Acetylation of Lysine 56 of Histone H3 Catalyzed by RTT109 and Regulated by ASF1 Is Required for Replisome Integrity*

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In budding yeast, acetylation of histone H3 lysine 56 (H3-K56) is catalyzed by the Rtt109-Vps75 histone acetyltransferase (HAT) complex, with Rtt109 being the catalytic subunit, and histone chaperone Asf1 is required for this modification. Cells lacking Rtt109 are susceptible to perturbations in DNA replication. However, how Asf1 regulates acetylation of H3-K56 and how loss of H3-K56 acetylation affects DNA replication are unclear. We show that at low concentrations the Rtt109-Vps75 complex acetylates H3-K56 in vitro when H3/H4 is complexed with Asf1, but not H3/H4 tetramers, recapitulating the in vivo requirement of Asf1 for H3-K56 acetylation using recombinant proteins. Moreover, the Rtt109-Vps75 complex interacts with Asf1-H3/H4 but not Asf1. In vivo, the Rtt109-Asf1 interaction is also dependent on the ability of Asf1 to bind H3/H4. Furthermore, the Rtt109 homolog in Schizosaccharomyces pombe (SpRtt109) also displayed an Asf1-dependent H3-K56 HAT activity in vitro. These results indicate that Asf1 regulates H3-K56 acetylation by presenting histones H3 and H4 to Rtt109-Vps75 for acetylation, and this mechanism is likely to be conserved. Finally, we have shown that cells lacking Rtt109 or expressing H3-K56 mutants exhibited significant reduction in the association of three proteins with stalled DNA replication forks and hyper-recombination of replication forks stalled at replication fork barriers of the ribosomal DNA locus compared with wild-type cells. Taken together, these studies provide novel insight into the role of Asf1 in the regulation of H3-K56 acetylation and the function of this modification in DNA replication.

Histones, the structural proteins of eukaryotic chromatin, are subjected to a variety of post-translational modifications such as acetylation, methylation, and ubiquitination (1–3). Distinct modifications regulate different cellular processes, including gene transcription, DNA repair, and DNA replication by modulating chromatin structure and/or recruiting effector proteins to chromatin (4–7). Lysine residues located in the N-terminal tail domains of histones H3/H4 are well known to be acetylated (8). Recently, several groups have reported the acetylation of lysine 56 of H3 (H3-K56) (9–13), a lysine residue located in the core domain of H3. Acetylation of H3-K56 is unique in that this modification is catalyzed by a novel histone acetyltransferase (HAT) complex, Rtt109-Vps75, with Rtt109 being the catalytic subunit (14–19). However, it is not clear how Asf1 regulates H3-K56 acetylation as Asf1 has no enzymatic activity (21), and Asf1 competes with Vps75 to interact with Rtt109 (17). Results from recent studies suggest that two different models explain how Asf1 regulates H3-K56 acetylation. First, Asf1 forms a complex with Rtt109 that is different from the Rtt109-Vps75 complex. Supporting this idea, it has been reported that Asf1 competes with Vps75 to interact with Rtt109 in vitro (17). Second, because cells expressing the Asf1(V94R) mutant that cannot bind to histone H3/H4 lose H3-K56 acetylation (22, 24), it is proposed that Asf1 functions to present H3/H4 dimers for Lys-56 acetylation by Rtt109. However, little if any biochemical evidence has accumulated to support this model. Moreover, unlike Asf1 that is not essential for cell viability in Saccharomyces cerevisiae, Asf1 in Schizosaccharomyces pombe is essential (25). Thus, it is not clear whether the Asf1-dependent H3-K56 acetylation is also conserved in S. pombe.

Acetylation of H3-K56 is restricted to newly synthesized histones and occurs predominantly during S phase of the cell cycle (9, 13), suggesting H3-K56 acetylation mediated by Rtt109 might be involved in DNA replication. Indeed, cells lacking Rtt109 or bearing H3 mutants deficient in H3-K56 acetylation

