as:

Biochemistry. 2010 August 3; 49(30): 6375–6385. doi:10.1021/bi100381y.

Kinetic mechanism of histone acetyltransferase and chaperone complex, Rtt109-Vps75†

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Abstract

Rtt109 is a histone acetyltransferase (HAT) involved in promoting genomic stability, DNA repair and transcriptional regulation. Rtt109 associates with the NAP1 family histone chaperone Vps75, and stimulates histone acetylation. Here we explore the mechanism of histone acetylation and report a detailed kinetic investigation of the Rtt109-Vps75 complex. Rtt109 and Vps75 form a stable complex with nanomolar binding affinity ($K_d = 10 \pm 2$ nM). Steady-state kinetic analysis reveals evidence for a sequential kinetic mechanism whereby Rtt109-Vps75, AcCoA and histone H3 substrates form a complex prior to chemical catalysis. Product inhibition studies demonstrate that CoA binds competitively with AcCoA and equilibrium measurements reveal AcCoA or CoA binding is not stimulated in the presence of H3 substrate. Additionally, Rtt109-Vps75 binds H3 substrates in the absence AcCoA. Pre-steady state kinetic analysis suggests the chemical attack of substrate lysine on the bound AcCoA is the rate-limiting step of catalysis, while the pH profile of k_{cat} reveals a critical ionization of $pK_a = 8.5$ that must be unprotonated for catalysis. Amino acid substitution at D287 and D288 did not substantially change the shape of the k_{cat} pH profile, suggesting these conserved residues do not function as base catalysts for histone acetylation. However, the D288N mutant revealed a dramatic 1000-fold decrease in k_{cat}/K_m for AcCoA, consistent with a role in AcCoA binding. Together, these data support a sequential mechanism in which AcCoA and H3 bind to Rtt109-Vps75 without obligate order, followed by the direct-attack of the unprotonated ϵ -amino group on AcCoA, transferring the acetyl-group to H3 lysine residues.

In eukaryotes, DNA is wrapped around an octamer of histones to form the fundamental unit of chromatin called the nucleosome. Post-translational modification of histones elicits effects on chromatin compaction, transcriptional control and replication modulation (1,2). Lysine 56 (K56), a residue located within the globular fold of histone H3, is abundantly acetylated in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (3-6). During replication-coupled nucleosome assembly, newly synthesized H3 molecules are marked with K56ac (lysine 56 acetylation), subsequently assembled into DNA to form chromatin, and then deacetylated in G2/M phase by the NAD-dependent activities of the sirtuin homologs Hst3 and Hst4 (5,7,8). Furthermore, K56ac predominates with exposure to DNA damage reagents, implicating a role for K56ac in genomic integrity and DNA repair (6,9-11). Specifically, K56ac is required for chromatin reassembly after double stranded DNA breaks, signals for the completion of DNA repair and increases the stability of replication forks (10,11). Replication-independent roles for K56 modification exist, as this acetylation and

[†]This work supported by grants from the NIH (GM059785) and a postdoctoral fellowship (EMK) from the American Heart Association (0920041G).

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Supporting Information Available: Additional experimental details, thermodynamic table, and isothermal titration calorimetry data results. This material is available free of charge via the Internet at <http://pubs.acs.org>.

increased histone turnover are prominent at active gene promoters, while the hypoacetylation of K56 marks regions of silent chromatin such as telomeres(12,13). In mammals, K56ac is elevated in response to DNA damage and is enriched in cancerous cell lines, suggesting that roles for K56ac are conserved (14). K56ac is also associated with genes bound by key regulators of pluripotency in embryonic stem cells, suggesting this histone modification is an epigenetic marker for the pluripotent state (15).

One particular family of proteins that bind histones and mediate chromatin dynamics are histone chaperones. Previously presumed only to function in preventing non-specific interactions of histones, these chaperones are now recognized as important regulators of histone assembly and nucleosome eviction (16). For example, the histone chaperone Asf1 is required for K56ac in replication-coupled nucleosome assembly upstream of the actions of histone chaperones CAF-1 and Rtt106(17). Asf1 also increases histone turnover in non-replicative processes such as transcriptional activation at gene promoters (12). Vps75 is a NAP (nucleosome assembly protein) histone chaperone homolog involved in transcription-related histone exchange and in stimulating the Rtt109-dependent acetylation of H3 tail residues (11,18-20).

Recently, the yeast protein Rtt109 was discovered as a novel lysine acetyltransferase (KAT11) that associates with histone chaperones and utilizes AcCoA to acetylate K56 of H3 (Scheme 1) (5,6,11,21).

Interestingly, Rtt109 shares no sequence homology with other mammalian HATs (histone acetyltransferases), although recently reported crystal structures reveal Rtt109 is structurally similar to the mammalian HAT p300 (KAT3)(21-25). Unlike other HATs, Rtt109 requires association with a histone chaperone, Asf1 or Vps75, for catalytic activation (11,19). In vivo, *asf1Δ* results in loss of K56ac and K9ac while *vps75Δ* diminishes K9ac and K23ac with little change in K56ac (6,11,26-28). In association with Asf1, Rtt109 acetylates H3K56 in vitro, but only in the presence of histone H4(11). In contrast, the high affinity complex Rtt109-Vps75 demonstrates broader substrate specificity and acetylates K56, K9, K14, and K23 on H3 alone, H3-H4, and H3 tail peptides(11,26). While Asf1-Rtt109 is responsible for K56ac and a portion of K9ac in vivo, Rtt109-Vps75 appears to function as an H3 tail acetyltransferase, although how the Rtt109-Vps75 complex directs the fate of histones at specific chromatin regions is not fully understood(11,18,26,28). Together, these data suggest distinct pathways for the regulation of Rtt109 by histone chaperones.

Recently, Rtt109 was demonstrated to be essential for the pathogenesis of *Candida albicans*, the primary fungal genus (*Candida*) responsible for opportunistic human infections (29). Loss of Rtt109 in *C. albicans* results in increased sensitivity to genotoxic agents, diminished pathogenicity in mice, and greater susceptibility to ROS-mediated cell death by macrophages(29). Given *C. albicans* poses a persistent public health problem and resistance to the current classes of antifungal agents is widespread, Rtt109 may be a novel therapeutic target in the treatment of fungal infections(30). Because Rtt109 is uniquely regulated by histone chaperones and the importance of developing new mechanism-based inhibitors for antifungal therapy, we sought to elucidate the catalytic mechanism of the yeast Rtt109-Vps75 complex. In this study, we have determined the binding affinity between Vps75 and Rtt109, elucidated the kinetic mechanism of Rtt109-Vps75, established the order of substrate binding and identified the rate-limiting step of the catalytic cycle. Employing various biochemical methods such as steady-state kinetic analysis, pre-steady state methods, pH rate-profile analysis and various binding assays, the results are consistent with a random sequential mechanism with rate-limiting chemical catalysis that is characterized by the direct attack of an unprotonated histone lysine on enzyme-bound AcCoA.

Experimental Procedures

Chemicals and Reagents

AcCoA, CoA, DTT, pyruvate dehydrogenase, pyruvic acid, and NAD⁺ were purchased from Sigma-Aldrich. Tris-Cl, P81 cellulose discs, and sodium chloride were available from Fisher. Most other reagents were of the highest grade available and purchased either from Fisher or Sigma-Aldrich. [³H]-Acetyl-CoA (2-25 Ci/mmol) was obtained from Morevek and acrylamide/bisacrylamide was purchased from Bio-Rad. Site-directed mutagenesis kits from Stratagene were utilized to introduce point mutations at D287 and D288 to Rtt109.

