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The Carboxyl Terminus of Rtt109 Functions in Chaperone Control of Histone Acetylation

Ernest Radovani,[a] Matthew Cadorin,[a] Tahireh Shams,[a] Suzan El-Rass,[a] Abdel R. Karsou,[a] Hyun-Soo Kim,[d] Christoph F. Kurat,[e] Michael-Christopher Keogh,[d] Jack F. Greenblatt,[b,c,e] Jeffrey S. Fillingham[a]

Department of Chemistry and Biology, Ryerson University, Toronto, Ontario, Canada[a]; Banting and Best Department of Medical Research, Donnelly Centre, University of Toronto, Toronto, Canada[b]; Department of Molecular Genetics, University of Toronto, Toronto, Canada[c]; Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York, USA[d]; The Donnelly Center, University of Toronto, Toronto, Ontario, Canada[e]

Rtt109 is a fungal histone acetyltransferase (HAT) that catalyzes histone H3 acetylation functionally associated with chromatin assembly. Rtt109-mediated H3 acetylation involves two histone chaperones, Asf1 and Vps75. *In vivo*, Rtt109 requires both chaperones for histone H3 lysine 9 acetylation (H3K9ac) but only Asf1 for full H3K56ac. *In vitro*, Rtt109-Vps75 catalyzes both H3K9ac and H3K56ac, whereas Rtt109-Asf1 catalyzes only H3K56ac. In this study, we extend the *in vitro* chaperone-associated substrate specificity of Rtt109 by showing that it acetylates vertebrate linker histone in the presence of Vps75 but not Asf1. In addition, we demonstrate that in *Saccharomyces cerevisiae* a short basic sequence at the carboxyl terminus of Rtt109 (Rtt109C) is required for H3K9ac *in vivo*. Furthermore, through *in vitro* and *in vivo* studies, we demonstrate that Rtt109C is required for optimal H3K56ac by the HAT in the presence of full-length Asf1. When Rtt109C is absent, Vps75 becomes important for H3K56ac by Rtt109 *in vivo*. In addition, we show that lysine 290 (K290) in Rtt109 is required *in vivo* for Vps75 to enhance the activity of the HAT. This is the first *in vivo* evidence for a role for Vps75 in H3K56ac. Taken together, our results contribute to a better understanding of chaperone control of Rtt109-mediated H3 acetylation.

Eukaryotic cells package their genomic DNA into chromatin. The basic unit of chromatin, the nucleosome, wraps 146 bp of DNA around a histone octamer and contains four different core histones: H2A, H2B, H3, and H4. Other factors, such as the linker histone, further pack nucleosomes into higher-order chromatin structures. One canonical function of linker histone is to bind DNA between nucleosomes, helping to condense chromatin to a 30-nm fiber (1). Chromatin remodeling is required to access DNA for essential cellular processes to occur such as transcription, replication, and repair. One mechanism used by the eukaryotic cell to remodel chromatin is through histone posttranslational modification (3). Such modifications include acetylation, methylation, and phosphorylation, and they can be carried out either at the nucleosomal level or, as in the case of acetylation, on newly synthesized histones prior to their deposition into chromatin.

Newly synthesized histone H4 is acetylated at lysines 5 and 12 (H4K5ac and H4K12ac) (4). This acetylation is evolutionarily conserved from yeast to metazoans and catalyzed by the Hat1 histone acetyltransferase (HAT) (5, 6). In the budding yeast *Saccharomyces cerevisiae*, Hat1 functions as part of a protein complex with two histone chaperones, Hat2 and Hif1 (7, 8). Newly synthesized histone H3 is also acetylated in *S. cerevisiae* on H3K9 and H3K56 (9, 10). H3K9ac is catalyzed by two HATs in yeast, Gcn5 and Rtt109 (11, 12). Gcn5 acetylates H3K9 at the nucleosomal level as part of the SAGA transcriptional coactivator complex (12) and may have an additional SAGA-independent role in acetylation of newly synthesized histone H3 (13). H3K56ac is catalyzed solely by the fungus-specific HAT Rtt109 in the *Ascomycota* yeasts *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans* (14–19). Unlike lysine 9, which is located within the N terminus of H3, K56 is the last residue of the αN-helix and precedes the histone fold domain (10). The positively charged H3K56 makes water-mediated contact with the phosphodiester backbone of DNA within the nucleosome (20). Acetylation of H3K56 has been proposed to weaken DNA-nucleosome interaction leading to more relaxed chromatin structure (10, 21). In addition, H3K56ac provides a binding surface for the histone H3-H4 chaperones Rtt106 and CAF-1 in replication-dependent chromatin assembly (22). Rtt109/H3K56ac function has been implicated in the regulation of retrotransposition, maintenance of genome stability, DNA damage repair, and transcription regulation (10, 23–27).

In *S. cerevisiae*, Rtt109 activity is associated with two histone chaperones, Asf1 and Vps75. The Asf1 histone chaperone is conserved from yeast to metazoans and is required by Rtt109 in *S. cerevisiae* to catalyze H3K56ac *in vivo* and *in vitro* and H3K9ac *in vivo* but not *in vitro* (11, 28). The Asf1 protein has a highly conserved 155-amino-acid N-terminal region (Asf1N) and a shorter, evolutionarily divergent carboxyl terminus (29). Structural studies have shown that Asf1 binds newly synthesized H3-H4 dimers through this N-terminal region and is believed to subsequently present them to Rtt109 for acetylation (30, 31). Furthermore, it has been shown that *in vivo* Asf1N is sufficient for H3K56ac by Rtt109 (32). In yeast the non-evolutionarily conserved carboxyl terminus of Asf1 is extremely acidic, whereas in humans the region is subject to cell cycle-dependent phosphorylation (33). In *S. cerevisiae*, functions of the Asf1 C terminus include mediating

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Address correspondence to Jeffrey S. Fillingham, jeffrey.fillingham@ryerson.ca.
M.C. and T.S. contributed equally to this article.
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telomeric silencing (34) and physical interactions with Rad53 (35) and CAF-1 (36).

Vps75 is a member of the NAP1 histone chaperone family with a preference for binding H3-H4 tetramers (2). *In vivo*, Vps75 is part of a stable protein complex with Rtt109 (37) and stabilizes the HAT (11). This interaction favors a catalytically active conformation of Rtt109 (38–40). *In vitro* Rtt109-Vps75 catalyzes efficient H3K56ac and H3K9ac in the absence of Asf1. *In vivo* Rtt109-mediated H3K9ac requires expression of both *ASF1* and *VPS75* (11). However, any *in vivo* role for Vps75 in H3K56ac is unclear since H3K56ac levels are not abolished in *vps75*Δ cells (2, 28). Despite significant interest in chaperone control of Rtt109 activity, the exact nature of interplay between Rtt109, Vps75, and Asf1 required to generate wild-type (WT) levels of H3K56ac and H3K9ac remains unclear. One hypothetical model used to describe this interplay has Rtt109-Vps75 acetylating H3K9 and H3K56 when H3 is bound to Asf1 as part of an H3-H4 dimer. Some support for this model is that Rtt109-Vps75 acetylates H3K56ac more efficiently on H3-H4 bound to Asf1 than H3-H4 dimers alone *in vitro* (41). Another possible model to describe the interplay proposes that Rtt109-Vps75 acetylates H3K9ac and H3K56ac on H3 bound to Vps75 before subsequent transfer to Asf1.