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** The abbreviations used are: H3-K56, histone H3 lysine 56; HAT, histone acetyltransferase; Asf1, anti-silencing factor 1; FACs, fluorescence activated cell sorting; ChiP, chromatin immunoprecipitation; ARS, autonomously replicating sequences; pol ε, DNA polymerase ε; PCNA, proliferating cell nuclear antigen; RFC3, replication factor C 3; rDNA, ribosomal DNA; ERC, extrachromosomal rDNA circles; CPT, camptothecin; HU, hydroxyurea; MMS, methylmethane sulfonate; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; DSP, dithiobis(succinimidyl propionate); IP, immunoprecipitation.
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Purification of Recombinant Histone H3/H4 Tetramers and Formation of Asf1-H3/H4 Complexes—Recombinant Drosophila H3/H4 tetramers were purified as described (31). Recombinant yeast Asf1-(1–167) was first purified using glutathione-Sepharose beads (Amersham Biosciences) and then eluted by digestion with precision protease. The eluted proteins were further purified using gel filtration chromatography. To purify Asf1-dH3/H4 complexes, purified Asf1 was incubated with recombinant Drosophila H3/H4 tetramers overnight at 4 °C, and the protein mixtures were separated using gel filtration chromatography.

HAT Assays—Rtt109-Vps75 complexes were purified as described (19). The HAT activities of Rtt109-Vps75 complexes were determined as described previously, with minor modifications (15). Briefly, samples were incubated at 30 °C for 30 min with 15-μl reaction mixtures that contained 50 mM Tris-HCl, pH 8.0, 5% (w/v) glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM PMSF, and 6 pmol of [3H]acetyl-CoA (4.3 mCi/mmol; Amersham Biosciences). When indicated, recombinant Drosophila H3/H4 tetramers (dH3/H4; 1 μg) or Asf1-dH3/H4 complexes (1.25 μg) were added as substrates. Whenever possible, we used the same amounts of H3 from either dH3/H4 tetramer or from the Asf1-dH3/H4 complexes, as determined by Western blot analysis for HAT assays. After incubation at 30 °C for 30 min, one-half of each reaction mixture (7.5 μl) was spotted onto P-81 phosphocellulose paper filters (Upstate Biotechnology, Inc.) and air-dried. After extensive washing, the radioactivity of air-dried filters was measured using a liquid scintillation counter. The second half of each sample was resolved using 15% SDS-PAGE, and the H3-labeled proteins were detected by autoradiography. To detect whether H3-K56 was acetylated, samples were incubated with unlabeled acetyl-CoA at 30 °C for 30 min, and Western blot analysis using antibodies that recognized acetylated lysine 56 of H3 was performed.

GST Pulldown Assays to Detect Interactions between Asf1 and Rtt109—GST-Rtt109-Vps75 complexes were formed by incubating recombinant Vps75 (10 μg) with GST-Rtt109 (2.5 μg) bound to glutathione-Sepharose beads in a binding buffer containing 25 mM Tris, pH 7.5, 1 mM EDTA, 0.01% Nonidet P-40, and 200 mM NaCl. The complexes were washed to remove unbound Vps75 before using for assays, and GST-Rtt109-Vps75 complexes were incubated with recombinant Asf1 or Asf1-dH3/H4 complexes (2.5 μg or 5.0 μg) in binding buffer overnight at 4 °C. After washing, the bound proteins were eluted using 1× SDS sample buffer, resolved using SDS-PAGE, and revealed by Coomassie Brilliant Blue staining. GST-REGα (2.5 μg), a proteosome binding protein, was used as a negative control. [35S]Methionine-labeled Asf1 was produced using in vitro transcription/translation coupled as described (32). In vitro translated Asf1 (5 μl) was incubated with 2.5 μg of H3/H4 tetramers for 2 h at 4 °C and then mixed with GST-Rtt109 or GST-Rtt109-Vps75 complex. The binding assays were described essentially above, and bound Asf1 was detected by autoradiography.

