Two factor authentication: Asf1 mediates crosstalk between H3 K14 and K56 acetylation

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ABSTRACT
The ability of histone chaperone Anti-silencing factor 1 (Asf1) to direct acetylation of lysine 56 of histone H3 (H3K56ac) represents an important regulatory step in genome replication and DNA repair. In Saccharomyces cerevisiae, Asf1 interacts functionally with a second chaperone, Vps75, and the lysine acetyltransferase (KAT) Rtt109. Both Asf1 and Vps75 can increase the specificity of histone acetylation by Rtt109, but neither alter selectivity. However, changes in acetylation selectivity have been observed in histones extracted from cells, which contain a plethora of post-translational modifications. In the present study, we use a series of singly acetylated histones to test the hypothesis that histone pre-acetylation and histone chaperones function together to drive preferential acetylation of H3K56. We show that pre-acetylated H3K14ac/H4 functions with Asf1 to drive specific acetylation of H3K56 by Rtt109–Vps75. Additionally, we identified an exosite containing an acidic patch in Asf1 and show that mutations to this region alter Asf1-mediated crosstalk that changes Rtt109–Vps75 selectivity. Our proposed mechanism suggests that Gcn5 acetylates H3K14, recruiting remodeler complexes, allowing for the Asf1-H3K14ac/H4 complex to be acetylated at H3K56 by Rtt109–Vps75. This mechanism explains the conflicting biochemical data and the genetic links between Rtt109, Vps75, Gcn5 and Asf1 in the acetylation of H3K56.

INTRODUCTION
Lysine acetylation is a reversible post-translational modification (PTM) that regulates eukaryotic transcription (1,2). The acetylation of histones alters the accessibility of chromatin and offers a platform for proteins to bind that provides a crucial step in transcription, replication and DNA repair (3,4). One of the critical sites of acetylation for DNA repair and cell cycle progression is lysine 56 of histone H3 (H3K56ac). The requirement for K56 to be modified is likely due to the location of this residue in the core of histone H3 at the nucleosome dyad, with acetylation of this site altering DNA accessibility (5–7).

In Saccharomyces cerevisiae, the KAT Rtt109 (KAT11) is responsible for acetylation of H3K56, as well as acetylation of H3-K9, K14, K23 and K27 (8,9). Functionally, Rtt109 requires two structurally distinct histone chaperones: Vps75 and Asf1 (3,10–13). The histone chaperone Vps75, a member of the Nap1 family, forms a stable and catalytically active complex with Rtt109 (11,14–17). The association of Rtt109 with Vps75 stabilizes the enzyme and increases the activity >100-fold. Moreover, the observation that in vivo both proteins are expressed in equimolar concentrations suggest a 1:1 complex (12,13,18). The second histone chaperone required for Rtt109 activity in yeast is Asf1, which binds to H3/H4 heterodimer, splitting the tetramer complex of (H3/H4)2 and effectively doubles the substrate concentration (9). Deletions of either Rtt109 or Asf1 in yeast cells eliminates H3K56 acetylation and impairs DNA damage repair (18,19).

The current understanding of Rtt109 KAT activity is a strong example of a major question in the field: what determines which residues on a histone get acetylated? The ability of a KAT to acetylate a specific residue relative to another is referred to as selectivity. Differences in specificity between two sites of acetylation are equal to selectivity, therefore if there is no difference in the specificity between two residues, then there won’t be any selectivity regardless of how high the specificity. One factor that has been reported to alter the selectivity of Rtt109 is Asf1, but this fact has been marked by several confusing observations: (i) In the absence of Asf1, Rtt109–Vps75 acetylates H3K9ac and H3K23ac. (ii) In vivo, the absence of Asf1 prevents H3K56...
acetylation. (iii) In vitro, addition of Atf1 to a reaction containing Rtt109 can promote selectivity for H3K56 but only when the histones used are extracted from eukaryotic cells possessing acetyl-lysine residue or other PTMs (3,9,11,13). (iv) In the absence of post-translationally modified histones, Atf1 increases the specificity of Rtt109–Vps75, but does not alter which residues are initially acetylated (H3K9 and H3K23) (9). Together, these data suggest that PTMs can alter the selectivity of Rtt109–Vps75. However, left unanswered is which PTMs are responsible for altering selectivity and how these modifications are being recognized?