In addition to acetylating H3, Rtt109 auto-acetylates itself at K290, and this modification has been shown to be important for its activity *in vitro* in the presence of Vps75 (42, 43). Rtt109 is required by the fungus *C. albicans* for pathogenicity (17). Rtt109 shows no sequence homology to any previously characterized HAT. However, when the crystal structures are compared, it is clear that Rtt109 and CBP/p300 share a structure (43–46). Although Rtt109 is, then, a distant homolog of CBP/p300, it is considered an important therapeutic target for pathogenic fungi (17, 47, 48). Thus, understanding its structure/function is of potential medical relevance.

In this study, we investigate the complex relationship of Rtt109, Vps75, and Asf1. First, we extend the *in vitro* substrate specificity of Rtt109 by showing that it acetylates linker histone in the presence of Vps75 but not Asf1. We also demonstrate that a lysine/arginine-rich sequence at the C terminus of Rtt109 (Rtt109C, consisting of amino acids 425 to 436) is required for H3K9ac *in vivo*. In addition, Rtt109C is required for optimal Rtt109-Asf1 H3K56ac *in vitro*. We show that in the absence of Rtt109C, Vps75 becomes essential for full H3K56ac activity, suggesting that Rtt109-Vps75 contributes *in vivo* to H3K56ac, a role which has not been documented before for the chaperone. Together, our results provide new insights into the mechanism by which the activity of Rtt109 is controlled by the two histone chaperones Asf1 and Vps75.

MATERIALS AND METHODS

Strains used in the study. The strains of *S. cerevisiae* used in this study were generated through standard molecular genetic procedures and are listed in Table S1 in the supplemental material.

Sequence alignments. Primary sequence alignments of predicted fungal Rtt109 sequences were performed as previously described (49). Briefly, sequences were obtained from NCBI and aligned using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2). Regions of similarity among the species were shaded using Boxshade, version 3.2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Protein expression. cDNA sequences of the *RTT109* gene encoding full-length protein or amino acids 1 to 424 [*RTT109*(*1–424*)], *VPS75*, *ASF1*, *ASF1N*, and *HHO1* were amplified from yeast genomic DNA (or in the case of *VPS75*, from plasmid GST-VPS75, where GST is glutathione *S*-transferase [2]) and then cloned in pET28a (Novagen) using NdeI and SalI restriction sites. Recombinant proteins were expressed and purified using a Ni-nitrilotriacetic acid (NTA) Fast Start kit (Qiagen) according to the manufacturer's instructions. Proteins were desalted by gel filtration chromatography using PD-10 prepacked columns (GE Healthcare). GST-Vps75 was expressed and purified as previously described (11).

HAT assays. HAT assays were performed using as the substrate a mixture of core histones and linker histone purified from chicken erythrocytes (Millipore), calf thymus H1 (Millipore), or Hho1 with six copies of a His tag (6×HIS-Hho1), as indicated in the figure legends. *In vitro* reactions using [^{14}C]acetyl-coenzyme A (CoA) were carried out as previously described (11). When unlabeled acetyl-CoA was used, the enzymes indicated in the figures were incubated for 30 min in 30-μl reaction mixtures that contained 5 μg of core histones, 50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1.8 μM acetyl-CoA. Reactions were stopped by boiling mixtures for 5 min in an equal volume of 2× SDS-PAGE buffer. Reaction products were subsequently separated by 15% SDS-PAGE, transferred onto nitrocellulose paper, and immunoblotted with antibodies as indicated in the figures and/or figure legends at the following concentrations: HIS probe (1:4,000; Santa Cruz), anti-H3K9ac (1:4,000; Abcam), anti-H3K56ac (1:4,000; a gift from J. Recht), anti-H3K56ac (1:2,500; Upstate), anti-H3K12ac (1:4,000; Active Motive), and anti-Hho1(1:2,000) (a gift from Pierre Defossez [50]). Protein concentrations were assessed as required using NanoDrop 2000c (Thermo Scientific) using the Protein A280 application.

Generation of WCE and Western blotting. Whole-cell extracts (WCE) were prepared using trichloroacetic acid (TCA) as previously described (51). Western blotting was performed as described above using the following antibodies at the indicated dilutions: H3K9ac (1:4,000; Abcam), H3K56ac (1:2,000; Upstate), H3K56ac (1:4,000; a gift from J. Recht), H3 (1:4,000; Active Motif), c-Myc (1:2,000; Santa Cruz), and TATA-binding protein ([TBP] 1:2,000; Santa Cruz).

FACS. Fluorescence-activated cell sorting (FACS) analysis was performed as described by Kurat et al. (52).

Spot tests. The strains indicated in Fig. 2B and Fig. S5 to S7 in the supplemental material were grown to an optical density at 600 nm (OD_{600}) of approximately 0.5. Five microliters of each culture was plated on plates of the indicated medium at four 10-fold serial dilutions.

IPs. Immunoprecipitations (IPs) were carried out as previously described (53) with modifications as follows. Cells were harvested from 200 ml of yeast cultures at an OD of ~1.0 and washed twice with ice-cold water and once with ice-cold IP buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.1% NP-40). Cells were resuspended in 0.85 ml of lysis IP buffer (50 mM Tris, pH 8, 150 mM NaCl, 1.5 mM magnesium acetate [MgOAc], 0.15% NP-40, 5 mM EDTA, protease inhibitor cocktail [Roche], 5 mM PMSF) and mechanically lysed with 1 ml of glass beads using a Mini-BeadBeater-8 (Biospec Products) at maximum speed at 4°C for 40 s for 10 cycles, with a 1-min ice break between cycles. After insoluble material was discarded by centrifugation (20 min at 14,000 rpm and 4°C), 0.75 ml of the supernatant was incubated with 25 μl of IgG-Sepharose beads (Amersham) for 2 h at 4°C. After incubation the beads were washed three times for 5 min using 0.75 ml of washing buffer (50 mM Tris, pH 8, 150 mM NaCl, 1.5 mM MgOAc, 0.15% NP-40, 5 mM EDTA). The proteins were eluted by boiling the beads in 50 μl of 2× SDS-PAGE loading dye. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose paper, and immunoblotted as described above.

RESULTS

Rtt109-Vps75, but not Rtt109-Asf1, acetylates histone H1 *in vitro*. We previously used the Vps75-Rtt109-TAP (where TAP is tandem affinity purification) purified protein complex in *in vitro* HAT assays to characterize its activity on H3 (11, 14). In these experiments we used as the substrate a histone preparation made

from chicken erythrocytes that includes the four core histones mixed with linker histones H1 and H5, along with ^{14}C-labeled acetyl-CoA as a substrate (data not shown). In these experiments we observed robust acetylation of the linker histone portion of the preparation in addition to H3. To investigate further, we performed HAT assays using recombinant proteins to assess whether the vertebrate linker histone-specific acetylation activity was specific to Rtt109-Vps75 or also catalyzed by Rtt109-Asf1. We performed *in vitro* HAT assays again using chicken erythrocyte core histones mixed with vertebrate linker histone as the substrate for recombinant Rtt109 (6×HIS-rRtt109) in the presence of either 6×HIS-Vps75 or 6×HIS-Asf1. Consistent with what we observed using TAP-purified Rtt109-Vps75, 6×HIS-Rtt109 catalyzed vertebrate linker histone acetylation only in the presence of 6×HIS-Vps75, not alone or with 6×HIS-Asf1 (Fig. 1A). Additionally, as expected, 6×HIS-Rtt109 with either 6×HIS-Vps75 or 6×HIS-Asf1 catalyzed H3K56ac while 6×HIS-Rtt109 and 6×HIS-Vps75 also catalyzed H3K9ac (Fig. 1A). We also noticed that 6×HIS-Rtt109-6×HIS-Vps75 acetylates H4 *in vitro*, consistent with recent results of Abshiru et al. (54). Thus, *in vitro* vertebrate linker histone acetylation is specific to Rtt109-Vps75.