Asf1-Rtt109 in Vivo Binding Assay—To test whether Asf1 binds to Rtt109 in vivo, the plasmids containing either Asf1–13myc-pRS414, Asf1 (V94R)–13myc-pRS414, Rtt109-2FLAG-pRS416, and vectors were transformed into the asf1Δ rtt109Δ double deletion mutant. To detect interaction between Asf1 and Rtt109, we used the cross-linker dithiobis[succinimidylpropionate] (DSP, Pierce) that cross-link proteins in close proximity as described (33). Briefly, yeast cells were washed and frozen in IP buffer (20 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM MgCl2, 1% Triton X-100, 1 mM PMSF, 1 mM benzamidine, and 1 mM Pefabloc). Frozen yeast cells were ground using a coffee grinder in the presence of dry ice. The resulting cell lysate was cross-linked with 3 mM DSP for 30 min at 4 °C with shaking. Cell lysate was diluted with the dilution buffer (150 mM KCl, 5 mM MgCl2, 1% Triton X-100) after quenching with 150 mM Tris-HCl, pH 7.5 (containing 1 mM PMSF, 1 mM benzamidine, and 1 mM Pefabloc SC), for 5 min on ice. After clarification by

exhibit spontaneous chromosome breaks mainly during the S/G2/M phase of the cell cycle (14, 15). In addition, the rtt109 mutant displays synthetic lethal phenotypes with mutations in several proteins involved in DNA replication, including PCNA, DNA polymerase α, and Cdc45 (15, 18, 26–28). Together, these phenotypes and genetic interactions suggest that H3-K56 acetylation mediated by Rtt109 is required for certain aspects of DNA replication. Supporting this idea, genetic interaction studies have indicated that Rtt101, a ubiquitin ligase that is involved in replication for damaged and natural pause sites on DNA (29), functions downstream of H3-K56 acetylation (18). However, how cells lacking H3-K56 acetylation are sensitive to perturbations in DNA replication is unclear. As mentioned above, Asf1 is required for the acetylation of H3-K56 in yeast cells, and interestingly, Asf1 mutants exhibit defects in the stability of proteins at stalled replication forks (30). Thus, H3-K56 acetylation catalyzed by Rtt109 and regulated by Asf1 may be required for replisome integrity.

Using in vitro HAT assays with purified protein complexes, we show at low protein concentrations that Rtt109-Vps75 complexes acetylate Asf1-H3/H4 but not H3/H4 in vitro. Furthermore, Asf1 interacts with Rtt109 or Rtt109-Vps75 complexes only in the presence of H3/H4. In yeast cells, the Rtt109-Asf1 interaction could be detected only in the presence of a cross-linker. More importantly, Rtt109 fails to bind the Asf1 (V94R) mutant that is defective in H3/H4 binding. Furthermore, similar to S. cerevisiae Rtt109, the sequence homolog of Rtt109 from Drosophila melanogaster displays HAT activity toward H3-K56, and this activity is enhanced by Asf1 in vitro. Thus, we have recapitulated the dependence of Asf1 for H3-K56 acetylation by Rtt109 using recombinant proteins, and we provide biochemical evidence supporting the idea that Asf1 functions to present H3/H4 to Rtt109-Vps75 HAT complexes for H3-K56 acetylation. Finally, we show at low protein concentrations that Rtt109-Vps75 complexes acetylate Asf1-H3/H4 but not H3/H4 in vitro. Furthermore, Asf1 interacts with Rtt109 or Rtt109-Vps75 complexes only in the presence of H3/H4. In yeast cells, the Rtt109-Asf1 interaction could be detected only in the presence of a cross-linker. More importantly, Rtt109 fails to bind the Asf1 (V94R) mutant that is defective in H3/H4 binding. Furthermore, similar to S. cerevisiae Rtt109, the sequence homolog of Rtt109 from Drosophila melanogaster displays HAT activity toward H3-K56, and this activity is enhanced by Asf1 in vitro. Thus, we have recapitulated the dependence of Asf1 for H3-K56 acetylation by Rtt109 using recombinant proteins, and we provide biochemical evidence supporting the idea that Asf1 functions to present H3/H4 to Rtt109-Vps75 HAT complexes for H3-K56 acetylation. Finally, we show that rtt109Δ and H3-K56R mutant cells exhibit reduced levels of replication proteins at stalled replication forks and elevated levels of recombination at forks paused at replication fork barriers at the rDNA locus. Together these results provide an explanation why cells lacking H3-K56 acetylation in cells lacking Rtt109 are sensitive to perturbations in DNA replication.
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centrifugation, the supernatant was loaded to pre-washed M2 beads (Sigma) and incubated for 2 h at 4 °C. After washing away unbound proteins, proteins bound to M2 beads (Sigma) were eluted using 1 × SDS sample buffer, resolved using SDS-PAGE, and detected using antibodies against the FLAG epitope (Sigma) (Rtt109-2FLAG), the MYC epitope (Asf1–13Myc), or against Vps75. Cell extracts were also analyzed using antibodies against H3 and H3 acetylated at lysine 56.