In this work, we demonstrate that Atf1 recognizes multiple specific acetylation states of H3, with the information provided by specific modifications allowing Atf1 to direct the subsequent acetylation preference of Rtt109–Vps75. We specifically show that Atf1 can improve selectivity toward H3K56, but only when H3K14 is pre-acetylated. This provides a mechanistic framework to explain the genetic linkages observed between Atf1, Rtt109 and Gcn5, as Gcn5 is the acetyltransferase mainly responsible for H3K14ac in vivo (20–23). Thus, these data help reconcile both the discrepancies between multiple biochemical reports and the genetic links between Gcn5 and Rtt109 in H3K56ac.

MATERIALS AND METHODS

Reagents

All Chemicals were purchased from Sigma-Aldrich or Fisher and were either the highest commercial grade or LC/MS grade. Ultrapure water was generated from a Millipore Direct-Q 5 ultrapure water system.

Protein preparation and purification

All recombinant histones, including wild-type (WT), single lysine acetylation, uniformly 13C and 15N isotopic labeled H3 and WT H4, were purified and provided by the Protein Purification Core at Colorado State University. Histone H3/H4 tetramerization was done using previously published methods (24). Saccharomyces cerevisiae Rtt109–Vps75 and Atf1 proteins were used. Site-directed mutagenesis of Atf1 constructs were purchased from GeneWiz. Rtt109–Vps75 complex, WT Atf1 and Atf1 mutants were expressed and purified following previous described procedures (25,26). All purified protein concentrations were determined by UV absorbance and calculated from the extinction coefficients (27).

k_cat from steady-state kinetic assays for Rtt109–Vps75

Steady-state kinetic assays for all H3/H4 were performed under the identical buffer condition (100 mM ammonium bicarbonate and 50 mM HEPES buffer (pH 7.2)) and with saturating H3/H4 (10 μM), Atf1 (12 μM) and acetyl-CoA (300 μM) at 37°C. The assays contained 0.04–3 μM Rtt109–Vps75 (varied with different substrates and histone concentrations). Under conditions where Rtt109–Vps75 is in excess to Atf1, several of the lysines reached a 100% acetylation in 5 s under the original experimental conditions (pH 7.2 and 37°C). To avoid this, we used pH 6.0 and 15°C to slow down the acetylation rate, so that we would be able to differentiate the acetylation rates between sites. The initial rates (v) of acetylation were calculated from the linear increase in acetylation as a function of time prior to a total 10% acetylation. For individual lysines, the steady-state parameters k_cat were determined by v/[E], where [E] is the concentration of Rtt109–Vps75 and histone substrate is saturated. In our previous paper (28), we demonstrated that the ratio of the apparent k_cat is a valid parameter to compare the specificity for multiple substrates/sites in a multi-substrate system, and therefore for this study we utilized the k_cat to compare different specificities at each observed site of acetylation.

Acetylated single-lysine H3/H4 titrations (k_cat conditions)

Titrations of different acetylated H3/H4 were carried out under the same, aforementioned buffer condition in the steady-state kinetic assays. Experiments were conducted using saturated 13C,15N-labeled H3/H4 (10 μM) and acetyl-CoA (300 μM) at 37°C. The concentrations of singly acetylated H3/H4 were varied between 1 and 10 μM, and Rtt109–Vps75 concentrations were utilized from 0.3 to 0.45 μM, accordingly.

Excess enzyme assays

Assays were performed under single turnover conditions to investigate the most likely Rtt109–Vps75-mediated acetylation on H3 of Rtt109–Vps75-mediated acetylation. The experimental condition were 1 μM H3/H4 (or any pre-acetylated H3/H4), 300 μM acetyl-CoA and 10 μM Rtt109–Vps75 in 100 mM ammonium bicarbonate and 50 mM HEPES buffer (pH 6.0) at 37°C. Buffer pH was adjusted from 7.2 to 6.0 for slowing down the acetylation reactions when Atf1 was present. For all the Rtt109–Vps75 kinetics experiments, the following sample preparations, including reaction quench in ice-cold trichloroacetic acid, post-reaction propionylation using propionic anhydride and tryptic digestion, were performed as in our previously published procedures (29).

UPLC-MS/MS analysis

A Waters Acquity H-class UPLC coupled with a Thermo TSQ Quantum Access triple quadrupole mass spectrometer was used to quantify the acetylated lysines on H3 peptides. The UPLC and MS/MS settings, solvent gradient and detailed mass transitions were reported in our previously published work (30). Retention time and specific mass transitions were both used to identify individual acetylated and propionylated peaks. The resolved peak integration was done using Xcalibur software (version 2.1, Thermo). Relative quantitative analysis was used to determine the amount of acetylation on individual lysines.