Expression, Purification and Quantification of Proteins

Expression and purification of co-expressed recombinant His-Vps75-untagged-Rtt109 and His-Vps75 were performed as detailed in Berndsen *et al.* (26). Untagged Rtt109 was further purified by separation of the complex via an HP-Phenyl sepharose column (GE) followed by purification over a Superdex 200 sizing column (GE) or a His-tagged construct of Rtt109 was purified by nickel affinity purification. These proteins freely exchange and do not form an irreversible complex. The same k_{cat} was achieved whether experiments were performed with (His-tagged or untagged) Rtt109 and Vps75 added as separate components or co-purified Rtt109-Vps75 complex. H3 protein and H4 protein from *Xenopus laevis* were recombinantly purified as previously described in Luger *et al.* (31). Rtt109 and Vps75 proteins were quantified by one of two methods: Bradford reagent assay or densitometry analysis using bovine serum albumin as the protein standard and Coomassie Blue R250 as the protein stain. Concentrations of co-purified Rtt109-Vps75 complex was determined from densitometry of Rtt109, as Vps75 was present in ~2-fold excess. These concentrations were confirmed by amino acid analysis performed by the UC-Davis Proteomics Core Facility. H3 protein was quantified by determining the absorbance at 276 nm using the extinction coefficient $\epsilon=4040 \text{ M}^{-1}\text{cm}^{-1}$. H3-H4 was reconstituted as previously described by Tanaka *et al.* (32).

Synthesis and analysis of H3 Peptide

The utilization of whole H3 protein substrate was problematic in some assays due to either high acetylation background activity of H3 or protein precipitation caused when H3 and Rtt109-Vps75 were combined at high concentrations. Because an H3 tail peptide has previously been shown to be a substrate for Rtt109-Vps75, the peptide substrate was utilized to circumvent the issues of using full length H3 (26). The H3 peptide (amino acid sequence ARTKQTARKSTGGKAPRKQL) was generated by the University of Wisconsin-Madison Biotechnology Center (UWBC) and purified over a preparative C18 HPLC column. Mass spectrometric identification of the peptide was performed on a Bruker REFLEX II: MALDI-TOF (matrix-assisted laser desorption/ionization-time-of-flight) instrument. Chromatographic purity of the purified peptide was found to be $\geq 95\%$ and peptide concentration was determined by amino acid analysis by the UC- Davis Proteomics Core Facility.

Fluorescein labeling of Vps75 and fluorescence polarization measurements

Vps75 was labeled with fluorescein-5-maleimide (Invitrogen) according to manufacturer's procedures in PBS (phosphate buffered saline) and resulted in 1:1 ratio of label to Vps75, suggesting that 1 of 2 cysteines in Vps75 was preferentially labeled. Excess fluorescein-5-maleimide was dialyzed away (PBS) from labeled Vps75 and protein concentrations were quantified as described above. HAT activity assays with labeled Vps75 showed less than a 2-fold loss in activity, indicating that the labeling procedure did not appreciably hinder the ability of Vps75 to activate Rtt109. For the fluorescence polarization studies, 125 μL

samples of 3.2 nM labeled Vps75_{dimer} (assumed as a nondissociating dimer) and 3.7-190 nM Rtt109 in PBS containing 0.2 mM AcCoA and 0.1 mg/mL BSA were prepared and equilibrated at 25°C for 15 minutes, prior to obtaining measurements using a Beacon™ 2000 Fluorescence Polarization System. The fluorescence polarizations measurements were performed in the presence of AcCoA, as Rtt109 alone (without AcCoA or Vps75) is unstable and precipitates at 25°C. Control experiments using BSA as the titrant showed no change in the FP signal. The fraction of bound labeled Vps75 (fraction of Rtt109-Vps75 complex formed) was determined by the following equation:

$$Fract.Bound = \frac{A_{obs} - A_f}{A_b - A_f} \quad (\text{Eq. 1})$$

where A_{obs} is the observed anisotropy, A_f is the anisotropy of free labeled Vps75_{dimer} and A_b is the anisotropy of maximally bound labeled Vps75_{dimer} with Rtt109. The concentration of bound complex was calculated by multiplying fraction bound by the [Vps75_{dimer}] used in the experiment. The total Rtt109 concentration was plotted against that of Rtt109-Vps75 (bound, labeled Vps75_{dimer}) and fitted to the following equation to yield the dissociation constant of the complex (K_d):

$$[Rtt109 - Vps75_{dimer}] = \frac{[Vps75_T] + K_d + [Rtt109_T] - \sqrt{([Vps75_T] + K_d + [Rtt109_T])^2 - 4[Vps75_T][Rtt109_T]}}{2} \quad (\text{Eq. 2})$$

where [Rtt109-Vps75_{dimer}] is the concentration of the complex, [Vps75_T] is the total concentration of labeled Vps75_{dimer} and [Rtt109_T] is the total concentration of Rtt109. Seven separate binding trials were performed in replicate with the K_d representing the average of the trials and the standard error reported.

Histone Acetyltransferase Assays for Rtt109-Vps75

Rtt109-Vps75 histone acetyltransferase activity was monitored by one of two methods at 25°C. In a continuous assay, Rtt109-Vps75 histone acetyltransferase activity was monitored by a coupled pyruvate dehydrogenase assay that recycles AcCoA (33,34). Briefly, CoA generated by the HAT reaction was converted to AcCoA by the pyruvate dehydrogenase complex in the presence of thiamine pyrophosphate (TPP), pyruvate, and NAD⁺. Reduction of NAD⁺ to NADH was monitored continuously at A340 using a Multiskan Ascent microplate reader in order to determine the initial rate of the reaction. In a discontinuous method, HAT assay components (enzyme, H3 substrate, and [³H]-AcCoA) were mixed and spotted at various time-points on P81 phosphocellulose discs as described previously (34). Discs were washed 3 × 500 mL for 5 minutes in 50 mM sodium bicarbonate solution (pH 9.0) and dried to remove unreacted AcCoA prior to liquid scintillation counting. An unwashed disc served to allow for the measurement of AcCoA specific activity. Minimally, two trials were performed ($n \geq 2$) in duplicate for all kinetic measurements, with error bars of data points representing the standard deviation. The standard error of each kinetic constant was determined. Each assay condition was empirically tested to ensure sufficient Vps75 was present so that $\geq 98\%$ Rtt109 was maximally activated.