Generation of S. pombe Rtt109 Expression Constructs and Purification of Recombinant Protein—The S. pombe Rtt109 open reading frame was amplified from a cDNA library using Pfu DNA polymerase (Stratagene) and the following primers: rtt109 forward (5’-AAAAGGTCAATACGCTGACTTGGTCAGAAG-3’) and rtt109 reverse (5’-AAAACCTGAGTTTATTTTTCACTTTTTCCGTGAGC-3’). The resulting Rtt109 PCR product was cloned into the pGEX-4T1 vector, sequenced, and used for protein expression. To express GST-tagged S. pombe Rtt109 (GST-Rtt109-S.P), the plasmid-transformed Escherichia coli cells were grown to an A600 of 0.6 at 22 °C, and 0.1 mm isopropyl β-D-thiogalactoside was added to induce expression of the recombinant protein overnight at 18 °C. Cells were collected by centrifugation, and lysates were prepared by passing cells through a French press in lysis buffer (25 mm Tris, pH 7.5, 150 mm NaCl, 1% Triton X-100, 1 mm PMSF, 1 mm benzamidin, 1 mm Pefabloc). The resulting lysate was cleared by centrifugation, and the supernatant was incubated with glutathione-Sepharose beads (Amersham Biosciences) at 4 °C for 3 h. After washing away unbound proteins, GST-Rtt109-S.P was eluted with 100 mm glutathione and dialyzed against TBS.

Chromatin Immunoprecipitation (ChIP) Assays—Preparation and immunoprecipitation (IP) of formaldehyde cross-linked chromatin were performed as described previously (13). For each sample, 25 ml of log phase cells were arrested in G1 using α-factor at 30 °C for 3 h. Cells were then collected by centrifugation and washed with H2O twice prior to releasing into 0.2 M HU at 30 °C. At the indicated times in the figures/figure legends following release into HU-containing media, 1% formaldehyde was added to each sample, and the samples were incubated on a shaker at low speed for 30 min at 30 °C. The cross-linking was then quenched with 125 mm glycine. Extracts were prepared by mechanical disruption with glass beads in 500 μl of ChIP lysis buffer (50 mm HEPES/KOH, pH 7.5, 140 mm NaCl, 1 mm EDTA, 1% Triton X-100, 0.1% deoxycholate, 1 mm PMSF, 1 mm Pefabloc, 1 μg/ml bacitracin, 1 mm benzamidin). The DNA was sheared by sonication to an average length of 500 bp to 1 kb. Cross-linked protein-DNA complexes were immunoprecipitated using polyclonal antibodies against either Rfc3 or PCNA and harvested with protein G beads. The immune complexes were then washed twice with ChIP lysis buffer, twice with ChIP lysis buffer containing 500 mm NaCl, and twice with Tris- LiCl buffer (10 mm Tris, pH 8.0, 250 mm LiCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 1 mm EDTA), and the DNA was isolated by adding 10% of Chelex-100 (Bio-Rad). The amount of DNA recovered from each IP was quantified using real time PCR (iQ Cycler, Bio-Rad) and normalized against DNA recovered from IPs where normal rabbit serum was used for IP. The sequences of primers used in this study are listed in supplemental Table S1.