Histone H3 acetylation with acid urea (AU) gel electrophoresis

A total of 30 μM H3/H4 (WT) or H3K14ac/H4, 300 μM acetyl-CoA, 0 or 36 μM Atf1 and 0 or 3 μM Rtt109–Vps75 were incubated at 37°C for 1 min. For each experiment, at
least ~0.2 μg of histones was loaded in each well of short AU gels. Short AU gels were prepared and run as previously described (31). Histone H3 in the gels was stained by Coomassie Brilliant Blue G-250.

Calculating the free energy of specificity and selectivity

We have previously shown that in a system where one substrate results in multiple products the ratio of $k_{cat}$ between products is equal to the ratio of $k_{cat}/K_m$ (9,28,30). This allows us to easily compare changes to specificity and selectivity by converting the ratio of $k_{cat}$ to a free energy

$$\Delta \Delta G^\circ_R = -RT \ln \left( \frac{k_{cat}}{k_{cat}^{200\mu M}} \right)$$

($\Delta \Delta G^\circ_R$) difference. We can also compare the free energy differences between $k_{cat}$ and the non-enzymatic rate ($k_{cat}$) at the concentration of acetyl-CoA that is saturating for Rtt109 (200 μM) or $k_{cat}$ 200 μM (9). There are residues that can be shown to be acetylated at longer times but not observed under steady-state conditions, where we discard all data above the time point where the total substrate (non-acetylated histone(s)) has decreased by 10% of the initial concentration. For the residues that aren’t observed under steady-state conditions, we can only set the upper limit of the rate of acetylation. We set this limit by finding the fastest rate that will not produce product (<0.005%) under saturating conditions before the faster rates have consumed more than 10% of the substrate. In most cases this rate is 2-3 orders of magnitude slower than the fastest or combination.

Fluorescence spectroscopy to determine H3/H4-Asf1 binding affinities

Fluorescence experiments were performed as previously described (32). Briefly, Asf1 was labeled with Alexa Fluor 546 by incubating Asf1 with 10× excess label overnight at 4°C in 10 mM Tris–HCl pH 7.5, 0.5 M NaCl, 0.5 mM TCEP, 10% glycerol, 0.05% Brij. Excess label was removed through dialysis. Fluorescent measurements were performed at 20°C using a PTI Quantmaster Fluorometer (Excitation 556/Emission 570, slit width 4 nm). Labeled Asf1 (1 nM) was added to both the sample and reference cuvette while unlabeled Xenopus H3-H4 was titrated into the sample cuvette and buffer was titrated into the reference cuvette. Each time point was allowed to come to equilibrium (incubation times varied from 3–10 min to ensure equilibrium was reached). The normalized change in fluorescence was determined using the following equation:

$$\text{Norm. Fraction Bound} = \frac{R_{obs} - R_{initial}}{R_{final} - R_{initial}}$$

Where $R_{obs}$ is the observed ratio of the fluorescent signal from the sample and reference (sample cuvette/reference cuvette), $R_{initial}$ and $R_{final}$ are the ratios of the initial (Asf1 only) and final (saturation reached) fluorescent signals. The data were analyzed with GraphPad Prism (version 8.01) using non-linear regression.

RESULTS

Asf1 promotes acetylation of H3K56ac only after an initial acetylation event

To investigate the role of Asf1 in the regulation of H3K56 acetylation we employed a multiplexed label-free high-throughput mass spectrometry assay (29). This approach allows simultaneous measurement of histone acetylation on each lysine found within H3/H4. Selectivity is typically measured by the ratio of $k_{cat}/K_m$ (termed the specificity constant), for two different products or substrates (33). However, under conditions in which one substrate (here, a histone complex) is converted into multiple products (different acetylation states), and the initial rates of all products are measured simultaneously, the ratio of the observed $k_{cat}$ for two residues is equal to the ratio of $k_{cat}/K_m$ (s) observed (28). Based on this approach, our initial experiments using recombinant histones surprisingly suggested that Asf1 did not promote acetylation of H3K56 or any change in selectivity when using H3/H4 (unmodified) under steady-state conditions (9).