Bisubstrate Kinetic Measurements

The bisubstrate analysis measurements were performed in 300 μ L reactions containing 50 mM Tris (pH 7.5), 1 mM DTT, 0.1 μ M Rtt109, 0.8 μ M Vps75, 2.4 mM TPP, 0.2 mM

NAD⁺, 0.2 mM pyruvate, 5 mM MgCl₂ and 0.05 U/ul of pyruvate dehydrogenase. H3 peptide was varied from 23-262 μM and AcCoA was varied from 0.25-2 μM. Data were fitted to equations for a sequential mechanism (1), ping pong mechanism (2) and the equilibrium ordered mechanism (3) using the program KinetAsyst (IntelliKinetics, State College, PA). K_a is the K_m for substrate A, K_b is the K_m for substrate B and K_{ia} is the dissociation constant substrate A. This data and all other kinetic data are shown using Kaleidagraph (Synergy Software, Reading, PA).

$$v = V_m AB / (K_{ia} K_b + K_a B + K_b A + AB) \quad (\text{Eq. 3})$$

$$v = V_m AB / (K_a B + K_b A + AB) \quad (\text{Eq. 4})$$

$$v = V_m AB / (K_a K_b + K_b A + AB) \quad (\text{Eq. 5})$$

Product Inhibition Kinetics

CoA, a product of the HAT reaction, was used as an inhibitor at varied levels of AcCoA. Using the filter binding assays, reaction conditions were carried out at 50 mM Tris (pH 7.5), 1 mM DTT, 20 nM Rtt109, 0.8 μM Vps75, 50 mM NaCl, 5 μM H3 protein (approximately K_m), 0.4 μM-14.4 μM AcCoA and 0-600 μM CoA. The datasets were fit to equations for competitive inhibition (4), noncompetitive inhibition (5), and uncompetitive inhibition (6) where K_{is} is the slope inhibition constant and K_{ii} is the intercept inhibition constant:

$$v = V_m S / [K_m (1 + I/K_{is}) + S] \quad (\text{Eq. 6})$$

$$v = V_m S / [K_m (1 + I/K_{is}) + S (1 + I/K_{ii})] \quad (\text{Eq. 7})$$

$$v = V_m S / [K_m + S (1 + I/K_{ii})] \quad (\text{Eq. 8})$$

Isothermal Calorimetry of AcCoA and CoA into Rtt109-Vsp75

Isothermal calorimetry (ITC) measurements were made using a VP-ITC Microcalorimeter from MicroCal, LLC (Northampton, MA). All binding measurements were performed in 50 mM Tris (pH 7.5 at 25°C), 5% glycerol (w/v), 50 mM NaCl, 2 mM β-mercaptoethanol, and 1 mM EDTA. Co-purified Rtt109-Vps75 complex was dialyzed against the above buffer and ligand solutions were suspended in the dialysis buffer. Data analysis was performed using Origin scientific plotting software and experimental data was fit to a one-site binding model where ΔH (enthalpy), K_a (binding constant) and n (number of binding sites) were flexible parameters. The thermodynamic parameters ΔG and ΔS were determined from the following equation:

$$\Delta G = \Delta H - T \Delta S = -RT \ln K_a \quad (\text{Eq. 9})$$

with standard error reported. For each experiment, 37-43 automatic injections of 1-8 μL of ligand were titrated into the cell (cell volume=1.42 mL) while being stirred at 300 RPM. 167 μM AcCoA were titrated into 5.8 μM co-purified Rtt109-Vps75 complex. For CoA binding, 2 mM CoA were titrated into 50 μM Rtt109-Vps75 co-purified complex. All experiments were also performed in the absence of Rtt109-Vps75 in order to account for thermodynamic changes as a result of ligand dilution.

Gel filtration analysis

Gel filtration chromatography was performed on a Superdex200 10/300GL column from GE and calibrated with Sigma protein standards comprised of albumin from bovine serum, alcohol dehydrogenase from yeast, β -amylase from sweet potato, carbonic anhydrase from bovine erythrocytes, and cytochrome c from horse heart. Protein samples (75 μM co-purified Rtt109-Vps75, and 50 μM H3-H4) were equilibrated on ice at 4 $^{\circ}\text{C}$ for 60 minutes in 50 mM Tris, pH 8.0, 215 mM NaCl and 5% glycerol prior to elution of protein on the column in 50 mM Tris, pH 8.0 and 215 mM NaCl. 100 μL samples were injected onto the columns, eluted at 0.5 ml/min and 0.5 mL fractions collected. The presence of protein was monitored by A280 absorbance and SDS-PAGE analysis.

Rapid-Quench Flow Analysis

Rapid-quench flow experiments were performed on an RQF-64 Rapid Quench Flow Apparatus from Hi-Tech Scientific experiments. In kinetic experiments with saturating substrate concentrations, co-purified Rtt109-Vps75 incubated with AcCoA was mixed with H3, quenched with 1% TFA, and analyzed by the filter binding assay. Final reaction conditions consisted of 50 mM Tris (pH 7.5), 1 mM DTT, 200 mM NaCl, 30 μM H3, 30 μM AcCoA and 1 μM of the co-purified Rtt109-Vps75 complex. Product formed over time best fit to a linear regression curve by least squares analysis. Single enzyme turnover experiments were performed under similar conditions as described for saturating substrate experiments, with the alteration of 0.4 μM of AcCoA in the final assay conditions. Product formed over time best fit to the single exponential equation (8) where A is the amplitude, k is the first order rate constant, and B is the total amount of product formed.

$$[\text{product}] = Ae^{kt} + B \quad (10)$$

pH analysis of the Rtt109-Vps75-Catalyzed HAT Reaction

Utilizing the filter-binding assay and under saturating substrate conditions (30 μM AcCoA, 577 μM of H3 peptide, 0.2 μM Rtt109 and 0.8 μM Vps75), the k_{cat} was determined from pH 7-10. To maintain constant ionic strength, 50 mM ACES, 25 mM Tris, and 25 mM ethanolamine was utilized. Measurements could not be made below pH 7 or above pH 10 because of protein activity loss at low pH units and high background rates caused by the H3 peptide at elevated pH units. It was verified that alterations in the k_{cat} were not due to protein inactivation or the hydrolysis of AcCoA between pH 7-10. Fits for the k_{cat} data were performed using equation 9 where C is the pH independent k_{cat} and K_a and is the ionization constant of an amino acid residue from either the substrate or the enzyme.

$$v = C / (1 + H/K_a) \quad (\text{Eq. 11})$$

To determine whether loss of activity at low pH was due to ionization of groups involved in forming the Rtt109-Vps75 complex, similar experiments were performed as above with the exception of 5 μ M of Vps75 in the HAT assay.

Rtt109 D287 and D288 mutant studies

Single (D287N and D288N) and double (D287A/D288A) point mutations were made utilizing a Stratagene site-directed mutagenesis kit, and recombinant protein was purified in a similar manner as wildtype His-Rtt109. H3 peptide saturation curves were performed in 50 mM Tris, pH 7.5 at 25 °C, 1 mM DTT, 1-2 μ M Rtt109 variant, 4 μ M Vps75, and 30 μ M AcCoA with H3 peptide varied from 50-500 μ M. AcCoA saturation curves were performed in 50 mM Tris, pH 7.5 at 25 °C, 1 mM DTT, 0.1-0.5 μ M Rtt109 variant, 4 μ M Vps75, and 400 μ M H3 peptide with AcCoA varied from 0.5-30 μ M. For pH rate analysis, reaction conditions were performed in 50 mM ACES, 25 mM Tris, 25 mM ethanolamine (pH 7-9), 1 mM DTT, 3 μ M Rtt109 D287A/D288A, 6 μ M Vps75, 400 μ M H3 peptide, and 60 μ M AcCoA.

Results

Measurement of the binding affinity between Rtt109 and Vps75

Previous studies demonstrated that histone acetyltransferase Rtt109 and histone chaperone Vps75 can be co-purified, resulting in a kinetic complex that is ~100-fold more efficient than Rtt109 alone (11). The ability to co-express and co-purify Rtt109 and Vps75 suggested the formation of a tight-binding stable complex. To determine their binding affinity, fluorescence polarization was performed with separately purified Rtt109 and Vps75.