Analysis of Extrachromosomal rDNA Circles (ERC)—To determine the effect of loss of H3-K56 acetylation on formation of extrachromosomal circles, total genomic DNA was prepared as described previously (34). Briefly, cells were digested with zymolyase in 500 μl of sorbitol solution (0.9 m sorbitol, 0.1 m Tris-HCl, pH 8.0, EDTA, 1% (v/v) 2-mercaptoethanol, 20 μg/ml zymolyase) for 30 min at 37 °C with gentle shaking. Cells were then lysed at 65 °C for 20 min by adding 80 μl of 10% SDS.
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After centrifugation for 5 min at 14,000 rpm, the DNA was precipitated using ethanol. The pellet was resuspended in 300 μl of TE containing 0.1 μg/ml RNase and incubated for 30 min at 37 °C. DNA (30 μg) was separated on a 0.8% 0.5 TBE-agarose, and Southern blot hybridization was performed with a 32P-labeled rDNA probe using randomly primed DNA labeling kit (Roche Applied Science). The probes against actin was pre-purified from E. coli and analyzed by SDS-PAGE followed by Coo massie Brilliant Blue (CBB) staining. B and C, Rtt109 from S. pombe migrates at a slightly lower molecular weight than recombinant Rtt109 from S. cerevisiae. GST-tagged recombinant S. pombe Rtt109 (Rtt109-S.P) and S. cerevisiae Rtt109 (Rtt109-S.C) were purified from E. coli and analyzed by SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining. B and C, Rtt109 from S. pombe migrates slightly lower than recombinant Rtt109, similar to Rtt109 from S. cerevisiae. Purified recombinant Drosophila H3/H4 tetramers alone (dH3/H4; black bars) or in a complex with Asf1 (Asf1-dH3/H4; white bars) were incubated with increasing amounts of recombinant Rtt109 from S. pombe (0.5, 1.0, and 1.5 μg) in the presence of [3H]acetyl-CoA. The incorporated [3H]acetate was detected by scintillation counting (B), or reaction mixtures were resolved using SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining to detect total proteins and autoradiography to detect 3H-acetylated proteins (C). Recombinant REG, a proteosome-binding protein, was used as a negative control. D, Asf1 stimulates the acetylation of histone H3 lysine 56 by Rtt109 from S. pombe. Experiments were performed as described in B and C, except using unlabeled acetyl-CoA. Samples were analyzed by Western blot (WB) using antibodies recognizing H3 acetylated at lysine 56 (H3-K56Ac) or total histone H3 (H3).

RESULTS

Reconstitution of Asf1-dependent H3-K56 Acetylation Using Recombinant Proteins—Asf1 is essential for H3-K56 acetylation in yeast cells (14–16, 18, 22, 30). Others have reported that in vitro Asf1 stimulates the enzymatic activity of Rtt109 but not Rtt109-Vps75 complexes (17). Because we have shown that Vps75 is an integral component of Rtt109-Vps75 complexes (19), we compared the ability of Rtt109-Vps75 complexes to acetylate H3 when presented as H3/H4 tetramers versus Asf1-H3/H4 complexes. For these in vitro assays, we first purified Asf1-H3/H4 complexes using recombinant Asf1 and recombinant Drosophila H3/H4 tetramers (dH3/H4) (Fig. 1A) and Rtt109-Vps75 complexes. As Rtt109-Vps75 complexes at high enough concentrations acetylate H3-K56 acetylation in vitro in the absence of Asf1 (19), three amounts of Rtt109-Vps75 complexes were used (0.2, 1.0, and 5.0 ng) for HAT assays. For all concentrations tested, Asf1 strongly stimulated the HAT activity of Rtt109-Vps75 complexes toward H3 (Fig. 1, B and C), whereas H4 was not acetylated. Moreover, Rtt109-Vps75 acetylated H3-K56 much more efficiently when H3 was present in the Asf1-H3/H4 complexes compared with H3/H4 tetramers (Fig. 1D). More importantly, when present at low protein concentrations, Rtt109-Vps75 complexes acetylated H3-K56 only when H3/H4 was in a complex with Asf1 but not as H3/H4 tetramers alone (Fig. 1, C and D). Thus, Asf1 also stimulates the activity of Rtt109-Vps75 complexes in vitro, and the Asf1-dependent acetylation of H3-K56 by Rtt109 can be recapitulated using recombinant proteins.

We have recently shown that the N-terminal tail domains of H3/H4 are required for efficient H3-K56 acetylation by Rtt109-Vps75 complexes (19) (Fig. 1E). Therefore, we next tested whether the tail domains of H3/H4 are also required for H3-K56 acetylation in the presence of Asf1. Full-length and “tailless” H3/H4 (sH3/H4) alone or in a complex with Asf1 were purified and used as substrates for HAT assays. As shown in Fig. 1E, Rtt109-Vps75 complexes acetylate H3-K56 on H3 present in Asf1-H3/H4 complexes much better than H3 in H3/H4 tetramers, irrespective of whether full-length or tailless H3/H4 was used. Moreover, Rtt109-Vps75 complexes acetylated H3-K56 more efficiently when full-length H3/H4 rather than tailless H3/H4 was in a complex with Asf1. Thus, the N-terminal tail domains of H3/H4 are required for efficient H3-K56 acetylation by Rtt109-Vps75 complexes, even in the presence of Asf1.