In order to determine if Rtt109-Vps75 catalyzed vertebrate linker histone acetylation occurs independent of the presence of other histones, we performed the above-described assay with a substrate of calf thymus H1 (Millipore) alone for either 6×HIS-Rtt109–6×HIS-Vps75 or 6×HIS-Rtt109–6×HIS-Asf1. As in the experiment described above, 6×HIS-Rtt109 acetylated the calf thymus H1 in the presence of 6×HIS-Vps75 and 6×HIS-Asf1 (Fig. 1B). Furthermore, the activity of 6×HIS-Rtt109 increased with increasing concentrations of the chaperone (Fig. 1B). Collectively, these experiments indicate that Rtt109-catalyzed vertebrate linker histone acetylation is enhanced *in vitro* in a chaperone-specific manner. Previous studies have also shown that human Gcn5 has *in vitro* H1 acetylation activity (55). We therefore tested recombinant yeast Gcn5 (yGcn5) for *in vitro* H1 HAT activity and observed that yGcn5 is also able to acetylate vertebrate linker histone *in vitro* (see Fig. S1 in the supplemental material), a result that is consistent with a variety of functional links that exist between Rtt109-Vps75 and Gcn5 (56, 57). In addition, yGcn5 showed acetylation activity on H3 and H4 (see Fig. S1). Recombinant Hat1 (6×HIS-Hat1), on the other hand, does not acetylate vertebrate linker histone (see Fig. S1), suggesting that linker histone acetylation is not a general property of chromatin assembly HATs. The *S. cerevisiae* Hho1 protein shares some sequence homology with vertebrate linker histones although they are not as evolutionary conserved in primary sequence as are core histones (58). We therefore next expressed and purified recombinant Hho1 (6×HIS-Hho1) and used it as a substrate for Rtt109-Vps75 *in vitro*, where we observed its acetylation although with somewhat lower efficiency than vertebrate linker histone (Fig. 1C). In histone H3, the two main substrates of Rtt109-Vps75, K9 and K56, both fall within KST sequences. Since Hho1 contains two KST tripeptide sequences at amino acid positions 5 to 7 and 25 to 27 (see Fig. S2 in the supplemental material), we hypothesized that the lysines acetylated in Hho1 were K5 and K25. To test this hypothesis, we expressed recombinant Hho1 that had both lysines mutated to arginines (rHho1K5R/K25R) and used it as a substrate for *in vitro* HAT assays. Our analysis showed that there was no decrease in acetylation levels for rHho1K5R/K25R compared to the level of WT rHho1 (see Fig. S3). Thus, yeast and vertebrate linker histone

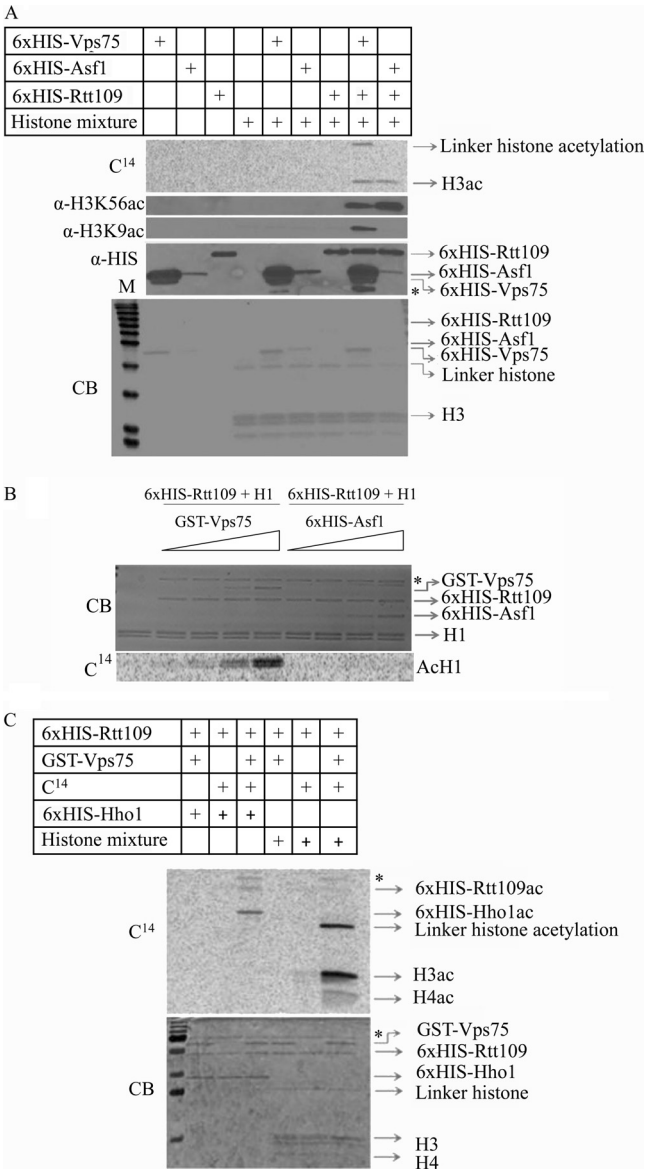

FIG 1 *In vitro* activity of Rtt109-Vps75 on linker histone H1. (A) Rtt109-Vps75 but not Rtt109-Asf1 acetylates vertebrate linker histone. HAT assays were carried out with [^{14}C]acetyl-CoA before being resolved by 15% SDS-PAGE. Gels were either transferred onto nitrocellulose and immunoblotted with anti-HIS, anti-H3K56ac, or anti-H3K9ac or stained with Coomassie blue (CB), saturated with Enlightning (PerkinElmer), and dried under vacuum, and then ^{14}C was imaged using a Typhoon phosphorimager. The asterisk indicates a breakdown product of 6×HIS-Vps75. (B) Increasing concentrations of Vps75 correlate with increasing acetylation activity of rRtt109 on bovine linker histone. Increasing concentrations of either 6×HIS-Vps75 or 6×HIS-Asf1 were incubated with a constant amount of full-length 6×HIS-Rtt109 and bovine H1. HAT assays were carried out and analyzed by SDS-PAGE/autoradiography as described for panel A. The asterisk indicates a probable dimeric form of 6×HIS-Rtt109. (C) Rtt109-Vps75 acetylates Hho1 *in vitro*. Recombinant proteins were incubated with either chicken core histones or 6×HIS-Hho1 as indicated. *In vitro* HAT assay reactions were carried out and analyzed as described for panel A. The asterisk indicates a probable dimeric form of 6×HIS-Rtt109. α, anti.

is an *in vitro* substrate for Rtt109-Vps75. Furthermore, this activity is shared by Gcn5 but not by Hat1.