The titration of Vps75_{dimer} (3.2 nM) with Rtt109 (3.7-190 nM) resulted in the formation of an Rtt109-Vps75 complex (Figure 1). The data were fitted to equation 2 and yielded a K_d value of 10 ± 2 nM. Throughout the detailed biochemical and kinetic experiments that follow, Rtt109 and Vps75 were maintained at concentrations which ensured that $\geq 98\%$ of Rtt109 was bound by Vps75, and thus the maximally activated complex was enzymatically characterized.

Steady-state kinetic analysis

Rtt109 lacks primary sequence homology with other well-studied HATs, such as GCN5, PCAF, p300, and Esa1(25,35-37). A striking feature of Rtt109-dependent acetylation is the requirement of a histone chaperone (Asf1 or Vps75) for efficient catalysis (11). Therefore, the mechanism of acetylation utilized by Rtt109 is unique, and whether Rtt109 employs similar or distinct catalytic strategies was uncertain. Previously characterized acetyl transfer enzymes bind substrates prior to direct-attack of lysine on the acetyl-moiety (sequential mechanism or Theorell-Chance) or form an enzyme-acyl intermediate prior to the transfer of the acyl-group onto lysine (ping-pong mechanism)(35,37,38). In the presence of histone chaperone Asf1 or Vps75, Rtt109 displays ~10-100 fold enhancement of histone acetyltransferase activity(11,26). With Vps75, Rtt109 forms a tight binding complex that can be co-purified, while Asf1 is only loosely associated with Rtt109 (11). Here, the detailed kinetic and binding analyses of the Rtt109-Vps75 complex was performed with H3 peptide, H3 protein or H3-H4 depending upon the technical limitations of each assay. Binding order was established with H3-H4, as this is the most physiological substrate; however, it is important to emphasize that the catalytic mechanism is unlikely to be different for the three substrates, especially given the observation that the k_{cat} varies only 5-fold between all three.

To distinguish between a sequential or ping-pong kinetic model, the initial rates of product formation were determined, utilizing the pyruvate dehydrogenase assay as described in

materials and methods, at varied concentrations of AcCoA and H3 peptide substrate. To circumvent issues of substrate inhibition and protein precipitation that was observed utilizing high concentrations of full-length H3 protein at low enzyme concentrations and low AcCoA, the bisubstrate kinetic analysis was performed with H3 peptide (amino acid sequence ARTKQTARKSTGGKAPRKQL), which did not exhibit these effects at high concentrations. The steady-state rates of product formation at varied substrate concentrations were fitted to several kinetic models and plotted in double-reciprocal format for visualization of the resulting patterns. As evident from the plots, a series of intersecting lines patterns consistent with a sequential mechanism were observed under varied concentrations of AcCoA at fixed, varied concentrations of H3 peptide, and vice-versa (Figure 2A and 2B). Furthermore, the clear absence of a parallel line pattern as determined by visual inspection and statistical analysis suggests Rtt109 does not utilize a ping-pong mechanism. The data were fitted to a sequential kinetic model (equation 3) and yielded the following kinetic constants: The $K_m=0.3 \pm 0.1 \mu\text{M}$ and $k_{\text{cat}}/K_m=3.9 \pm 0.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for AcCoA, while the $K_m=112 \pm 11 \mu\text{M}$ and $k_{\text{cat}}/K_m=9.8 \pm 0.9 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ for the H3 peptide (Table 1). The k_{cat} value of Rtt109-Vps75 ($0.11 \pm 0.01 \text{ s}^{-1}$) towards H3 peptide substrate is approximately 5-fold lower than that of Rtt109-Vps75 towards full-length H3 substrate ($0.62 \pm 0.03 \text{ s}^{-1}$) (Table 1). Together, these data provide evidence for a sequential mechanism in which both substrates must be bound in the active site before chemical catalysis.

CoA product inhibition of Rtt109-Vps75

To establish the order of substrate binding and product release, inhibition studies were performed using CoA as a product inhibitor. Here, we varied AcCoA at several fixed concentrations of CoA and fixed full-length H3 ($5 \mu\text{M}$) concentration, and plotted the rate data in double reciprocal format. The resulting line pattern intersects at the y-axis, which is diagnostic of competitive inhibition. Parallel or an intersecting line pattern in the 2nd or 3rd quadrants would have demonstrated uncompetitive and noncompetitive inhibition, respectively (Figure 2C). Fitting the data to the equation for competitive inhibition (equation 6) yielded a K_{is} value of $110 \pm 20 \mu\text{M}$ for CoA. The lack of a y-intercept effect ($1/v$) indicates that AcCoA and CoA both compete for the same enzyme form. This result supports a mechanism where Rtt109-Vps75 can bind to AcCoA prior to H3, though does not exclude random binding of substrates. Because Rtt109-Vps75 can acetylate multiple residues on the tail and globular fold of H3, it was not technically feasible to generate a fully acetylated H3 peptide sufficiently soluble form to perform product inhibition analysis (26). Creating an acetylated H3 full-length product inhibitor was also not a viable option, as Rtt109-Vps75 is able to acetylate numerous lysines, albeit at slower rates. Next, we sought to directly measure binding affinities of AcCoA and CoA to the Rtt109-Vps75 complex.

AcCoA and CoA binding to Rtt109-Vps75

To provide direct evidence for AcCoA and CoA binding to the free Rtt109-Vps75 complex, isothermal titration calorimetry (ITC) was utilized. In each experiment, AcCoA or CoA was titrated into Rtt109-Vps75 and heats of binding were measured (Figure 3A and 3B). The binding isotherms revealed saturable binding consistent with a one-site model where AcCoA bound to the Rtt109-Vps75 complex with a K_d value of $1.4 \pm 0.3 \mu\text{M}$, while CoA yielded a K_d value of $43 \pm 5 \mu\text{M}$ (Figure 3 and Supplemental Table 1). These values are in good agreement with the dissociation constants determined from fluorescence measurements (22). Additionally, the K_d value for CoA is similar to the inhibition constant obtained for CoA ($K_{is} = 110 \pm 20 \mu\text{M}$). The decreased binding affinity of CoA to Rtt109-Vps75 compared to that of AcCoA reveals the contribution of the acetyl group to overall binding.

Binding of CoA to Rtt109-Vps75 in the presence of H3 substrate

If H3 bound first and promoted AcCoA binding to Rtt109-Vps75, one would predict an increase in affinity for AcCoA in the presence of an H3 substrate. Because the presence of AcCoA and H3 substrate would result in product turnover by Rtt109-Vps75 and interfere with equilibrium binding measurements, we performed ITC by titrating CoA into Rtt109-Vps75 in the presence of saturating concentrations of H3 peptide (577 μM), leading to the formation of a dead-end substrate-product complex. The presence of H3 peptide did not enhance the binding of CoA to Rtt109-Vps75 ($K_d = 44 \pm 3 \mu\text{M}$ compared to $43 \pm 5 \mu\text{M}$ without peptide) (Supplemental Table 2 and Supplemental Figure 1). Together, the product inhibition and binding studies provide support for a kinetic mechanism whereby AcCoA can bind prior to H3 substrate, and the presence of H3 peptide does not alter the binding affinity of AcCoA. It should be noted that H3 peptide yields a K_m value that is 2-orders of magnitude higher than that for full-length H3 and 3-orders of a magnitude higher than that observed with AcCoA. Therefore, when H3 peptide is the substrate, AcCoA binding may precede H3 peptide binding.

