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,a Christoph F. Kurat,a Michael-Christopher Keogh,a,b Jack F. Greenblatt,a,b,c,e Jeffrey S. Fillingham,a

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

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 modifications (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 α-N-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...
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 vitro role for Vps75 in H3K56ac is unclear since H3K56ac levels are not abolished in vps75Δ 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 Ndel and Sal 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 [14C]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 MgCl2, 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 Motive), c-Myc (1:2,000; Santa Cruz), and TATA-binding protein (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 (OD600) 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 optical density at 600 nm (OD600) of 1–4 with ice-cold IP buffer (10 mM Tris, pH 8, 150 mM NaCl, 1.5 mM MgOAc, 0.1% NP-40). Cells were resuspended in 0.85 ml of 10 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 protease inhibitor cocktail (Roche). Cells were lysed with 1 ml of glass beads using a Mini-Bead-Beater-8 (Biospec Products) at maximum speed at 4°C for 5 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 washing buffer (50 mM Tris, pH 8, 150 mM NaCl, 1.5 mM MgOAc, 0.15% NP-40, 5 mM EDTA) was added to the supernatant, which 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

May 2013 Volume 12 Number 5 ec.asm.org 655

ec.asm.org 655
from chicken erythrocytes that includes the four core histones mixed with linker histones H1 and H5, along with 14C-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-Rtt109) 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 evolutionarily 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 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-
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 (424KPRKKAKALPKT436 in \textit{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 \textit{rtt109} \textit{gcn5} 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 \textit{rtt109Δ} \textit{gen5Δ} strain (Fig. 2B, the rescued strain grows at the same rate as \textit{gen5Δ}), as well as H3K56ac and some H3K9ac (Fig. 2C). In contrast, the deletion mutant \textit{12Myc-Rtt109Δ}(1–424), which rescued the slow-growth phenotype of \textit{rtt109Δ} \textit{gen5Δ} cells (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) \textit{gcn5Δ} strain resembles that of a \textit{vps75Δ} \textit{gcn5Δ} 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 \textit{in vivo} and acetylate H3K9 \textit{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 and 12Myc-Rtt109(1–424) in a \textit{rtt109} \textit{gcn5} strain and examined their ability to interact with Vps75. As expected, 12Myc-Rtt109 expressed under the control of the ADH1 promoter on a CEN-based plasmid rescued the slow-growing phenotype of \textit{rtt109Δ} \textit{gen5Δ} cells. However, 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) \textit{gcn5Δ} strain resembles that of a \textit{vps75Δ} \textit{gcn5Δ} 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.

**FIG 2** The carboxyl terminus of fungal Rtt109s contain a Lys/Arg-rich sequence which is essential for H3K9ac in \textit{S. cerevisiae}. (A) Using the ClustalW algorithm, \textit{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 \textit{rtt109Δ} \textit{gen5Δ} 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.
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
to 424 from Rtt109 contacts Vps75 in the Rtt109-Vps75 (59). We next tested whether 6\textit{HIS-Rtt109}(1–424) is functional in HAT assays performed in the presence of 6\textit{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\textit{HIS-Rtt109}, 6\textit{HIS-Rtt109}(1–424), and 6\textit{HIS-Vps75} and performed in vitro HAT assays. We observed that in the presence of 6\textit{HIS-Vps75}, 6\textit{HIS-Rtt109}(1–424) catalyzed H3K56ac, H3K9ac (Fig. 3B), and vertebrate linker histone acetylation (see Fig. S4 in the supplemental material) similarly to 6\textit{HIS-Rtt109}.