The Lys/Arg-rich sequence at the carboxyl terminus of Rtt109 is essential for H3K9ac *in vivo*. In order to discover crit-

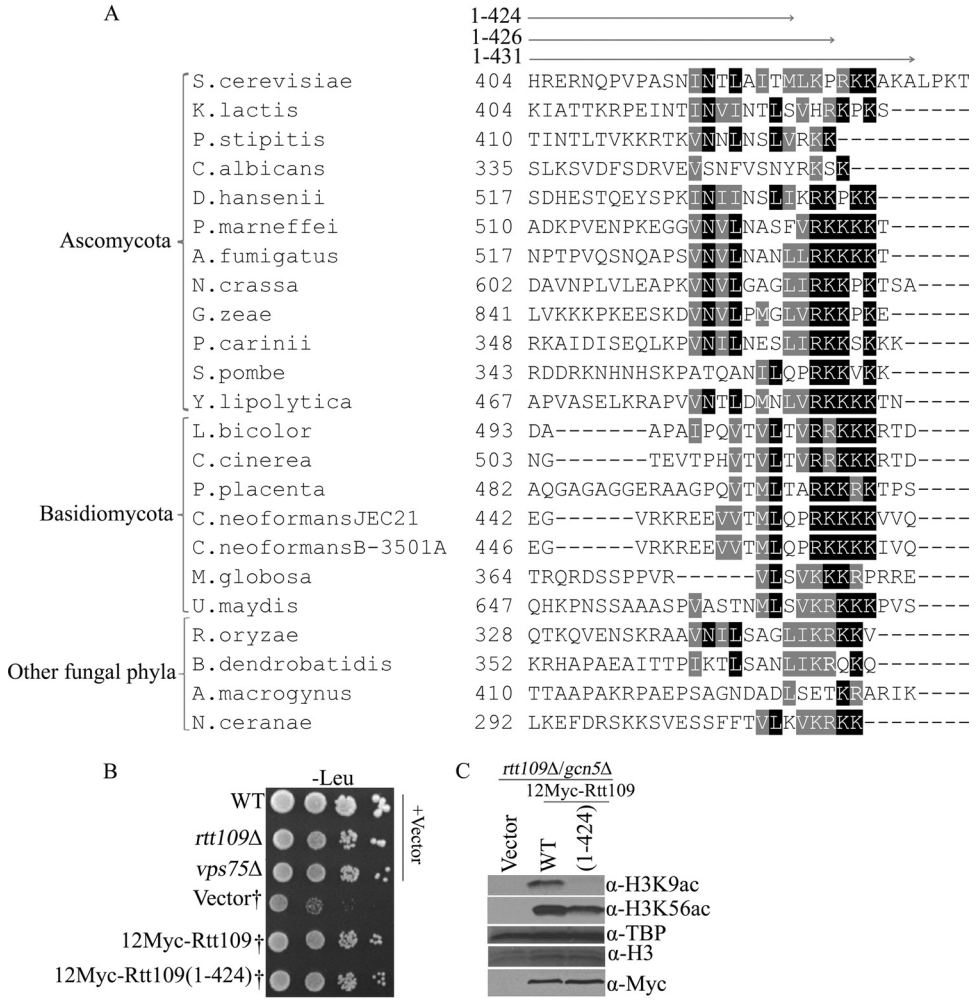

FIG 2 The carboxyl terminus of fungal Rtt109s contain a Lys/Arg-rich sequence which is essential for H3K9ac in *S. cerevisiae*. (A) Using the ClustalW algorithm, *S. cerevisiae* Rtt109 was aligned with predicted Rtt109 sequences from fungi of the *Ascomycota*, *Basidiomycota*, *Zygomycota* (*Rhizopus oryzae*), *Chytridiomycota* (*Batrachochytrium dendrobatidis* and *Allomyces macrogynus*), and *Microsporidia* (*Encephalitozoon cuniculi* and *Nosema ceranae*). Identical amino acid residues are shaded in black, whereas similar amino acid residues are shaded in gray using Boxshade, version 3.2. (B) The *12MYC-RTT109* and *12MYC-RTT109*(*1–424*) mutants expressed under the control of the ADH1 promoter on a CEN plasmid (pRB415A-12Myc) rescue the slow-growth phenotype of *rtt109*Δ *gcn5*Δ cells. †, vectors containing indicated genes were transformed into *rtt109*Δ *gcn5*Δ strains. (C) The basic carboxyl terminus of Rtt109 is absolutely required for H3K9ac but not for full H3K56ac. WCE from the indicated strains were resolved by 15% SDS-PAGE, transferred onto nitrocellulose paper, and immunoblotted with the antibodies indicated on the right of each blot.

ical amino acids for Rtt109 function, we performed a comparative analysis of predicted Rtt109 amino acid sequences from representative fungal species (Fig. 2A). From this analysis we noticed that almost every predicted fungal Rtt109 has a short sequence enriched with lysine and arginine amino acids at the extreme carboxyl terminus (Fig. 2A). Because of its high degree of conservation, we hypothesized that this short Lys/Arg-rich sequence (^{424}K PRKKAKALPKT^{436} in *S. cerevisiae*) could be important to Rtt109 function. Two well-characterized functions of Rtt109 are H3K9ac and H3K56ac. To test the importance of the short Lys/Arg-rich sequence to these two Rtt109 functions, we expressed a C-terminal deletion mutant, 12Myc-Rtt109(1–424), along with full-length 12Myc-Rtt109 under the control of the ADH1 promoter on a CEN-based plasmid in a *rtt109*Δ *gcn5*Δ strain previously shown to be null for H3K56ac and H3K9ac (11). As expected, 12Myc-Rtt109 rescues a portion of the slow-growing phenotype of the *rtt109*Δ *gcn5*Δ strain (Fig. 2B, the rescued strain grows at the same rate as *gcn5*Δ), as well as H3K56ac and some H3K9ac (Fig. 2C). In contrast, the deletion mutant 12Myc-Rtt109(1-424), which rescued the slow-growth phenotype (Fig. 2B) and expressed at a similar level to the wild type (Fig. 2C), did not rescue H3K9ac (Fig. 2C). Antibodies against TBP and histone H3 were used as loading controls (Fig. 2C). In addition, we noticed that the deletion mutant rescued H3K56ac at a reproducibly slightly lower level than 12Myc-Rtt109 (Fig. 2C). Thus, the phenotype of the *12Myc-Rtt109*(*1-424*) *gcn5*Δ strain resembles that of a *vps75*Δ *gcn5*Δ strain (11) where there is no Vps75 to bind Rtt109 and enhance H3K9ac but there is still H3K56ac since the chaperone is not essential for the modification.

Rtt109(1–424) and Vps75 physically interact *in vivo* and acetylate H3K9 *in vitro*. To determine the functional role of the Rtt109 C terminus, we first assessed whether it is required for the physical interaction of Rtt109 with Vps75. Therefore, we expressed 12Myc-Rtt109(1–424) and 12Myc-Rtt109 in an *rtt109*Δ