Analysis of H3 substrates binding to Rtt109-Vps75

Given that Rtt109 forms a tight complex with Vps75 and that the histone chaperone is capable of binding H3, we determined whether Rtt109-Vps75 is able to bind H3 in the absence of AcCoA. First, H3 peptide was titrated into Rtt109-Vps75 and heats of binding were monitored by ITC (Supplemental Figure 2). These binding measurements yielded a K_d value of $103 \pm 7 \mu\text{M}$, which is in agreement with the K_m value of $112 \pm 11 \mu\text{M}$ for H3 peptide (Supplemental Table 1 and Table 1). We were unable to perform direct binding studies of H3 protein to Rtt109-Vps75 because protein precipitation occurred at concentrations necessary for ITC measurements. To provide further support for the ability of H3 substrate to bind Rtt109-Vps75 prior to AcCoA binding, we performed a binding analysis utilizing gel-filtration, a technique that allows for the resolution of protein complexes on the basis of relative size/shape. Preparations of Rtt109-Vps75 alone, H3-H4 alone, and a mixture containing Rtt109-Vps75 (in stoichiometric excess) with H3-H4 were resolved on a gel filtration column (Superdex 200 10/300 GL from GE). We attempted to analyze full-length H3 and Rtt109-Vps75 mixtures, but were unable to combine these proteins in sufficient quantities without resulting in protein precipitation. As shown by SDS-PAGE analysis of fractions eluted from the gel filtration column, Rtt109-Vps75 eluted at $\sim 13 \text{ mL}$ and H3-H4 eluted at $\sim 15 \text{ mL}$ (Figure 4). Preparations of H3-H4 and Rtt109-Vps75 co-elute at $\sim 11 \text{ mL}$ as expected for the formation of a larger complex than either H3-H4 or Rtt109-Vps75 alone (Figure 4). Because Rtt109-Vps75 is able to form a stable complex with H3 substrates in the absence of AcCoA, substrate binding is likely random, or at the very least not strictly ordered.

Rapid-quench kinetics

Rapid-quench kinetic analysis was used to determine whether substrate binding, chemical catalysis or product release is the rate-limiting step. This rapid kinetic approach permits millisecond quenching of the reaction, and allows one to measure the rate of product formation during the first enzyme turnover. If a burst of product (acetylated H3) is formed followed by a slower steady-state rate of product formation, then product release is likely rate-limiting in turnover. Alternatively, the presence of a lag phase early in the reaction can be indicative of substrate binding (i.e. substrate-induced conformational change) as rate-limiting. Lastly, linear product formation is suggestive of chemical catalysis as a slow step in the kinetic cycle. To distinguish the kinetic behavior for Rtt109-Vps75 during the early phase of the reaction (millisecond range), we measured the amount of product formed with excess (multiple-turnover) concentrations of AcCoA and H3 protein substrate. Specifically, Rtt109-Vps75, pre-incubated with AcCoA, was rapidly reacted with H3 substrate, and the

reaction was quenched with 1% TFA at varied times between 60 ms to 1 second. The amount of acetylated product was then quantified via a filter binding HAT assay. The amount of product formed over time produced a linear curve yielding a rate of $0.53 \pm 0.02 \text{ s}^{-1}$, which is in good agreement with the steady-state k_{cat} value of $0.62 \pm 0.03 \text{ s}^{-1}$ (Figure 5A and Table 1). A burst or lag was undetectable, providing compelling evidence that the chemical step of acetyl transfer from AcCoA to H3 is the rate-limiting step (Figure 5A).

To provide additional support for rate-limiting acetyl-transfer, single turnover experiments were performed in which sub-stoichiometric AcCoA was pre-incubated with Rtt109-Vps75 and reacted with saturating amounts of H3 protein (final conditions of $1 \mu\text{M}$ Rtt109-Vps75, $0.4 \mu\text{M}$ AcCoA, and $30 \mu\text{M}$ H3). The amount of acetylated product formed over time was fitted to a first-order exponential equation (equation 10) where $k = 0.59 \pm 0.05 \text{ s}^{-1}$, which is in excellent agreement with the value of $0.53 \pm 0.02 \text{ s}^{-1}$ determined from the rapid-quench experiments under multiple turnover conditions (Figure 5B). Together, these data indicate that chemical catalysis is rate-limiting for the overall Rtt109-Vps75 catalyzed reaction.

pH profile analysis

To identify ionizations that are critical for catalysis, a k_{cat} pH profile analysis of Rtt109 ($0.2 \mu\text{M}$) with Vps75 ($0.8 \mu\text{M}$) was performed. To monitor the pH dependence of k_{cat} , initial rates of product formation were measured under saturating concentrations of AcCoA and H3 peptide and at varied pH (7-10). The observed rates increased with increasing pH until a plateau of activity was reached above pH 8.5 (Figure 6A). The data were fitted to a curve that described a single critical ionization that must be unprotonated for activity (equation 11), yielding a $\text{p}K_a$ value of 8.5 ± 0.1 and a pH independent k_{cat} value of $0.53 \pm 0.05 \text{ s}^{-1}$. To verify that the ionization with a $\text{p}K_a$ of 8.5 was not due to an ionizable residue that caused dissociation of Rtt109 and Vps75 at low pH, the ability of increased levels of Vps75 to activate Rtt109 ($0.2 \mu\text{M}$) was evaluated at pH 7 and pH 8. The addition of 6.25-fold extra Vps75 ($5 \mu\text{M}$ compared to $0.8 \mu\text{M}$) did not increase initial rates of product formation at pH 7 or pH 8 (Figure 6B). The lack of rescue of Rtt109-Vps75 activity by adding additional Vps75 suggests the critical ionization observed in the pH profile is not due to an ionizable group that affects association of the Rtt109-Vps75 complex. Thus, the pH rate analysis indicates a group with a $\text{p}K_a$ of 8.5 must be unprotonated for catalysis.

Analysis of Rtt109 D287 and D288 variants

Previously, kinetic analysis of several HATs such as GCN5, PCAF and Esa1 indicated the involvement of a general base (glutamate or aspartate) that facilitates the deprotonation of the ϵ -amino group of the lysine substrate, which is required for the nucleophilic attack of bound AcCoA (35,36,39). In Esa1, mutation of the general base catalyst Glu-338 (E338Q) resulted in an upward shift of the $\text{p}K_a$ (1.4 pH-units) of an ionizable group (35). Although the E338Q enzyme displayed severe catalytic impairment at neutral and low pH values, at pH values >9 , the E338Q variant is nearly as efficient as the wildtype enzyme. It was concluded that E338 acts to facilitate the removal of a proton from lysine, and this function is dispensable at high pH values where the ϵ -amino group is largely unprotonated. The crystal structure of Rtt109 ($\Delta 130$ -179) did not reveal obvious ionizable groups in close proximity to the acetyl-group of bound AcCoA(22). However, D287 and D288 are conserved amino-acid residues that reside in the active site, ~ 8 -11 Å from AcCoA, and could potentially have roles in catalysis (Figure 7)(22).