The S. pombe Rtt109 Homolog Displays Asf1-stimulated HAT Activity toward H3-K56—Sequence homologs of S. cerevisiae Rtt109 (scRtt109) were identified in several fungal species (15). In addition, acetylation of H3-K56 has been reported in S. pombe (22). Therefore, we next tested whether the sequence homolog of scRtt109 from S. pombe (gene name SPBC342.06) also functions as an H3-K56 HAT. We cloned the gene, which we have termed spRTT109, and sub-
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Rtt109-Vps75 binds to Asf1-H3/H4 Complexes but not Asf1 Alone—Rtt109 from both yeast species acetylated H3-K56 more efficiently in the presence of Asf1. Asf1 may directly modulate the activity of Rtt109 by forming a complex with Rtt109. Alternatively, Asf1 regulates H3-K56 acetylation through its effect on histone H3/H4. To differentiate these two models, we analyzed the binding of Asf1 to GST-tagged Rtt109 and Rtt109-Vps75 complexes in the presence and absence of H3/H4. First we tested whether in vitro translated [35S]methionine-labeled Asf1 bound to GST-Rtt109 or GST-Rtt109-Vps75 complexes in the presence or absence of H3/H4. Asf1 alone did not associate with Rtt109 or Rtt109-Vps75 complexes under a variety of conditions tested. However, Rtt109 or Rtt109-Vps75 complex could pull down Asf1 in the presence of H3/H4 (Fig. 3A and data not shown), suggesting Rtt109 and Rtt109-Vps75 complexes bind to Asf1 only when Asf1 is in Asf1-H3/H4 complexes. To test this idea, we analyzed the interaction between Asf1 and Rtt109-Vps75 complexes using recombinant Asf1 purified from bacteria, alone or in a complex with H3/H4. As shown in Fig. 3B, GST-Rtt109-Vps75 complexes bound to Asf1-H3/H4 complexes, and under the same conditions, no interaction was detected with Asf1 alone. These results suggest that Asf1 regulates H3-K56 acetylation mainly through its effect on histones H3/H4.

While our manuscript was in preparation, Tsubota et al. (17) reported that Asf1 interacted with Rtt109 in vitro and proposed that Asf1 formed a distinct complex with Rtt109 than the Rtt109-Vps75 complex. These results directly contradict our results presented in Fig. 3, A and B. One possible explanation for these conflicting results is that different recombinant Rtt109 was used in both in vitro studies. In this regard, we noticed that GST-Rtt109 that we used for binding assay displays intrinsic H3-K56 HAT activity (15), whereas the recombinant Rtt109 that Tsubota et al. (17) used had no detectable H3-K56 HAT activity. Because of the concerns that different recombinant proteins may yield different results, we translated Asf1 in vitro for performing in vitro HAT assays. As compared with GST-tagged scRtt109 (scRtt109), spRtt109 migrated at a slightly lower molecular weight when analyzed using SDS-PAGE (Fig. 2A). Importantly, spRtt109 displayed HAT activity toward H3, but not H4 (Fig. 2, B and C), and acetylated H3-K56 in vitro. While our manuscript was under review, others have shown that the sequence homolog of Rtt109 in S. pombe is an H3-K56 HAT (20). The Asf1 in S. pombe is essential for cell viability, whereas Asf1 in S. cerevisiae is not. Therefore, we next asked whether Asf1 is also involved in the regulation of H3-K56 acetylation in S. pombe by comparing the ability of spRtt109 to acetylate H3 in Asf1-H3/H4 to that in H3/H4 tetramers. Interestingly, spRtt109 acetylated H3-K56 more efficiently when H3 was present in Asf1-H3/H4 complexes compared with H3 in H3/H4 tetramers (Fig. 2, B–D). Together, these results indicate that the scRtt109 homolog in S. pombe is also an H3-K56 HAT and that H3-K56 acetylation in S. pombe is likely to be regulated by Asf1.
We reasoned that the inability to detect interactions between Rtt109 and Asf1 in yeast cells was because of the transient nature of this interaction. Therefore, we tested whether Asf1 interacts with Rtt109 in the presence of the cross-linker DSP. DSP is a homobifunctional and membrane-permeable cross-linker that cross-links proteins in close proximity. Asf1 co-immunoprecipitated with Rtt109 and Vps75 after cross-linking with DSP (Fig. 3, C and D). The interactions between Asf1 and Rtt109 were specific as Asf1 could not be detected in control samples in which Rtt109 was not expressed. It has been reported that the Asf1(V94R) mutant cannot bind to H3 and H4 (22, 36, 37). Moreover, H3-K56 acetylation is lost in the Asf1(V94R) mutant cells (22). Therefore, we tested whether Asf1(V94R) binds to Rtt109-Vps75 complexes. More importantly, under the same conditions to detect interactions between Asf1 and Rtt109-Vps75, Asf1(V94R) could not co-immunoprecipitate with Rtt109. These results indicate that the ability of Asf1 to bind H3/H4 is a prerequisite for Rtt109 to interact with Asf1 in yeast cells and to provide an explanation why the Asf1(V94R) mutant loses H3-K56 acetylation. Taken together, these in vitro and in vivo binding studies provide strong support for the idea that Asf1 serves as a component of substrates to regulate H3-K56 acetylation by Rtt109-Vps75 HAT.