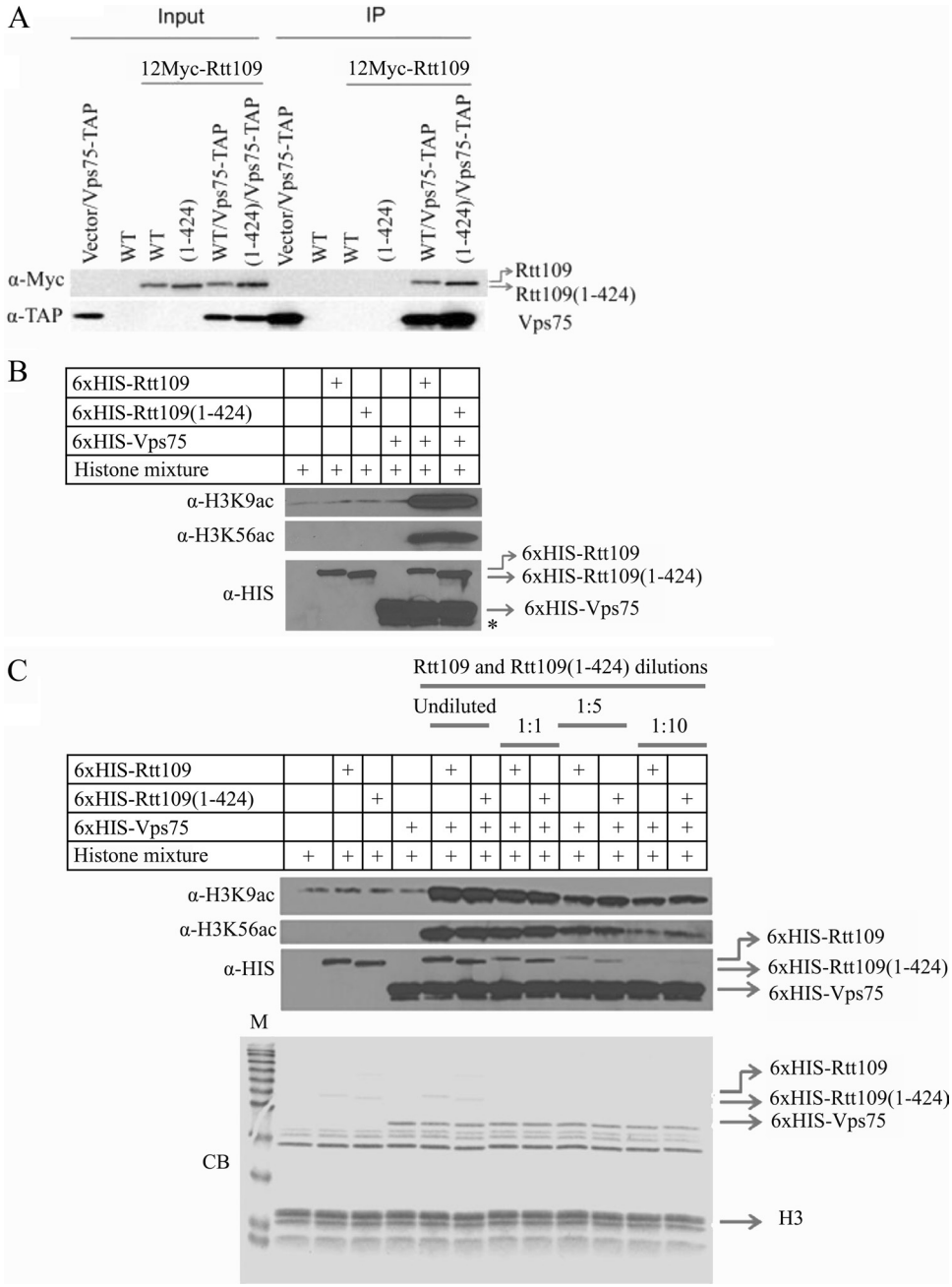

FIG 3 Rtt109(1–424) physically interacts with Vps75 *in vivo* and can acetylate H3K9 in the presence of Vps75 *in vitro*. (A) 12Myc-Rtt109(1–424) coimmunoprecipitates with Vps75-TAP no differently than 12Myc-Rtt109. Vps75-TAP was immunoprecipitated from the indicated strains with IgG-Sepharose beads as described in Materials and Methods. WCE and IPs were resolved by 12% SDS-PAGE, transferred onto nitrocellulose paper, and immunoblotted with anti-Myc and anti-TAP. (B) Rtt109(1–424)-Vps75 is able to acetylate H3K9 *in vitro*. HAT assays were performed using indicated recombinant proteins with acetyl-CoA and chicken core histones for 30 min, resolved by 10% and 15% SDS-PAGE, and transferred onto nitrocellulose. The 10% gel was immunoblotted with anti-6×HIS, whereas the 15% gel was immunoblotted with anti-H3K56ac and anti-H3K9ac. The asterisk indicates a breakdown product of 6×HIS-Vps75. (C) Rtt109(1–424)-Vps75 catalyzes *in vitro* H3K9ac at the same efficiency as Rtt109-Vps75. Serial dilutions of equal amounts of 6×HIS-Rtt109 and 6×HIS-Rtt109(1–424) were incubated with a constant amount of 6×HIS-Vps75. HAT reactions were carried out as described for panel B using as substrate a mixture of core and linker histones purified from chicken erythrocytes. Reactions were resolved by 15% SDS-PAGE and Coomassie stained or transferred onto nitrocellulose and immunoblotted as described for panel B. Lane M, molecular mass marker.

VPS75-TAP strain, immunoprecipitated Vps75-TAP from whole-cell extracts (WCE) made using these strains, and then used Western blotting with antibodies against Myc to assess interaction with 12Myc-Rtt109(1–424). We observed that the truncated version of Rtt109 copurified with Vps75-TAP no differently than the WT (Fig. 3A). Thus, the deletion of Rtt109C does not prevent *in vivo* Rtt109-Vps75 physical interaction, consistent with a study that shows structural evidence that an α-helix containing residues 412

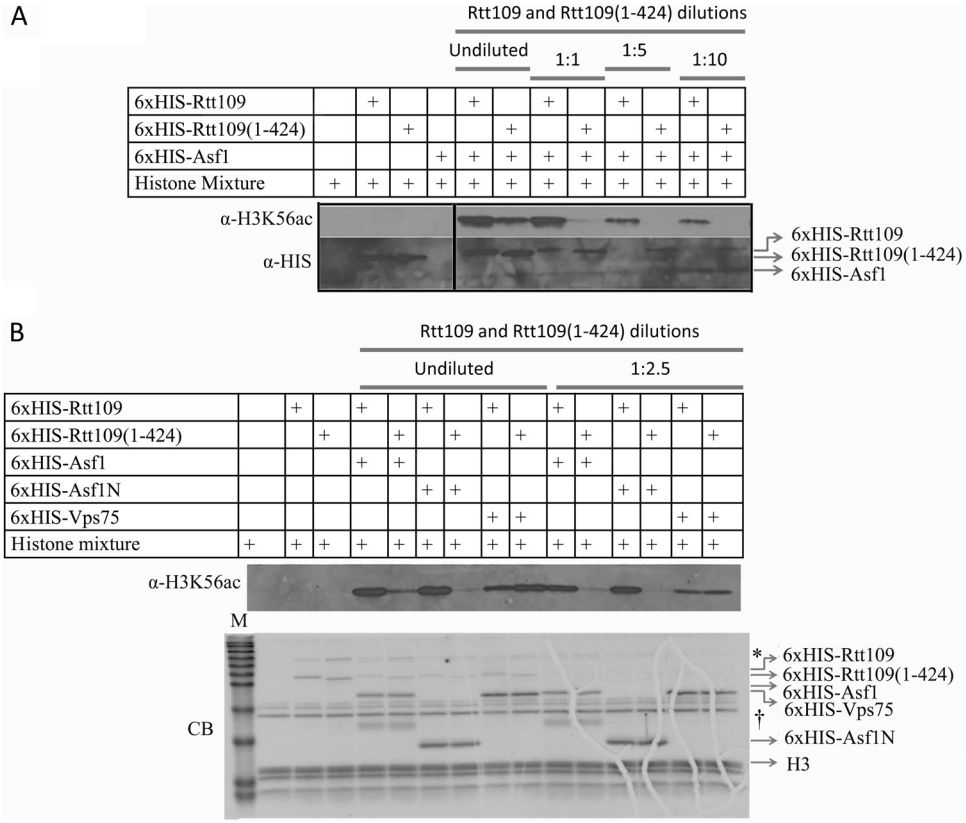

FIG 4 Rtt109C is required by Asf1 to enhance H3K56ac. (A) Rtt109(1–424) is not efficient in acetylating H3K56 in the presence of Asf1 *in vitro*. HAT assays were performed as described in the legend of Fig. 3C except that the same series of dilutions of full-length 6×HIS-Rtt109 and 6×HIS-Rtt109(1–424) were incubated with a constant concentration of 6×HIS-Asf1. Reaction products were separated by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with anti-HIS and anti-H3K56ac. The two boxed images were grouped together from the same SDS-PAGE gel. (B) *In vitro*, Asf1 and Asf1N but not Vps75 require Rtt109C to enhance the *in vitro* activity of Rtt109. Serial dilutions of equal amounts of full-length 6×HIS-Rtt109 and 6×HIS-Rtt109(1–424) were incubated with either of 6×HIS-Asf1, 6×HIS-Asf1N, or 6×HIS-Vps75. HAT assays were performed and analyzed as described in the legend of Fig. 3C. The asterisk indicates a probable dimeric form of 6×HIS-Rtt109 and 6×HIS-Rtt109(1–424); the dagger (†)indicates a breakdown product of 6×HIS-Asf1.