To dissect the importance of these residues, single glutamine variants (D288N and D288N) and a double alanine variant of Rtt109 (D287A/D288A) were generated, and steady-state kinetic analyses were performed in the presence of Vps75. H3 peptide saturation curves showed an ~ 50 fold loss of activity for the D288N Rtt109 variant ($k_{\text{cat}} = 2.1 \pm 0.1 \times 10^{-3}$

s^{-1}) compared to wildtype enzyme ($k_{cat} = 1.1 \pm 0.1 \times 10^{-1} s^{-1}$) (Table 1). The D287N variant ($k_{cat} = 8.7 \pm 0.4 \times 10^{-3} s^{-1}$) was less affected with an ~ 10 fold decrease in activity (Table 1). Minimal alteration was noted for the peptide K_m values for these variants ($K_m = 62 \pm 8 \mu M$ for Rtt109 D287N and $K_m = 55 \pm 11 \mu M$ for Rtt109 D288N compared to $K_m = 112 \pm 11 \mu M$ for wildtype Rtt109, Table 1). A ~ 16 -fold increase in K_m for AcCoA was observed for the D288N mutant ($K_m = 4.9 \pm 0.9 \mu M$) compared to wild type enzyme ($K_m = 0.3 \pm 0.1 \mu M$) and strikingly, the k_{cat}/K_m decreased by ~ 3 -orders of magnitude ($k_{cat}/K_m = 3.9 \pm 0.5 \times 10^5 M^{-1}s^{-1}$ for wild type versus $6 \pm 1 \times 10^2 M^{-1}s^{-1}$ for D288N). Little change in K_m AcCoA was observed for D287N ($K_m = 0.7 \pm 0.3 \mu M$). To investigate if the remaining aspartic acid could compensate for the mutated residue in the single amino-acid variants, a double mutant (D287A/D288A) was analyzed. The D287A/D288A variant yielded an ~ 100 -fold k_{cat} decrease ($k_{cat} = 1.1 \pm 0.1 \times 10^{-3} s^{-1}$) compared to wildtype enzyme. Taken together, these results argue against the ability for D287 or D288 to compensate in catalysis (Table 1). Moreover, the D288 substitution appears to account for most of the activity loss of the D287A/D288A variant (Table 1). Similar to the single-site variants, the D287A/D288A variant displayed minimal change in the K_m for H3 peptide ($K_m = 44 \pm 7 \mu M$, Table 1). To determine if D287 or D288 in Rtt109-Vps75 act as a base-catalyst, we monitored the pH dependence of k_{cat} with the D287A/D288A variant, revealing a slight lowering of the pK_a (7.5 ± 0.1) compared to wildtype complex (Fig 6A). This is in contrast to other HAT enzymes that utilize general-base catalysis and display an increase in the pK_a of an ionizable group with mutation of the general base. These data argue against a role for D287 or D288 in general base catalysis.

Discussion

Rtt109 is a novel HAT (KAT) that lacks sequence similarity to other well-characterized CoA-dependent acetyltransferases and requires association with a histone chaperone for efficient catalysis. In this study, we sought to understand the catalytic mechanism for this unique high affinity histone chaperone-HAT complex and performed detailed kinetic and binding analyses that support a random sequential (direct-attack) mechanism (Figure 8). We propose that AcCoA and H3 (or H3-H4) bind to the Rtt109-Vps75 complex without obligate order, forming a ternary complex (enzyme•AcCoA•H3), whereby the ϵ -amino group of lysine directly attacks the acetyl group of bound AcCoA (Figure 8). After acetyl-group transfer to the substrate lysine, acetylated H3 and CoA are released either in ordered or random fashion (Figure 8). Using rapid-quenching techniques, we provided evidence consistent with chemical catalysis as the rate-limiting step in turnover. From pH-rate analyses, we propose that the ionizable group which must be unprotonated for catalysis reflects the lysine ϵ -amine of bound substrate.

Several lines of evidence support the conclusion in which Vps75 and Rtt109 form a reversible, high-affinity interaction that represents the catalytically maximized complex. First, direct binding measurements yielded nanomolar K_d values (~ 10 nM) of the complex. Furthermore, similar k_{cat} values were attained whether micromolar (i.e. RQF) or sub-micromolar proteins concentrations were utilized (i.e. steady-state analysis), consistent with low nanomolar K_d values and a Rtt109-Vps75 complex that was maximally activated. We also observed that co-expression of Rtt109-Vps75, followed by resolution over nickel-affinity, anion exchange and gel filtration maintained the complex, and that only a strong hydrophobic interaction column (phenyl-sepharose) could disrupt their interaction. After separating Rtt109 and Vps75 by hydrophobic interaction chromatography, recombining the two proteins produced fully-activated complex, demonstrating the reversibility of binding. Consistent with a high affinity complex, endogenous Rtt109 co-purifies with Vps75 in yeast (5,27,40,41). Previously, two other labs reported a lower affinity interaction between Vps75 and Rtt109 ($K_d = 2.7$ to $\sim 5 \mu M$) (20,42). While it is unclear what this low affinity interaction

represents, here, we observed no significant changes in catalytic efficiency at higher protein concentrations ($\sim 1 \mu\text{M}$), suggesting that this binding event does not markedly alter catalysis. Alternatively, this discrepancy may be the result of different methods, protein constructs or binding conditions used by others. For example, we find that Rtt109 alone is an unstable protein and is highly prone to aggregation, and inclusion of AcCoA during the binding assays stabilized Rtt109 and prevented aggregation.

Unlike other HAT enzymes that require obligate-ordered substrate binding (AcCoA followed by histone substrates) (25,36,37), a unique feature of Rtt109-Vps75 is the random binding of substrates (Fig 8). In such a mechanism, it is predicted that one substrate should be able to bind independently of the other, and the binding of substrate will not significantly enhance the binding of the second. From product inhibition studies, CoA exhibited competitive inhibition against AcCoA, indicating AcCoA and CoA can bind to the same enzyme form, yielding an Rtt109-Vps75•AcCoA or Rtt109-Vps75•CoA complex (Fig 8). Equilibrium binding studies revealed both AcCoA and CoA bind to Rtt109-Vps75, and no increase in binding affinity is observed when CoA binding is measured in the presence of H3 peptide. Additionally, H3-H4 formed a tight-binding complex with Rtt109-Vps75 in the absence of AcCoA, as demonstrated by gel filtration analysis. Also consistent with a random substrate-binding model, the H3 peptide is able to bind Rtt109-Vps75 in the absence of AcCoA. Interestingly, p300, the likely mammalian structural ortholog of Rtt109, requires binding of AcCoA prior to binding histone substrates, revealing that although Rtt109 and p300 share roles in acetylating lysine 56 of H3, their kinetic mechanisms differ (25,43). Other key differences between Rtt109 and p300 are highlighted by the fact that p300 has efficient HAT activity in the absence of histone chaperones and likely displays wider substrate specificity, including both histone and non-histone substrates(43,44).

The pre-steady state kinetic analysis provides strong support for acetyl group transfer as the rate-limiting step in catalysis at pH 7.5. The observed rates in both multiple- and single-turnover experiments are in agreement with the steady-state k_{cat} value. The lack of a discernable lag or burst phase during multiple-turnover conditions argues against substrate binding or product release as rate-limiting steps in catalysis. Given that the k_{cat} reflects the chemical step of catalysis, the effect of pH on the k_{cat} reveals information on how chemical catalysis is accomplished by the Rtt109-Vps75 complex.