Cells Lacking Rtt109 Exhibit Reduced Levels of Replication Proteins at Stalled Replication Forks—Cells lacking Asf1 or Rtt109 exhibit no detectable levels of H3-K56 acetylation and are sensitive to DNA-damaging agents (14–16, 28). Epistatic analysis of asf1/H9004 and rtt109/H9004 mutant cells indicate that the asf1/H9004 mutant cells toward DNA-damaging agents is because of...
loss of H3-K56 acetylation (14, 18). We have now confirmed this result as asf1Δ rtt109Δ double deletion mutant cells displayed sensitivity toward DNA-damaging agents (camptothecin, HU, methylmethane sulfate, and ultraviolet (UV) radiation) to a similar degree as cells for each single deletion alone (supplemental Fig. S1). These results suggest that Asf1 and Rtt109 function in the same genetic pathway to suppress the sensitivity of yeast cells toward DNA-damaging agents. Because Asf1 is required for the maintenance of replication fork integrity (30), we hypothesized that loss of H3-K56 acetylation may affect replisomes, the protein machine present at replication forks (38, 39). To test this idea, we first analyzed whether deletion of RTT109 affects the association of HA-tagged DNA polymerase ε with stalled replication forks using ChIP assays, as depicted in Fig. 4A. Cells were first synchronized at G1 with α-factor and then released into fresh media containing HU. The fractions of cells were then either collected for analysis of DNA content using FACS (Fig. 4B) or fixed using formaldehyde and processed for ChIP assays at 15-min intervals following release into HU-containing media. As reported previously (40), pol ε was enriched at the early replication origins ARS305 (Fig. 4C) and ARS607 (Fig. 4D) after wild-type cells were released into S phase for 30 or 45 min in the presence of HU, whereas relatively little pol ε was detected at these two origins 15 and 60 min after release into HU-containing media. In addition, relatively little pol ε was detected at sites 12 and 14 kb from ARS305 (Fig. 4C) and ARS607 (Fig. 4D) at any time point following release into media containing HU. Thus, the presence of HU appears to stall replication forks and block pol ε from reaching distal sites. Remarkably, the binding of pol ε to stalled replication forks was reduced substantially in rtt109Δ mutant cells, as compared with wild-type cells (Fig. 4, C and D).

The reduction of pol ε at stalled replication forks in rtt109Δ mutant cells could be due to a defect in the initiation of DNA replication. To test this idea, we analyzed whether ARS305 replicated normally in the presence of HU in wild-type and rtt109Δ mutant cells. DNA was isolated from cells synchronized at G1 using α-factor or cells released into HU for 30 min after synchronizing at G1. The relative amount of DNA from HU-blocked cells versus that from α-factor-arrested cells was then quantified using real-time PCR with primers for amplification of the early replication origin ARS305 and late replication origin ARS501. As shown Fig. 4E, 2-fold more DNA was recovered from the early replication origin ARS305 than the late replication origin ARS501 for both wild-type and rtt109Δ mutant cells. These data are consistent with the idea that DNA replication initiates normally at early replication origins in the presence of HU in both wild-type and rtt109Δ mutant cells, whereas the firing of late replication origins is blocked by HU. Thus, the reduction of pol ε at stalled replication forks in rtt109Δ mutant cells is not likely due to defects in the initiation of DNA replication. Instead, we suggest that Rtt109 functions at a step other than initiation of DNA replication.