To rigorously compare in vitro HAT activities of full-length 6\textit{HIS-Rtt109} and 6\textit{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\textit{HIS-Vps75}. Western blot analysis of the products of the HAT assays showed that even at low concentrations, 6\textit{HIS-Rtt109}(1–424) appears as efficient as full-length 6\textit{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 \textit{in vitro} for full Rtt109-Asf1 activity. Since Rtt109(1–424) showed a slight but reproducible decrease in H3K56ac \textit{in vivo} (Fig. 2C), we tested whether Asf1 synergized any differently with Rtt109(1–424) than with full-length Rtt109 in \textit{in vitro} HAT assays. Again, we performed HAT assays using several dilutions of 6\textit{HIS-Rtt109} and 6\textit{HIS-Rtt109}(1–424) with a constant amount of 6\textit{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\textit{HIS-Rtt109} and 6\textit{HIS-Rtt109}(1–424) with a constant concentration of either 6\textit{HIS-Asf1}, 6\textit{HIS-Asf1N}, or 6\textit{HIS-Vps75}. Both versions of Asf1 enhanced the activity of full-length 6\textit{HIS-Rtt109} equally (Fig. 4B). Similar to what we observed above (Fig. 4A), again we observed reduction in H3K56ac activity when 6\textit{HIS-Rtt109}(1–424) was incubated with 6\textit{HIS-Asf1} (Fig. 4B). However, in the presence of 6\textit{HIS-Asf1N}, 6\textit{HIS-Rtt109}(1–424) was even more reduced in H3K56ac activity (Fig. 4B), suggesting that the carboxyl terminus of 6\textit{HIS-Asf1} could function in H3K56ac catalysis. As observed above (Fig. 3C), we again observed no difference in 6\textit{HIS-Vps75}-stimulated H3K56ac activity between 6\textit{HIS-Rtt109} and 6\textit{HIS-Rtt109}(1–
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 rtt109Δ vps75Δ 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) vps75Δ 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 rtt109Δ vps75Δ strain expressing two additional Rtt109 deletion clones, the 12MYC-Rtt109Δ(1–426) and 12MYC-RTT109Δ(1–431) mutants (Fig. 2A). We observed that, when expressed in the rtt109Δ vps75Δ strain, the 12MYC-RTT109Δ(1–431) mutant resulted in WT levels of H3K56ac, but the 12MYC-RTT109Δ(1–426) mutant showed a decrease in H3K56ac identical to that of the 12MYC-RTT109Δ(1–424) mutant (Fig. 5C). Because the Lys/Arg-rich sequence is present in 12MYC-Rtt109Δ(1–431) but not in 12MYC-Rtt109Δ(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 rtt109Δ vps75Δ strain expressing the 12MYC-RTT109Δ(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 rtt109Δ vps75Δ strain expressing the 12MYC-RTT109Δ(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 asf1Δ gcn5Δ 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, we also observed the 12MYC-ASF1N mutant in asf1Δ vps75Δ cells, we observed no rescue of H3K56ac (Fig. 5F). In addition, 12MYC-ASF1N vps75Δ 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 H3K56ac 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 rtt109Δ gcn5Δ cells the 12MYC-RTT109ΔK290R mutant encoding a K290R change in Rtt109 that prevents acetylation but retains the positive charge of the residue and the 12MYC-RTT109ΔK290Q 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-Rtt109ΔDDAA, 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 rtt109Δ cells. When we expressed either 12MYC-RTT109Δ(1–424)K290R or 12MYC-RTT109Δ(1–424)K290Q mutant in rtt109Δ cells, we observed very little rescue of H3K56ac compared to when we expressed the 12MYC-RTT109Δ(1–424) and 12MYC-RTT109Δ 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-RTT109ΔK290Q rtt109Δ and 12MYC-RTT109ΔK290R rtt109Δ 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-RTT109Δ(1–424)K290Q rtt109Δ and 12MYC-RTT109Δ(1–424)K290R rtt109Δ 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 H3K36ac 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 in vivo evidence, we support the idea that K290ac could act as a “switch” to control Vps75-mediated H3 acetylation by Rtt109.

ACKNOWLEDGMENTS

We thank Judith Recht and Julianne Kus for critical reading of the manuscript. We also thank Roberto Botelho for pB415A-12Myc, Lucy Pemberton for plasmids encoding wild-type and mutant HA-VPS75, and Pierre Defoix for anti-Hho1 antibody. We also thank Adam Rosebrook (University of Toronto) for assistance with FACS analyses. We thank Brenda Andrews (University of Toronto) for providing laboratory space and reagents to C.F.K.

J.S.F. is supported by an NSERC Discovery grant and by a grant from the Banting Research Foundation. T.S. was supported by an NSERC USRA (Undergraduate Student Research Awards). M.-C.K. is supported by National Institutes of Health grant 1R21 ES019966-01. C.F.K. is supported by an EMBO long-term fellowship.