The limitation to steady-state kinetic analysis is that results are limited to those observed with <10% of total substrate turnover, and therefore such an assay provides data only on the initial acetylation event. If H3K56 acetylation was dependent on the acetylation of other residues, we would not observe it under these conditions. Thus, we hypothesized that once one residue is acetylated it will alter which residue is acetylated next. To test this, we used enzyme concentrations in excess of H3/H4 ([E]>[S]) in the presence and absence of Asf1 and allowed the reaction to proceed until acetylation plateaued. The time it took the reactions to plateau varied from ~3 to 90 min (Figure 1). Under these conditions, Rtt109 is capable of acetylating multiple residues on a single histone H3. The sum of the fraction acetylated for each residue approximates the number of acetylations per histone, and in the absence of Asf1 we observe ~2.2 acetylations per histone while we observe ~3.5 acetylations per histone in the presence of Asf1.

Consistent with our hypothesis that Asf1 functions with pre-acetylation to drive H3K56 acetylation, we observed a rapid increase in acetylation of H3K56 after other residues are acetylated when Asf1 is present. This is demonstrated by a lag in H3K56 acetylation as other lysine residues are acetylated (Figure 1A). Once initial acetylation is obtained, Asf1 tripled the amount of H3K56ac observed, taking this PTM from the lowest abundance to the highest and reduced the fraction of H3K9ac by more than 2-fold. We hypothesize that an exosite (a secondary binding site distinct from the active site) may exist within the Rtt109–Vps75/H3/H4-Asf1 complex that drives selectivity through recognition of pre-acetylated H3.

Pre-acetylation can alter the selectivity of Rtt109–Vps75

In order to identify which acetylation sites might be functioning with Asf1 to drive H3K56ac, we made a library of singly acetylated histones (H3K9ac, H3K14ac, H3K23ac, H3K27ac and H3K56ac) using a strain of Escherichia coli containing an orthogonal Nα-acetylsyl-tRNA synthetase/tRNA_CUA pair (34). This approach al-
lows us to make a histone with a specific site of acetylation. Each singly acetylated histone H3 was then refolded with histone H4 to form the histone tetramer \((\text{H3K}(x)\text{ac}/\text{H4})_2\). Using enzyme concentrations in excess of H3/H4 \([E] > [S]\) with each of the singly acetylated histone H3 in the presence and absence of Asf1 and allowed the reaction to proceed until acetylation plateaued, all possible additional acetylation sites can be observed (Figure 1B–F).

To determine which acetylated residues can alter the selectivity of Rtt109–Vps75, we performed steady-state \([E] < [S]\) kinetics to determine which, if any, of the singly acetylated histone complexes can alter residue selectivity (Figure 2A and Supplementary Table S1). H3K9 and K23 have the highest specificities \((k_{\text{cat}}\) values) for WT histones. When either one was pre-acetylated, it broadened the selectivity to other residues and with the addition of Asf1 the specificities increased (Figure 2A and Supplementary Table S1). Rtt109–Vps75 acetylates H3K14ac/H4 (H3/H4 at pre-acetylated at lysine 14 on the H3 tail) at H3K23 and K27, but this modification suppresses K9 acetylation. The largest change in selectivity came with the acetylation of Asf1-H3K14ac/H4 by Rtt109–Vps75, where selectivity was completely shifted to H3K56. Under conditions where Rtt109–Vps75 is in excess to Asf1-H3K14ac/H4, the changes are even more apparent, with H3K56ac being completely acetylated in <5 s, even when the pH (6.5) and temperature (15°C) were lowered (Figure 1C). In contrast, incubation of mono-acetylated H3K27 caused Rtt109–Vps75 to acetylate H3K23 and H3K56 (Figure 2A and Supplementary Table S1), but Asf1 had no impact on selectivity.

Among the monoacetylated substrates, pre-acetylated H3K56 produced the smallest turnover rate of additional lysine residues, and only modestly promoted Rtt109–Vps75 acetylation of H3K14 and H3K23 (Figure 2A and Supplementary Table S1). Overall, the apparent \(k_{\text{cat}}\) of H3K14ac and H3K56ac without Asf1 was \(\sim 2–3\) orders of magnitude less than the \(k_{\text{cat}}\) from other forms of H3/H4 (Figure 2A and Supplementary Table S1).

In addition to testing the singly acetylated histones in this assay, lysine (K) to glutamine (Q) mutations, which are often used to mimic acetyl-lysine (35,36), were also detected to determine whether these mutations would alter Rtt109–Vps75 selectivity. However, by comparing data from the pre-acetylated histones to data generated with H3K9Q and H3K14Q mutations, it is clear that these mutations do not effectively mimic lysine acetylation (Supplementary Figure S1 and Table S2).