The pH k_{cat} analysis of Rtt109-Vps75 revealed an ionizable group ($pK_a=8.5$) that must be unprotonated for efficient catalysis. Due to the proximity of two conserved aspartic acid residues (D287, D288) near the putative active site, we investigated whether these residues have a role in deprotonating the ϵ -amino group of lysine (Figure 7) (22-24). The mutational analysis revealed there were substantial effects on catalysis, which were reflected by the large ~ 50 -100-fold decrease in the k_{cat} of the D288N and D287A/D288A variants. However, pH k_{cat} analysis of D287A/D288A did not eliminate nor cause an upward shift in the pK_a value as would be expected for the loss of a general base catalyst and the unassisted deprotonation of the lysine. In fact, the apparent pK_a value for the D287A/D288A mutant was shifted downward by ~ 1 pH unit. These results are in contrast to other previously characterized HATs that utilize a general base mechanism, where the pK_a value of ~ 8 represents the hydrogen-bonded ion pair between the N- ϵ -lysine and the general base glutamate/aspartate (35,36,39).

Since D287 or D288 are not likely responsible for the ionization in the k_{cat} pH profiles, we propose that the pH-dependent ionization reflects the enzyme-bound lysine of H3 substrate, which is perturbed when bound in the hydrophobic active site. The crystal structures of Rtt109 reveal a hydrophobic active site that creates an environment favoring the neutralized, unprotonated state of the substrate lysine (Figure 7). Residues that line the hydrophobic

“tunnel” include F84, V85, and the aliphatic side chain of K87 on β sheet 4, as well as L191, F192, and the aliphatic side chain of R194 on β sheet 5 of Rtt109 (Figure 7). Rtt109 may increase the nucleophilicity of the substrate lysine by perturbing the pK_a of lysine downwards to ~ 8.5 within the hydrophobic tunnel of the enzyme•AcCoA•H3 complex. Perturbation of the pK_a values of ionizable groups arise from the energetically unfavorable process of transferring a charged group from a high dielectric constant environment (i.e. water) into a less polar environment, such as the burial of the charged group into the interior of a protein environment. For example, a V66K mutant in staphylococcal nuclease displayed a pK_a value of 10.2 in the denatured state compared to the pK_a value of 6.4 in the native state (45,46). This downward shift of pK_a is suggested to favor the uncharged form of lysine 66 within the context of the hydrophobic interior of the folded protein. Interestingly, p300, the closest structural homolog of Rtt109 in mammals also appears to lack a putative general base (25). Like Rtt109, catalysis by p300 revealed the requirement of an ionizable group ($pK_a = 8.4$) and the x-ray structure revealed a very hydrophobic active site(25).

Our steady-state analysis revealed a critical role for aspartate 288 of Rtt109, as mutation resulted in both k_{cat}/K_m and k_{cat} defects. An ~ 16 -fold increase in K_m for AcCoA and a ~ 50 fold decrease in k_{cat} was observed for the D288N mutant compared to wild type enzyme. Thus, the k_{cat}/K_m for AcCoA was decreased by ~ 3 -orders of magnitude. This large k_{cat}/K_m effect may reflect a role for D288 in AcCoA binding. Surprisingly, D288 of Rtt109 lies ~ 8 Å from AcCoA in the active site, suggesting that direct interaction between AcCoA and D288 is unlikely(22-24).

One potential role for D288 may be linked to its interaction with acetylated K290 of Rtt109. As shown by mass spectrometric analysis, previous studies demonstrated that K290 is nearly completely acetylated in both affinity purified TAP-tagged Rtt109 from yeast, and Rtt109 (Rtt109 $\Delta 130$ -179) expressed in bacteria (Fig 7) (22). Three independently solved crystal structures of Rtt109 revealed electron density at lysine 290 consistent with an acetyl group, while genetic analysis indicated that mutation of this residue produced defective Rtt109 in vivo (22-24). Additionally, wildtype Rtt109 used in our analyses is fully acetylated at K290, while the D287A/D288A mutant is mostly unacetylated at K290 (BNA and JMD unpublished data). Together, these studies suggest a role for D288 in autoacetylation. The structures of Rtt109 show D288 and K290 residing on α -helix 6, forming a “lid” domain that interacts with the hydrophobic core domain of Rtt109, including β -sheet 5 which partially houses the acetyl group of AcCoA (Figure 7) (22-24). The interaction of α -helix 6 with the core domain results in the burial of acetylated K290 within the hydrophobic core and formation of a hydrogen bond interaction between D288 and acetylated K290 (22-24). D288 might properly position acetylated K290 or facilitate the chemistry of autoacetylation (i.e. general base), whereupon mutation of this residue prevents K290 acetylation. An unfavorable interaction of positively charged K290 with the core interior could perturb a local rearrangement of the acetyl binding pocket of AcCoA, as reflected by the large k_{cat}/K_m defect. Interestingly, Rtt109-Vps75 displays a 30-fold higher binding affinity for AcCoA over CoA, indicating that the acetyl group significantly contributes to overall AcCoA binding, and this acetyl-specific interaction might be disrupted in the D288N Rtt109 mutant. A multi-faceted layer of regulation for Rtt109 exists, governed by interactions with histone chaperones and autoacetylation of K290. Current efforts are directed at providing a detailed understanding of the functional role of K290 autoacetylation and the involvement of D288 in this modification, as well as the potentially distinct mechanisms of activation mediated by histone chaperones Asf1 and Vps75.

Supplementary Material

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Acknowledgments

This work was supported by NIH Grant GM059785 (to JMD) and a postdoctoral fellowship (to EMK) from the American Heart Association (0920041G). We thank all members in the lab of John Denu for helpful discussions.

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Abbreviations used in this work

HAT	Histone Acetyltransferase
DTT	dithiothreitol
Rtt109 and Rtt106	Regulator of Ty1 Transposition 109 and 106
CAF-1	Chromatin Assembly Factor 1
Vps75	Vacuolar Protein Sorting 75
Asf1	Anti-Silencing Function 1
Hst3 and Hst 4	Homolog of SIR Two 3 and 4
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Esal	essential Sas2-related acetyltransferase 1
PCAF	p300/CBP associated factor
LC-MS	liquid chromatography mass spectrometry
AcCoA	acetyl-Coenzyme A
CoA	Coenzyme A

FIGURE 1. Binding of Rtt109 to Vps75

Fluorescein labeled Vps75_{dimer} (3.2 nM) was titrated with 3.7-190 nM of Rtt109 in PBS containing 0.2 mM AcCoA and 0.1 mg/mL BSA at 25°C and anisotropy measured by fluorescence polarization. Assays were performed 7 times in replicate and a representative figure shown. Data were fitted to equation 2, yielding a K_d value of 10 ± 2 nM assuming Vps75 exists as a non-dissociating dimer.