to 424 from Rtt109 contacts Vps75 in the Rtt109-Vps75 (59). We next tested whether 6×HIS-Rtt109(1–424) is functional in HAT assays performed in the presence of 6×HIS-Vps75. From previous studies, we know that *in vitro*, in the presence of Vps75, Asf1 is not necessary for Rtt109 to perform either H3K9ac or H3K56ac, thus allowing us to examine the relationship between Rtt109(1–424) and Vps75. We therefore expressed and purified 6×HIS-Rtt109, 6×HIS-Rtt109(1–424), and 6×HIS-Vps75 and performed *in vitro* HAT assays. We observed that in the presence of 6×HIS-Vps75, 6×HIS-Rtt109(1–424) catalyzed H3K56ac, H3K9ac (Fig. 3B), and vertebrate linker histone acetylation (see Fig. S4 in the supplemental material) similarly to 6×HIS-Rtt109. To rigorously compare *in vitro* HAT activities of full-length 6×HIS-Rtt109 and 6×HIS-Rtt109(1–424), we performed a HAT assay using several dilutions of either full-length or C-terminal deletion mutant versions of Rtt109 with a constant amount of 6×HIS-Vps75. Western blot analysis of the products of the HAT assays showed that even at low concentrations, 6×HIS-Rtt109(1–424) appears as efficient as full-length 6×HIS-Rtt109 in both Vps75-catalyzed H3K9ac and H3K56ac (Fig. 3C). Taken together, these results suggest that *in vivo* Rtt109(1–424)-Vps75 has the potential to catalyze H3K9ac.

The carboxyl terminus of Rtt109 is required *in vitro* for full Rtt109-Asf1 activity. Since Rtt109(1–424) showed a slight but reproducible decrease in H3K56ac *in vivo* (Fig. 2C), we tested whether Asf1 synergized any differently with Rtt109(1–424) than with full-length Rtt109 in *in vitro* HAT assays. Again, we performed HAT assays using several dilutions of 6×HIS-Rtt109 and 6×HIS-Rtt109(1–424) with a constant amount of 6×HIS-Asf1. Importantly, for each concentration tested, we observed that full-length Rtt109 catalyzed H3K56ac more efficiently than Rtt109(1–424) (Fig. 4A), suggesting that there exists a functional interaction between Rtt109C and Asf1.

We next performed HAT assays using two dilutions of both full-length 6×HIS-Rtt109 and 6×HIS-Rtt109(1–424) with a constant concentration of either 6×HIS-Asf1, 6×HIS-Asf1N, or 6×HIS-Vps75. Both versions of Asf1 enhanced the activity of full-length 6×HIS-rRtt109 equally (Fig. 4B). Similar to what we observed above (Fig. 4A), again we observed reduction in H3K56ac activity when 6×HIS-Rtt109(1–424) was incubated with 6×HIS-Asf1 (Fig. 4B). However, in the presence of 6×HIS-Asf1N, 6×HIS-Rtt109(1–424) was even more reduced in H3K56ac activity (Fig. 4B), suggesting that the carboxyl terminus of 6×HIS-Asf1 could function in H3K56ac catalysis. As observed above (Fig. 3C), we again observed no difference in 6×HIS-Vps75-stimulated H3K56ac activity between 6×HIS-Rtt109 and 6×HIS-Rtt109(1–

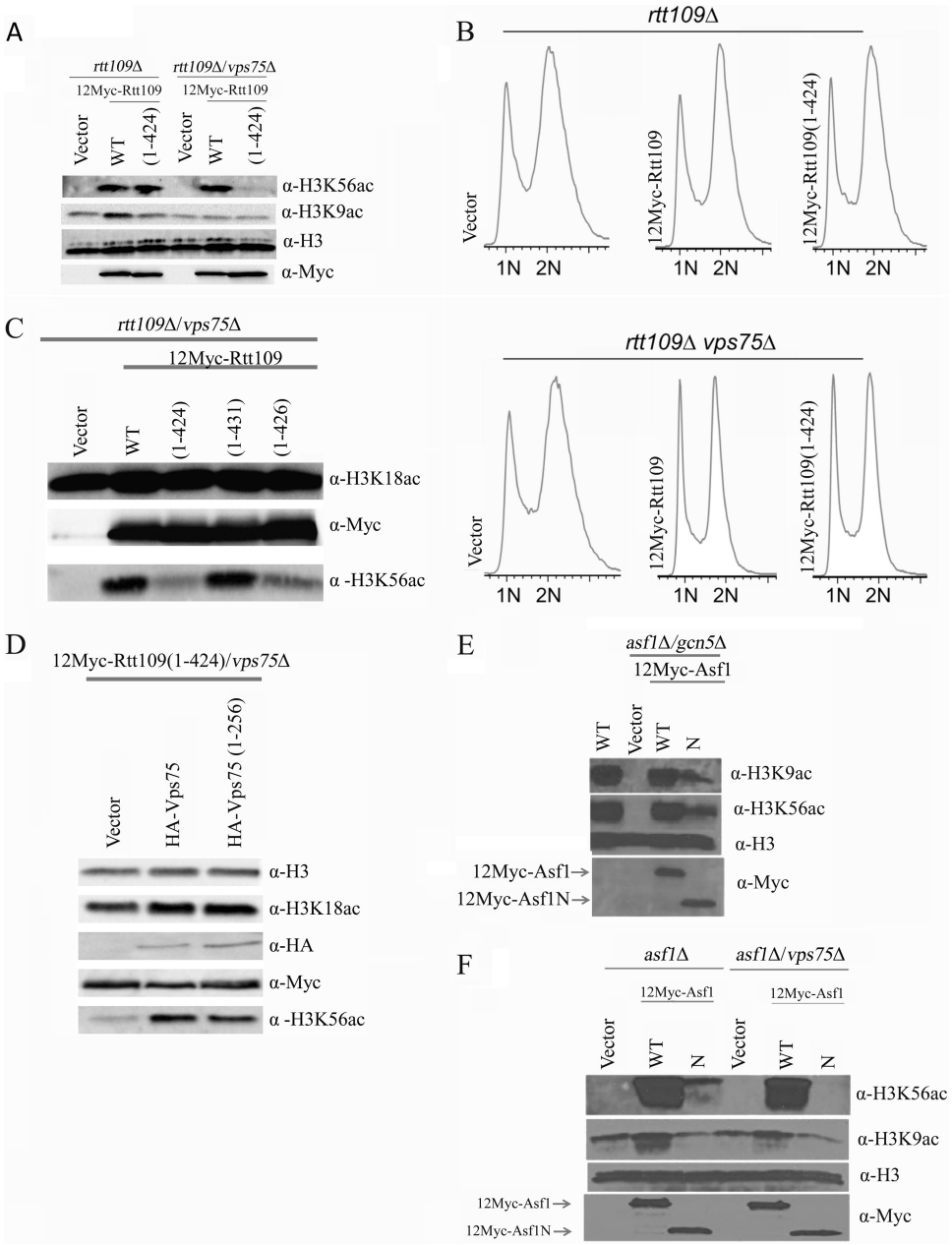

FIG 5 The role of Vps75 and the carboxyl terminus of Asf1 in H3K56 acetylation *in vivo*. (A and C to F) WCE were prepared from the indicated strains, separated by 15% SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with indicated antibodies. (B) FACS analysis indicates no significant change in cell cycle profile of the *rtt109*Δ or *rtt109* Δ*vps75*Δ strain expressing either the full-length *12MYC-RTT109* or the *12MYC-RTT109*(*1–424*) mutant.