From these data, we conclude that in the absence of Asf1, H3K27ac was the only residue capable of driving Rtt109–Vps75 acetylation of H3K56. However, each premonoacetylated histone drove distinct sequential acetylation patterns by this enzyme.

**Asf1-H3K14ac drives acetylation of H3K56ac by Rtt109–Vps75**

To confirm the observation that Rtt109–Vps75 only acetylates H3K56 in the presence of Asf1-H3K14ac/H4, we used
Figure 2. Analysis of Asf1-dependent changes to residue selectivity as a function of pre-acetylated states of histone H3. (A) Comparison of site-specific $k_{cat\,app}$ of H3/H4 (with and without singly acetylated mark) by Rtt109–Vps75 acetylation in the absence or presence of Asf1. The error bar represents the standard error in $k_{cat\,app}$. The apparent $k_{cat}$ are summarized in Supplementary Tables S1 and 2. (B) Free difference for residue acetylation as compared to non-enzymatic acetylation with and without Asf1. Changes in the apparent free energy of residue selectivity due to Asf1 with varying states of histone H3. (C) The apparent free energy changes to selectivity due to different acetylation states of histone H3 with and without Asf1. In these heat maps, the right side of the diagonal is the selectivity between residues in the absence of Asf1 and the left side is in the presence of Asf1, and the horizontal and vertical black bars represent the sites of pre-acetylation. If Asf1 has no impact on selectivity, then both sides of the diagonal will be mirror images of each other as is the case with no pre-acetylation and H3K27ac. All other changes represent changes in selectivity due to Asf1.

Comparing changes in specificity and selectivity due to pre-acetylation state and Asf1

In order to fully understand how Asf1 mediates crosstalk between acetylated residues to influence specificity and selectivity, we applied a recently developed method to analyze data that compensated for the fact that many of the acetylated residues are acetylated at a rate too slow to be observed before 10% of the substrate has been consumed. By comparing the fastest possible rates of acetylation of an individual lysine residue to the rate of acetylation under the same assay conditions, but in the absence of enzyme ($k_{cat\,E200}/H9262M$) (9), we can calculate the free energy for each residue that can be acetylated (Figure 2; Supplementary Table S1 and 3). By this method, the free energy difference ($\Delta G^\circ_R$) between the acetylation rate containing enzyme and the non-enzymatic acetylation rate was determined. In the absence of Asf1 these values ranged from 4.2 to $−3.7$ kcal mol$^{−1}$, and with Asf1, the values ranged from $−0.6$ to $−5.4$ kcal mol$^{−1}$ (Figure 2B). These values represent the tendency of the enzyme to target a site (specificity) and their difference can be used to represent their relative selectivity.

The $\Delta G^\circ_R$ values in the absence of Asf1 are much less favorable than in the presence of Asf1 and many are even less favorable than non-enzymatic acetylation (a positive value). While Asf1 increased the favorability of acety-
An Asf1 acidic patch functions as an exosite and interacts with the tail of H3 to mediate acetylation

Based on these data, Asf1 interacts with specific lysine residues on pre-acetylated H3 to facilitate a broad range of acetylation patterns induced by Rtt109–Vps75, but the mechanism of these interactions remains unclear. We hypothesized that an exosite (a secondary binding site distinct from the active site) may exist within the Rtt109–Vps75–H3/H4-Asf1 complex to drive selectivity. Electrostatic interactions are essential for many protein–protein and protein–ligand interactions. Structural analysis of the Asf1 histone complex using APBS Electrostatics plugin PyMol 2.0 \((37,38)\) revealed an acidic patch on Asf1 (Figure 3A). We hypothesized that interactions between this negatively charged region of Asf1 and the positively charged tail of H3 may be crucial for Asf1 to mediate Rtt109–Vps75 selectivity. This interaction is likely altered by acetylation of H3 and would therefore affect the interaction between Asf1 and the H3 tail.