FIGURE 2. Bisubstrate kinetic analysis and CoA product inhibition of Rtt109-Vps75

Experiments were performed two or three times in with a representative figure shown. The average of a representative duplicate dataset is displayed in double reciprocal format. Bisubstrate reactions (figures A and B) were comprised of 25 mM Tris (pH 7.5 at 25°C), 1 mM DTT, 0.1 μM Rtt109, 0.8 μM Vps75. Initial rates were monitored by the pyruvate dehydrogenase assay and data fitted to equation 3 (see Table 1 for kinetic constants). CoA product inhibition reactions (figure C) consisted of 50 mM Tris (pH 7.5) at 25°C, 1 mM DTT, 20 nM Rtt109, 0.8 μM Vps75 utilizing the filter binding assay and data fitted to equation 6, yielding K_{is} value of 110 ± 20 . A: Varied [AcCoA] at fixed, varied [H3 peptide]. Shown [H3 peptide]: 23 μM (circles), 38 μM (squares), 58 μM (diamonds), 96 μM

(triangles), and 262 μM (inverted triangles). B: Varied [H3 peptide] at fixed, varied [AcCoA]. Shown [AcCoA]: 0.25 μM (circles), 0.45 μM (squares), 0.9 μM (diamonds), and 2 μM (triangles). C: Varied [AcCoA], at fixed, [CoA] and fixed [H3] (5 μM); 0 μM (circles), 250 μM (squares), and 600 μM (diamonds).

FIGURE 3. ITC profile of Rtt109-Vps75 with AcCoA and CoA

A: AcCoA was titrated into Rtt109-Vps75 complex. Top Graph: Data obtained for 37 automatic injections (1-8 μ L) of AcCoA into co-purified Rtt109-Vps75. Bottom Graph: Integrated curve showing experimental points (squares) and fitted to a one-site binding curve (line). 167 μ M of AcCoA was injected into 5.8 μ M of Rtt109-Vps75, yielding a K_d value of 1.4 ± 0.3 μ M. Buffer conditions: 50 mM Tris (pH 7.5 at 25 $^{\circ}$ C), 5% glycerol (w/v), 50 mM NaCl, 2 mM β -mercaptoethanol, and 1mM EDTA. B: CoA was titrated into the Rtt109-Vps75 complex, yielding a K_d value of 43 ± 5 μ M. 2 mM of CoA was injected into 50 μ M of co-purified Rtt109-Vps75. Buffer conditions are same as in A.

FIGURE 4. Gel-filtration analysis of Rtt109-Vps75 and H3-H4

Shown are elution profiles of protein complexes from gel-filtration chromatography. Samples from elution fractions were analyzed by SDS-PAGE followed by Ruby-Sypro staining of 12% SDS-PAGE gels. mL = approximate volume of protein elution from column chromatography. A: SDS-PAGE gel of co-purified Rtt109-Vps75 (75 μ M) elution profile. B: SDS-PAGE gel of H3-H4 (50 μ M) elution profile. C: SDS-PAGE gel of the elution profile for a mixed sample of co-purified Rtt109-Vps75 (75 μ M) and H3-H4 (50 μ M).

FIGURE 5. Rapid quench kinetics of Rtt109-Vps75

A: Reactions were performed in 50 mM Tris-Cl (pH 7.5), 200 mM NaCl, and 1 mM DTT. Using a Hi-Tech quench flow device, co-purified Rtt109-Vps75 (1 μM) and AcCoA (30 μM) were rapidly mixed with H3 (30 μM) (concentrations given after mixing). Reactions were quenched with 1% TFA after 60 ms to 10 s. Data were fitted to a linear regression equation to determine turnover rate. Error bars represent standard deviation. Experiments were performed twice in duplicate, yielding a rate of $0.53 \pm 0.02 \text{ s}^{-1}$. B: Reaction conditions were similar to multiple turnover RQF experiments with AcCoA (0.4 μM) limiting enzyme turnover. Reactions were quenched from 120 ms to 50 s. Experiments were performed twice in duplicate and fit to equation 10, yielding a rate of $0.59 \pm 0.05 \text{ s}^{-1}$.

FIGURE 6. k_{cat} pH analysis of wildtype and D287A/D288A Rtt109-Vps75 complexes

A: Profiles of the pH dependence of k_{cat} . Reactions conditions consisted of 50 mM ACES, 25 mM Tris, and 25 mM ethanolamine buffer (ATE buffer) at 25 °C, 1 mM DTT, and saturating AcCoA and H3 peptide concentrations. Initial rates of product formation were measured by a filter-binding HAT assay. Experiments were performed twice in duplicate and data fit to equation 11. For wildtype enzyme (circles), 0.2 μM Rtt109 and 0.8 μM Vps75 were utilized, yielding a pK_a value of 8.5 ± 0.1 . For experiments with Rtt109 D287A/D288A (triangles), 2 μM Rtt109 D287A/D288A and 4 μM Vps75 were utilized, yielding a pK_a value of 7.5 ± 0.1 . B: Conditions are similar as in A. Vps75 (0.8 or 5 μM) was added to Rtt109 (0.2 μM) at pH 7 or pH 8 to determine whether increasing [Vps75] increases the rate of acetylation of H3 peptide by Rtt109. Experiments performed 3 times in duplicate, with error bars representing standard deviation.

FIGURE 7. Putative lysine binding site of Rtt109 bound to AcCoA

Ribbon model of Rtt109 overall structure and zoomed-in view of the active site (PDB code 3D35). AcCoA (orange), residues of hydrophobic pocket (blue), D287 (red), D288 (red) and K290 (green) are shown as stick models. α -helix 6, β -sheet 4 and β sheet 5 are noted.

FIGURE 8. Proposed kinetic mechanism of acetylation of H3 substrate by Rtt109-Vps75 complex

A: The Cleland diagram of the kinetic mechanism is shown. RV = Rtt109-Vps75 complex. H3 = H3 substrate. B: Chemistry of catalysis. Lysine of H3 attacks AcCoA with acetyl-transfer following.

Scheme 1.

Table 1Summary of steady state kinetic constants for Rtt109-Vps75^a

Rtt109 Enzyme	Substrate (s ⁻¹)	<i>k</i> _{cat} (s ⁻¹)	<i>K</i> _m (μM)	<i>k</i> _{cat} / <i>K</i> _m (M ⁻¹ s ⁻¹)
Wildtype	AcCoA	0.11 ± 0.01	0.3 ± 0.1	3.9 ± 0.5 × 10 ⁵
	H3 peptide	0.11 ± 0.01	112 ± 11	9.8 ± 0.9 × 10 ²
	Full length H3 ^b	0.62 ± 0.03	7 ± 1	9 ± 1 × 10 ⁴
D287N	AcCoA	1.2 ± 0.1 × 10 ⁻²	0.7 ± 0.3	1.7 ± 0.7 × 10 ⁴
	H3 peptide	8.7 ± 0.4 × 10 ⁻³	62 ± 8	1.4 ± 0.2 × 10 ²
D288N	AcCoA	2.8 ± 0.2 × 10 ⁻³	4.9 ± 0.9	6 ± 1 × 10 ²
	H3 peptide	2.1 ± 0.1 × 10 ⁻³	55 ± 11	3.8 ± 0.8 × 10 ¹
D287A/D288A	H3 peptide	1.1 ± 0.1 × 10 ⁻³	44 ± 7	2.5 ± 0.4 × 10 ¹

^aStandard errors are reported. All assays were performed in the presence of H3 peptide with the exception of when H3 full-length was utilized as indicated.

^bFull-length H3 saturation curve was performed in 50 mM Tris, 1 mM DTT, 0.2 μM Rtt109, 1.6 μM Vps75, 50 AcCoA and 2-32 μM full-length H3.