424). Taken together, the *in vitro* results suggest that Rtt109C is important for Asf1-stimulated but not Vps75-stimulated catalysis.

Both Vps75 and the C terminus of Asf1 are important for enhancing H3K56ac *in vivo*. Rtt109(1-424) in combination with Asf1 showed *in vitro* reduced H3K56ac (Fig. 4A), suggesting that Rtt109C is required for Asf1 to fully enhance the activity of the HAT. *In vivo*, however, Rtt109(1-424) does not show a significant decrease in levels of H3K56ac (Fig. 2C), suggesting that the truncated HAT is not solely dependent on Asf1 to enhance H3K56ac. Because *in vitro* Vps75 enhances H3K56ac by Rtt109 independently of the Rtt109C (Fig. 3C and 4C), we hypothesized that *in vivo* Rtt109(1-424) is partially depending on Vps75 for full H3K56ac catalysis. We therefore expressed the *12MYC-RTT109*(*1-424*) mutant in the *rtt109*Δ *vps75*Δ strain and, consistent with this hypothesis, we observed very small amounts of H3K56ac in contrast to the results with the full-length Rtt109 control (Fig. 5A). Interestingly, despite the fact that H3K56ac levels were low, the *12MYC-RTT109*(*1-424*) *vps75*Δ strain did not show significantly slow growth or sensitivity to hydroxyurea (see Fig. S5 in the supplemental material). The identical FACS profiles of the WT and Rtt109(1-424) indicate that the observed decrease in H3K56ac is not a consequence of altered cell cycle kinetics of Rtt109(1-424) (Fig. 5B). The C terminus of Vps75 also has a sim-

ilar Lys/Arg-rich sequence at its C terminus (60). Although its deletion did not affect H3 acetylation levels (60), we were interested to determine whether the two Lys/Arg-rich sequences could function in a redundant manner. We first ensured that it is the Lys/Arg-rich sequence in Rtt109 (and not the C-terminal five amino acids) that is important by assessing H3K56ac levels in an *rtt109*Δ *vps75*Δ strain expressing two additional Rtt109 deletion clones, the *12MYC-Rtt109*(*1-426*) and *12MYC-RTT109*(*1-431*) mutants (Fig. 2A). We observed that, when expressed in the *rtt109*Δ *vps75*Δ strain, the *12MYC-RTT109*(*1-431*) mutant resulted in WT levels of H3K56ac, but the *12MYC-RTT109*(*1-426*) mutant showed a decrease in H3K56ac identical to that of the *12MYC-RTT109*(*1-424*) mutant (Fig. 5C). Because the Lys/Arg-rich sequence is present in 12Myc-Rtt109(1-431) but not in 12Myc-Rtt109(1-426), we conclude that it is the Lys/Arg-rich sequence that is important for function. Next, to investigate possible redundancy of the Lys/Arg-rich sequence with that of Vps75, we first expressed the full-length *HA-VPS75* in the background of the *rtt109*Δ *vps75*Δ strain expressing the *12MYC-RTT109*(*1-424*) mutant. Importantly, we complemented H3K56ac levels (Fig. 5D), which confirms the *in vivo* importance of Vps75 for normal levels of H3K56ac when Rtt109(1-424) is present. Next, we tested the ability of the *HA-VPS75*(*1-256*) mutant to complement the H3K56ac defect of the *rtt109*Δ *vps75*Δ strain expressing the *12MYC-RTT109*(*1-424*) mutant. Similar to what we observed for cells expressing the full-length *HA-VPS75*, we complemented the defect in H3K56ac with the *HA-VPS75*(*1-256*) mutant (Fig. 5D), which lacks the Lys/Arg-rich-containing C terminus of Vps75 (60). Thus, the Lys/Arg-rich sequence of Rtt109 is not redundant with that of Vps75.

Our *in vitro* assays suggested that the carboxyl terminus of Asf1 functions in H3K56ac catalysis (Fig. 4B). Therefore, we next expressed the *12MYC-ASF1N* mutant in *asf1*Δ *gcn5*Δ cells and, despite the fact we saw rescue of the slow-growth phenotype (see Fig. S6 in the supplemental material), again we observed only partial rescue of both H3K56ac and H3K9ac (Fig. 5E) compared to expression of *12MYC-ASF1*, suggesting that the carboxyl terminus of the chaperone is involved in H3 acetylation. Consistent with the C terminus of Asf1 having a role in H3K56ac, when we expressed the *12MYC-ASF1N* mutant in *asf1*Δ *vps75*Δ cells, we observed no rescue of H3K56ac (Fig. 5F). In addition, *12MYC-ASF1N vps75*Δ cells were slow growing and sensitive to hydroxyurea (see Fig. S7). Taken together, these experiments suggest that *in vivo* Asf1 and Vps75 are both important for full H3K56 acetylation.

K290 in Rtt109 is important for Vps75-dependent activities. Although previous *in vitro* studies have shown that auto-acetylation of Rtt109 at K290 is important for its activity, the functional role of the lysine is still unclear *in vivo*. To test whether K290 is important for H3K9ac catalysis, we expressed in *rtt109*Δ *gcn5*Δ cells the *12MYC-RTT109K290R* mutant encoding a K290R change in Rtt109 that prevents acetylation but retains the positive charge of the residue and the *12MYC-RTT109K290Q* mutant encoding a K290Q change in Rtt109 that mimics constitutive acetylation of the residue. Interestingly, unlike H3K56ac which showed little change, both mutants showed low levels of H3K9ac (Fig. 6A) compared to full-length Rtt109 even though their interaction with Vps75 was not significantly affected (Fig. 6B). Thus, K290 of Rtt109 appears important for H3K9ac catalysis. 12Myc-Rtt109DDAA, which has both D187 and D188 mutated to alanines, was also used as a negative control since it mimics a previously described putative catalytically inactive Rtt109 (14, 16). Because H3K9ac is a Vps75-related activity of Rtt109, we tested whether K290 is also important for *in vivo* Vps75-dependent H3K56ac (demonstrated in Fig. 5A). Therefore, we deleted the carboxyl terminus of Rtt109K290R and Rtt109K290Q and expressed them in *rtt109*Δ cells. When we expressed either *12MYC-RTT109*(*1-424*)*K290R* or *12MYC-RTT109*(*1-424*)*K290Q* mutant in *rtt109*Δ cells, we observed very little rescue of H3K56ac compared to when we expressed the *12MYC-RTT109*(*1-424*) and *12MYC-RTT109* mutants (Fig. 6C). Because the physical interaction of the two mutants with Vps75 was not significantly affected (see Fig. S8 in the supplemental material), we attribute the decrease of K56ac to the mutation of K290. Collectively, our results show that *in vivo* K290 is important for Vps75-related activities by Rtt109.