To further investigate how Asf1 and pre-acetylated H3K14ac/H4 co-mediate H3K56 acetylation by Rtt109–Vps75, point mutations were made individually to charged residues (aspartic acid or glutamic acid) within the acidic patch \((D37, E39, D54, D58, E88 and E105)\). These residues were either converted to alanine to eliminate the charge, or to arginine to reverse the charge (Figure 3B). Steady state kinetic measurements, using methods described above, were performed with each of the Asf1 mutants with either H3/H4 or H3K14ac/H4. Most Asf1 mutations altered the selectivity of Rtt109–Vps75 with H3/H4 as substrate, and lead to broad acetylation at four lysine residues \((H3-K9, K14, K23 and K27 or K56)\) (Figure 4A and Supplementary Table S4). However, the selectivity varied depending on the location of the mutated residue on Asf1. Asf1 mutant D37A promoted Rtt109–Vps75 acetylation on H3-K9, H3K14, H3K23 and H3K27. Similarly, Asf1 E39A promoted acetylation was detected on H3K9, H3K14, H3K23 and H3K56. The only alanine mutant to behave like WT Asf1 was E105A. Interestingly, arginine mutants E39R, D54R and D58R also behaved like WT Asf1 with no observable changes to selectivity. With D58A, E88R and E105R mutants, acetylation was detected at H3K9, H3K23 and H3K56, whereas D37R facilitated acetylation at H3K9, H3K14 and H3K23. Unlike WT Asf1, each mutated Asf1 derivative that utilized H3K14ac/H4 as substrate no longer mediated acetylation solely on H3K56 (Figure 4B and Supplementary Table S5). Alanine mutations to either D37 or D54 of Asf1 resulted in acetylation primarily being observed at H3K9, H3K23 and H3K27. Introducing a positive charge (Supplementary Tables S4 and S5), however, did not lead to conserved acetylation patterns between the two mutants. D37R acetylation was observed at H3K9 and H3K23. Acetylation at H3K23, H3K27 and H3K56 was detected for D54R. Acetylation at H3K27 and H3K56 was observed for Asf1 mutants E39A, E39R, D58R and E88R. Rtt109–Vps75 primarily acetylates H3K9, H3K23 and H3K56 with the E88A mutant. Consistent with WT histones without Asf1, only H3K23 acetylation was observed with the E105A mutant. However, upon introducing an arginine residue at E105, acetylation was observed at H3K27 and H3K56.

Of all of the mutated derivatives of Asf1, only E105A completely lost the ability to mediate crosstalk between H3K14ac and K56ac, while resulting in little changes to the specificity of H3/H4. To further examine the impact of mutations at E105, steady state kinetics were performed with Asf1 mutants E105A/R with singly acetylated histones at H3K9, K23, K27 and K56 (Figures 5 and 6; Supplementary Table S6). When H3K9ac/H4 was used as a substrate, acetylation was observed at H3K23 and H3K56 for E105A. Interestingly, for E105R, only H3K23 acetylation was detected. With pre-acetylated H3K23 and H3K56 for E105A, acetylation was only observed at H3K9. Conversely, E105R displayed more broad selectivity with acetylation at H3K9, H3K14 and H3K56. Like H3/H4, when H3K27ac/H4 was used as substrate with E105A, acetylation was detected at H3K9 and H3K23. Positive mutant E105R with H3K27ac/H4 displayed acetylation only
Asf1, and the changes to free energy due to mutations to Asf1 with H3 at H3K14. H3K56ac.

Mutations of Asf1 can suppress the Asf1-mediated cross-talk between H3K14ac and H3K56ac. Comparison of site-specific $k_{cat(app)}$ of (A) H3/H4 or (B) H3K14ac/H5 by Rtt109–Vps75 acetylation in the presence of Asf1 mutants. The error bar represents the standard error in $k_{cat(app)}$. The apparent $k_{cat}$ are summarized in Supplementary Tables S3 and 4. (C) Free energy differences due to the addition of K14ac (right column) with various mutations to Asf1, and the changes to free energy due to mutations to Asf1 with H3/H4 (middle column) and H3K14ac/H4 (left column).

at H3K14. H3K56ac/H4 was the only pre-acetylated H3 to not drive any additional acetylation with both E105A/R.

### E105A suppresses selective binding of H3K14ac/H4 to Asf1

Our results suggested that Asf1 could be a histone modification ‘reader’. In order to test this hypothesis, we carried out binding assays looking at the affinity of H3/H4 and H3K14ac/H4 to Asf1 and Asf1E105A. Using labeled Asf1, $K_d$ values of 40 ± 4 nM for WT Asf1 and 50 ± 2 nM for the E105A mutant were determined (Figure 7). These results are consistent with previous work that observed minimal differences in histone binding between Asf1 and Asf1(E105A) (39). However, when we tested the impact of H3K14ac/H4 on binding to Asf1, we found an apparent increase in affinity of ~2-fold as compared to H3/H4 with WT Asf1 ($K_d$ values of H3/H4 40 ± 4 nM, H3K14ac/H4 14 ± 2 nM). This increased affinity was lost with the introduction of the E105A mutation ($K_d$ values of H3/H4 50 ± 2 nM, H3K14ac/H4 40 ± 2 nM). These results were consistent with those using labeled H3/H4E63C and titrating Asf1 (data not shown). Together this suggests that H3K14ac can alter the interaction with Asf1, and may explain the observed ability of Asf1 to mediate cross-talk between H3K14ac and H3K56 acetylation.