REFERENCES

1. Graziano V, Gerchman SE, Schneider DK, Ramakrishnan V. 1994. Histone H1 is located in the interior of the chromatin 30-nm filament. Nature 368:351–354.
2. Selth L, Svejstrup JQ. 2007. Vps75, a new yeast member of the NAP histone chaperone family. J. Biol. Chem. 282:12358–12362.
3. Bannister AJ, Kouzarides T. 2011. Regulation of chromatin by histone modifications. Cell Res. 21:381–395.
4. Solbe RE, Cook RG, Perry CA, Annunziato AT, Allis CD. 1995. Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. Proc. Natl. Acad. Sci. U.S.A. 92:1237–1241.
5. Parthun MR, Widom J, Gottschling DE. 1996. The major cytoplasmic histone acetyltransferase in yeast: links to chromatin replication and histone metabolism. Cell 87:85–94.
6. Verreault A, Kaufman PD, Kobayashi R, Stillman B. 1998. Nucleosomal DNA regulates the core-histone-binding subunit of the human Hat1 acetyltransferase. Curr. Biol. 8:96–108.
7. Poveda A, Pambianco M, Tafrov S, Tordera V, Sternglanz R, Sendra R. 2004. Hif1 is a component of yeast histone acetyltransferase B, a complex mainly localized in the nucleus. J. Biol. Chem. 279:16033–16043.
8. Ruiz-Garcia AB, Sendra R, Galiana M, Pambianco M, Perez-Otín JE, Tordera V. 1998. HAT1 and HAT2 proteins are components of a yeast nuclear histone acetyltransferase enzyme specific for free histone H4. J. Biol. Chem. 273:12599–12605.
19. Kuo MH, Brownell JE, Sobel RE, Ranalli TA, Cook RG, Edmondson DG, Roth SY, Allis CD. 1996. Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines. Nature 383:269–272.

20. Masumoto H, Hawke D, Kobayashi R, Verreault A. 2007. Yeast Gcn5 functions in two multisubunit complexes to acetylate cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. Nat. Cell Biol. 9:1229–1236.

21. Filillingham J, Recht J, Silva AC, Suter B, Emili A, Saglaj I, Krogan NJ, Allis CD, Keogh MC, Greenblatt JF. 2008. Chaperone control of the activity and specificity of the histone H3 acetyltransferase Rtt109. Mol. Cell. Biol. 28:4342–4353.

22. Grant PA, Duggan L, Cote J, Roberts SM, Brownell JE, Candau R, Ohba Masumoto H, Hawke D, Kobayashi R, Verreault A. 2012. Histone acetylase Rtt109 is required for proper H3K56 acetylation: a chromatin mark associated with S phase in mitosis and meiosis. Proc. Natl. Acad. Sci. U. S. A. 109:6988–6993.

23. Sijlje HH, Ning EA. 2001. Identification of human Asf1 chromatin assembly factors as substrates of Tousled-like kinases. Curr. Biol. 11:1068–1073.

24. Tamburini BA, Carson JJ, Linger JG, Tyler JK. 2006. Dominant mutants of the Saccharomyces cerevisiae Asf1 histone chaperone bypass the need for CAF-1 in transcriptional silencing by altering histone and Sir protein recruitment. Genetics 173:599–610.

25. Jiao Y, Seeger K, Lauterwasser A, Gaubert F, Gauvois R, Mann C, Ochsenbein F. 2012. Surprising complexity of the Asf1 histone chaperone-Rad53 kinase interaction. Proc. Natl. Acad. Sci. U. S. A. 109:2866–2871.

26. Blennow J, Almén H, Kestilä S, Lehtomäki P, Juvonen K, Jokitalo E, Mollby R, Carlsson G, Lundgren J. 2010. Histone h3 lysine 56 acetylation as an antifungal therapeutic strategy. Nat. Med. 16:745–750.

27. Antczak AJ, Tsubota B, Kaufman PD, Berger JM. 2006. Structure of the yeast histone H3–Asf1 interaction: implications for chaperone mechanism, species-specific interactions, and epigenetics. BMC Struct. Biol. 6:26. doi: 10.1186/1423-0120-6-26.

28. English CM, Adkins MW, Carson JJ, Churchill ME, Tyler JK. 2006. Structural basis for the histone chaperone activity of Asf1. Cell 127:495–508.

29. Recht J, Tsubota T, Tanny JC, Diaz RL, Berger JM, Zhang X, Garcia BA, Shabanowitz J, Burlingame AL, Hunt DF, Kaufman PD, Allis CD. 2006. Histone chaperone Asf1 is required for histone H3 lysine 56 acetylation, a modification associated with S phase in mitosis and meiosis. Proc. Natl. Acad. Sci. U. S. A. 103:6998–6993.

30. Xhemalce B, Miller KM, Driscoll R, Masumoto H, Jackson SP, Kouzrides T, Verreault A, Arcangioli B. 2007. Regulation of histone H3 lysine 56 acetylation in Schizosaccharomyces pombe. J. Biol. Chem. 282:15040–15047.

31. Davey CA, Sargent DF, Luger K, Maeder AW, Richmond TJ. 2002. Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. J. Mol. Biol. 319:1097–1113.

32. Neumann H, Hancock SM, Buning R, Routh A, Chapman L, Somers J, English CM, Adkins MW, Carson JJ, Churchill ME, Tyler JK. 2001. Identification of human Asf1 chromatin assembly factors as substrates of Tousled-like kinases. Curr. Biol. 11:1068–1073.