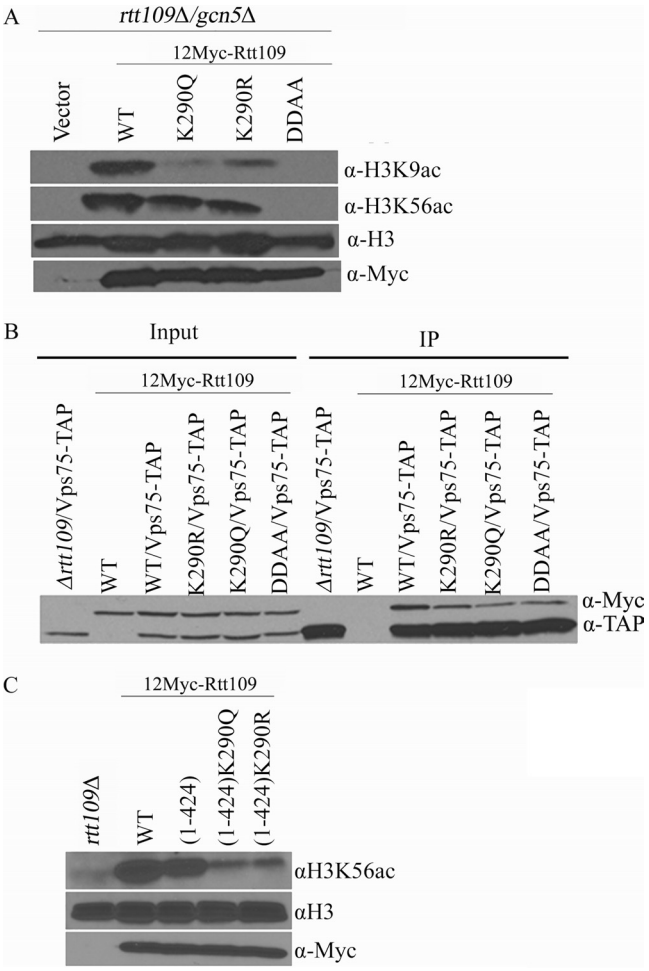

FIG 6 Lysine 290 of Rtt109 is important for *in vivo* H3K56ac and H3K9ac. (A) *12MYC-RTT109K290Q rtt109*Δ and *12MYC-RTT109K290R rtt109*Δ strains show significantly decreased levels of H3K9ac but not H3K56ac *in vivo*. Western blotting was performed as described in the legend of Fig. 5A. (B) 12Myc-Rtt109K290Q and 12Myc-Rtt109K290R are able to interact with Vps75-TAP. Immunoprecipitations were done as described in the legend of Fig. 3A. (C) *12MYC-RTT109*(*1–424*)*K290Q rtt109*Δ and *12MYC-RTT109*(*1–424*)*K290R rtt109*Δ strains have significantly decreased levels of H3K56ac *in vivo*. WCE were prepared from the indicated strains, separated by 15% SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with anti-Myc, anti-H3K56ac, and anti-H3.

DISCUSSION

In this study, we have investigated chaperone regulation of the fungal HAT Rtt109. We have shown that *in vitro* linker histone H1 acetylation is a chaperone-specific regulated activity of Rtt109. Consistent with previously demonstrated functional links between Rtt109-Vps75 and Gcn5 (24), *in vitro* linker histone acetylation provides another common substrate for the two HATs. The Vps75 chaperone has homology to the Nap1 histone chaperone. Yeast Nap1 has been shown to mediate assembly of H1 onto chromatin (61, 62). Since Vps75 is a member of the NAP family of histone chaperones, this provides a mechanistic basis to Rtt109-Vps75 linker histone acetylation. Is this a relevant *in vivo* activity? Future studies will address this, but it is worth mentioning that a recent study showed that human Gcn5 acetylates H1.4 at K34ac during transcription activation (63). Keeping in mind its evolutionary relationship with Rtt109, it will be interesting to determine whether p300/CBP also acetylates linker histone.

Additionally, in this study we have demonstrated that a small basic patch at the C terminus of Rtt109 is required *in vivo* for optimal H3K56ac. Our data suggest that in the absence of Rtt109C, Vps75 becomes important for H3K56ac catalysis by Rtt109. Finally, we show that K290 in Rtt109 is required for Vps75-related H3K56ac activities of the HAT. Taken together, our data provide new insights into the chaperone control of Rtt109.

Different models have been proposed to account for the complex interplay between Rtt109, Vps75, and Asf1 with respect to H3 acetylation. For example, Rtt109-Vps75 could acetylate H3 bound as an H3-H4 dimer to Asf1. Some evidence for this arises from the simple fact that expression of *ASF1* is essential for Rtt109-based H3K9 and H3K56 acetylation *in vivo* and that Rtt109-Asf1 catalyzes H3K56ac *in vitro* in the absence of *VPS75*. In addition, Rtt109-Vps75 acetylates H3K56ac more efficiently *in vitro* on H3-H4 prebound to Asf1 than on H3-H4 dimers alone (41). Our data could be reconciled with this model if the function of the Lys/Arg-rich sequence at the Rtt109 C terminus is to synergize with Asf1, as is suggested by our *in vitro* data. According to this model, Vps75 would functionally substitute for the Rtt109 carboxyl terminus in Rtt109(1–424), and both Vps75 and Rtt109C would therefore have redundant roles in mediating H3K56ac. Further, we also showed that Rtt109C is essential for H3K9ac *in vivo*. *In vitro*, however, in the presence of Vps75, Rtt109(1–424) appears to catalyze H3K9ac as efficiently as full-length Rtt109 (Fig. 3C). These data could be described by the model if Asf1 has an inhibitory role on Rtt109-mediated H3K9ac and if Rtt109C, in addition to Vps75, is required to overcome the inhibition. Asf1 has been previously shown to function like this, blocking H3 and H4 acetylation by the SAS complex *in vitro* (64). A hypothetical function for this type of inhibitory activity of acetylation of N-terminal tails could be to protect acetylated histones from the action of nuclear histone deacetylases (HDACs) before their assembly into chromatin (32). At this point, there exists no clear evidence of this ternary complex other than the fact that the three proteins can be copurified in the presence of H3-H4 and a cross-linker (41).

Alternatively, based on clear *in vivo* and *in vitro* requirements of Vps75 for Rtt109-based H3K9ac, the transfer model proposes that Rtt109-Vps75 acetylates H3K9ac and H3K56ac on H3 bound to Vps75 before subsequent transfer to Asf1, which would mediate its nuclear transport and passage in replication-dependent chromatin assembly pathways. Our data can be reconciled with this model, again if we envision the C terminus of Rtt109 physically interacting with Asf1. Our *in vitro* assays that suggest that the carboxyl terminus of Rtt109 functions in H3K56ac catalysis (Fig. 4A and B) are consistent with this although further work using *in vitro* protein interaction assays will be required to test whether deleting the carboxyl terminus of Rtt109 affects the interaction with Asf1. According to this model, when the ability of Rtt109-Vps75 to acetylate H3 is abolished through either *VPS75* deletion or the Rtt109 K290R mutation, the yeast relies on Rtt109 acetylating histone H3 bound to Asf1, and in the case of Rtt109(1–424), this acetylation would occur with the low efficiency we observed *in vitro*. Although we favor this second model, the resolution of Rtt109, Asf1, and Vps75 interplay clearly requires further analysis. For example, it will be informative to determine structurally exactly how Vps75 physically interacts with H3. In addition, it will be informative to clarify the relative contribution of Rtt109-Vps75's cytoplasmic and nuclear roles (60) and the *in vivo* contribution of the Asf1 C terminus to CAF-1 (and other possible) interaction(s) that exist (36).

We have also shown that K290 in Rtt109 is important for Vps75-related H3 acetylation by Rtt109. Albaugh et al. (51) showed that Rtt109 auto-acetylation of K290 enhances *in vitro* activity of the HAT in the presence of Vps75. Based on their *in vitro* and our *in vivo* evidence, we support the idea that K290ac could act as a "switch" to control Vps75-mediated H3 acetylation by Rtt109.

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