### Discussion

The data presented in this work demonstrates that Asf1 will alter the selectivity of Rtt109–Vps75 by working synergistically with H3K14ac and allowing us to map acetylation of H3 by Rtt109–Vps75 with and without Asf1 (Figure 8). Thus, Asf1 and H3K14 acetylation are both required for the efficient acetylation of H3K56. Early studies suggest that Asf1 could induce the acetylation of H3K56, but further research showed that K9 and K23 were the primary targets for in vitro and in vivo (3,7,11,12,18,19). The mechanism proposed herein resolves these discrepancies, and explains genetic data suggesting the importance of conserved residues within Asf1, opposite the histone binding face (32). These discoveries were facilitated by the new approaches to data analysis used in this paper.

The combination of targeted mass spectrometry, enzymology and kinetic modeling allowed us to dissect the mechanism of Asf1. This method is applicable to other histone chaperones, which may possess similar abilities, as well as other known acetyl-binding proteins. What separates the unique function of Asf1 from classical acetyl-binding domains is that Asf1 has a high affinity for histones, independent of their acetylation state. The histone tail recognition region is contained in an exosite which contains an acidic patch on the opposite face of the histone binding region and is tuned to recognize specific acetylation states. This exosite allows for another currently unique function of Asf1, as it can mediate cross-talk or direct the residue acetylated by Rtt109–Vps75 depending on the previous acetylation site(s). The ability of Asf1 to mediate cross-talk explains many of the differences observed in prior biochemical and genetic studies. Such discrepancies include: observations by Li et al. that Gcn5 is required for H3K56 acetylation while, work by Burgess et al. that Rtt109 is the HAT responsible for H3K56 acetylation, not Gcn5. What is clear is that H3K56ac is essential for DNA damage repair and transcription as cells lacking either HAT results in high sensitivity to DNA damaging agents and changes in nucleosome disassembly (40–43).

The ability of Asf1 to alter the selectivity of Rtt109–Vps75 is completely dependent on the pre-acetylation status of the histone substrate. In the absence of pre-acetylation, Asf1 increases the rate of acetylation of H3K9 and K23 by Rtt109–Vps75, but it does not alter the degree to which residues get acetylated (9,44). If either H3K9 or K23
Figure 5. Analysis of E105 mutations impact of Asf1 acidic patch. (A) Electronic potential surface for Asf1 (PDB ID: 2io5), calculated using PyMOL plugin APBS electrostatics and default settings. Asf1 E105A and E105R mutations were computationally made in PyMol using mutagenesis wizard and APBS electrostatics were calculated as for WT. (B) Free energy changes to selectivity due to mutation to Asf1, where the right side of the diagonal is the selectivity between residues with WT Asf1 and the left side is in the presence of Asf1 with E105A or R mutations.

are acetylated in the presence of Asf1, the specificity increases by almost three orders of magnitude and the selectivity is broadened. Neither H3K27ac or K56ac in complex with Asf1 have large impacts on selectivity, although they retain their ability to increase the rate of acetylation by Rtt109–Vps75. Only Asf1-H3K14ac/H4 completely focused Rtt109–Vps75 to acetylate H3K56. This fact explains the observation that Asf1 has a greater ability to drive the acetylation to H3K56 when using histones from biological sources such as chicken erythrocytes (9,45,46).

In order to understand how the Asf1-H3K14ac/H4 complex was able to focus the acetylation of Rtt109–Vps75, we studied the available structures of Asf1-H3/H4 and identified an acidic patch on the opposite face of the histone binding site. Many of the residues in this region are conserved among species and have been identified in genetic studies as being critical for Asf1 function (32,39). These same amino
Acids are conserved in human Asf1A and Asf1B (Supplementary Figure S3), and the majority of these amino acids have been found to be mutated in cancers exhibiting defects in DNA damage repair (47,48). This suggests a conserved mechanism where subtle changes to the charged surface of Asf1A and Asf1B could result in unique crosstalk.