33. Shabanowitz J, Burlingame AL, Hunt DF, Kaufman PD, Allis CD. 2006. A method for genetically installing site-specific acetylation in recombinant histones de novo. Nucleic Acids Res. 34:2999–3004.

34. Tang Y, Holbert MA, Wurtele H, Meeth K, Rocha W, Gharib M, Jiang EH, Thibault P, Verreault A, Raymond M. 2010. Chaperone control of the histone H3 lysine 56 acetylation as an antifungal therapeutic strategy. Nat. Med. 16:774–780.
49. Fillingham JS, Thing TA, Vythilingum N, Keuroghlian A, Bruno D, Golding GB, Pearlman RE. 2004. A non-long terminal repeat retrotransposon family is restricted to the germ line micronucleus of the ciliated protozoan Tetrahymena thermophila. Eukaryot. Cell 3:157–169.

50. Veron M, Zou Y, Yu Q, Bi X, Selmi A, Gilson E, Defossez PA. 2006. Histone H1 of Saccharomyces cerevisiae inhibits transcriptional silencing. Genetics 173:579–587.

51. Kao CF, Osley MA. 2003. In vivo assays to study histone ubiquitylation. Methods 31:59–66.

52. Kurat CF, Lambert JP, van Dyk D, Tsui K, van Bakel H, Kaluarachchi S, Friesen H, Kainth P, Nislow C, Figys D, Fillingham J, Andrews BJ. 2011. Restriction of histone gene transcription to S phase by phosphorylation of a chromatin boundary protein. Genes Dev. 25:2489–2501.

53. Kobor MS, Venkatasubrahmanyan S, Meneghini MD, Gin JW, Jennings JL, Link AJ, Madhani HD, Rine J. 2004. A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS Biol. 2:E131. doi:10.1371/journal.pbio.0020131.

54. Abshiru N, Ippersiel K, Tang Y, Yuan H, Marmorstein R, Verreault A, Thibault P. 2012. Chaperone-mediated acetylation of histones by Rtt109 identified by quantitative proteomics. J. Proteomecs [Epub ahead of print.] doi:10.1016/j.jprot.2012.09.026.

55. Herrera JE, Bergel M, Yang XJ, Nakatani Y, Bustin M. 1997. The histone acetyltransferase activity of human GCN5 and PCAF is stabilized by corepressors. J. Biol. Chem. 272:27253–27258.

56. Burgess RJ, Zhang Z. 2010. Roles for Gcn5 in promoting nucleosome assembly and maintaining genome integrity. Cell Cycle 9:2979–2985.

57. Tjeertes JV, Miller KM, Jackson SP. 2009. Screen for DNA-damage-responsive histone modifications identifies H3K9Ac and H3K56Ac in human cells. EMBO J. 28:1878–1889.

58. Kasinsky HE, Lewis JD, Dacks JB, Ausio J. 2001. Origin of H1 linker histones. FASEB J. 15:34–42.

59. Su D, Hu Q, Zhou H, Thompson JR, Xu RM, Zhang Z, Mer G. 2011. Structure and histone binding properties of the Vps75-Rtt109 chaperone-lysine acetyltransferase complex. J. Biol. Chem. 286:15625–15629.

60. Keck KM, Pemberton LF. 2011. Interaction with the histone chaperone Vps75 promotes nuclear localization and HAT activity of Rtt109 in vivo. Traffic 12:826–839.

61. Kepert JF, Mazurkiewicz J, Heuvelman GL, Toth KF, Rippe K. 2005. NAP1 modulates binding of linker histone H1 to chromatin and induces an extended chromatin fiber conformation. J. Biol. Chem. 280:34063–34072.

62. Mazurkiewicz J, Kepert JF, Rippe K. 2006. On the mechanism of nucleosome assembly by histone chaperone NAP1. J. Biol. Chem. 281:16462–16472.

63. Kamieniarz K, Izzo A, Dundr M, Tropper P, Ozretic L, Kirfel J, Scheer E, Tropel P, Wisniewski JR, Tora L, Viville S, Buettner R, Schneider R. 2012. A dual role of linker histone H1.4 Lys 34 acetylation in transcriptional activation. Genes Dev. 26:797–802.

64. Sutton A, Shia WJ, Band D, Kaufman PD, Osada S, Workman JL, Sternglanz R. 2003. Sas4 and Sas5 are required for the histone acetyltransferase activity of Sas2 in the SAS complex. J. Biol. Chem. 278:16887–16892.