We designed a set of mutations to test the hypothesis that the acidic patch regulates the ability of Asf1 to mediate crosstalk between acetylation states of H3. We chose residues that would impact this charge and assayed them with either H3/H4 or H3K14ac/H4 to identify any residue that drives the Asf1-H3K14ac/H4-dependent change in selectivity. Mutants E105A, E39R and D58R all behaved like WT Asf1 with H3/H4 as substrate, but when H3K14ac was used, these mutants no longer drove exclusive H3K56 acetylation. While E39R and D58R still retain the ability to acetylate H3K56, they also acetylate H3K27. The E105A mutant no longer acetylated K56 and selectivity was shifted completely to H3K23. It was also 10-fold slower than either Asf1-H3/H4 or Asf1(E105A)-H3/H4. Pre-acetylated histone
tones (except H3K9) with E105A/R had lower specificity and primarily acetylated H3K9 and/or H3K23. By examining the electrostatic potential of each of the mutants, we can gain insight into how each of them impact the charged surface of Asf1. Interestingly, the E105A and E39R mutations have similar charge surfaces (Figure 5A; Supplementary Figures S5 and 6). Residue E39 is located on a beta strand adjacent to E105, thus introducing an arginine residue at E39 leads to an increase in positive charge across the two beta strands effectively neutralizing E105 and mimicking the E105A mutation.

Furthermore, in vivo experiments as well as histone binding assays examining mutation to D37R, E39R and E105R of Asf1 have previously shown that mutations to the core of Asf1 lead to increases in gene silencing while having no impact on H3/H4 binding (32,39). Taken together, one possible explanation is that the H3K14ac is guided into the small neutrally charged pocket near E105 and that expanding this neutral region increased the dynamics of the peptide and broadens the selectivity. Our binding data support this explanation as H3K14ac has a 2-fold higher affinity for WT Asf1 that is lost upon E105A mutation. These findings suggest the importance of these residues, specifically E105 for binding to pre-acetylated histone and build upon previous in vivo work that demonstrates the importance of E39 and E105 for Asf1 anti-silencing function.

The data also resolve some questions raised by early genetic work that demonstrated the importance of the E105 residue to Asf1 anti-silencing function (32) and a genetic link between Asf1 and H3K14ac in DNA repair (49–51). Multiple studies also support the conclusion that H3K14ac and H3K56ac both function in the DNA repair pathway (4,52). By combining our studies with the known genetic studies, we can propose a model where H3K14 is acetylated within the nucleosome by an acetyltransferase such as SAGA (20,30,53–54); which then recruits the SWI/SNF remodeler complex (52,55,56). This facilitates access to Asf1, thus allowing Rtt109–Vps75 to acylate H3K56.
Recently, a crystal structure of Rtt109–Asf1-H3/H4 complex was solved for a pathogenic fungus, *Aspergillus fumigatus* (AfRtt109) (57). The AfRtt109 differs from the *S. cerevisiae* Rtt109, which was used in this study, in that the AfRtt109 is missing an insertion loop required for Vps75 binding (Supplementary Figure S4A) (14,17). The AfRtt109 also has a slightly different specificity than the ScRtt109 preferring H3K27ac (57). From the AfRtt109–Asf1-H3/H4 complex structure it is clear that the H3 tail is positioned perfectly to wrap around Asf1 to bind the acidic patch (Figure 3). Furthermore, Asf1-H3/H4 binds Rtt109 on the opposite side of the binding site of Vps75 (Supplementary Figure S4B) suggesting that Asf1 may occupy a similar location in the Rtt109–Vps75-Asf1-H3/H4 complex.

The level of crosstalk between acetylation sites helps explain some of the limited complexity of the observed combinatorial histone modification patterns seen in vivo. We demonstrate that under conditions where sequential acetylation by Rtt109–Vps75 is observed, Asf1 can drive H3K56ac after at least one round of acetylation. These experiments suggest that Asf1 functions with the histone acetylation state to alter the selectivity of Rtt109–Vps75. To date, little, if any, work has been done to assess how pre-acetylated histones impact Rtt109 selectivity, nor if histone chaperones are also influenced by PTMs. The results in this study provide insight into how different histone acetylation marks and histone chaperones concerted or individually impact Rtt109–Vps75 histone acetylation. We also propose the mechanistic details of how Asf1 mediates the crosstalk between H3K14ac to drive H3K56ac by Rtt109–Vps75. Together these data demonstrate how one PTM can regulate subsequent PTMs, which raises the question: can histone acetylation function to coalesce multiple inputs from the cell to coordinate dynamic changes to chromatin?

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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