To gain additional insight into how cells lacking H3-K56 acetylation affect DNA replication proteins at stalled replication forks, we determined whether the association of PCNA and Rfc3, two other proteins present at replication forks, was affected by mutations in Rtt109, as well as an H3 mutant unable to be acetylated at lysine 56 (H3-K56R), using ChIP assays. PCNA is essential for DNA replication and serves as the clamp for DNA polymerases. Rfc3 is a subunit of replication factor C, which loads PCNA onto primer-template junctions (38, 39, 41). PCNA and Rfc3 were enriched at the early replication origins (ARS305 and ARS607) in wild-type cells that were synchronized in G1, using α-factor and released into S phase in the presence of HU, whereas relatively little PCNA and Rfc3 were detected at sites 12 and 14 kb from ARS305 and ARS607 (Fig. 5, A–D). Importantly, the binding of PCNA and Rfc3 to stalled replication forks was reduced substantially in rtt109Δ and H3-K56R mutant cells (Fig. 5, A–D), and this reduction was not because of reduced PCNA and Rfc3 protein levels in the mutant
Regulation of Acetylation of H3 Lysine 56 by Asf1

A Loss of H3-K56 Acetylation Results in Accumulation of Extrachromosomal rDNA Circles—To study the consequences of loss of H3-K56 acetylation on replication fork integrity, we analyzed how a loss of H3-K56 acetylation affects replication at the rDNA locus. In budding yeast, the rDNA locus consists of about 150 copies of actively transcribed repeats. Each repeat contains one origin, but only 15% of the origins are active in a given S phase. Replication of these 150 tandem copies proceeds bidirectionally from activated origins in the beginning and then the leftward-moving replication forks stall at the replication fork barrier near 3’ end of the 35S transcription unit (42). Fork instability at this locus or loss of silencing results in accumulation of ERCs (29, 34). It has been shown that cells lacking Rtt101, a ubiquitin ligase that functions downstream of Rtt109, accumulates ERCs because of defects in replication (29). We monitored formation of rDNA circles in wild-type, rtt109Δ, H3-K56R, or as a control in rtt101Δ mutant cells (Fig. 6). In agreement with published results, significantly more ERCs have been accumulated in the rtt101Δ mutant cells compared with wild-type cells. Importantly, accumulation of ERCs was observed in the rtt109Δ and H3-K56R mutant cells compared with wild-type cells, suggesting hyper-recombination of the rDNA locus (Fig. 6). Because the rtt101Δ mutant cells have no detectable defect in silencing,3 the observed accumulation of rDNA circles is not likely because of compromised rDNA silencing in the mutant cells. Instead, we suggest that hyper-recombination at the rDNA locus is because of instability of forks in the rtt109Δ mutant cells. Interestingly, less ERCs were observed in cells lacking H3-K56 acetylation than the rtt101Δ mutant cells that have H3-K56 acetylation (Fig. 6). These results suggest that loss of H3-K56 acetylation results in hyper-recombination of replication forks stalled at the replication fork barrier and that Rtt109 may function differently from Rtt101 to maintain genome stability, at least at the rDNA locus.

DISCUSSION

Here we demonstrate that Asf1 is required for the efficient acetylation of H3-K56 by Rtt109-Vps75 HAT complexes in vitro and, furthermore, that Rtt109-Vps75 complexes only bind Asf1-H3/H4 complexes but Asf1 alone in vitro. In yeast cells, in contrast to stable interaction between Rtt109 and Vps75, the Asf1-Rtt109 interaction is only detectable in the presence of a chemical cross-linker. More importantly, the Asf1(V94R) mutant defective in binding to H3/H4 could not bind to Rtt109 in vivo. These results support the idea that Asf1 functions mainly as a component of substrates to regulate H3-K56 acetylation by Rtt109-Vps75 complexes. The function of Asf1 in the regulation of H3-K56 acetylation is likely conserved in the S. pombe as we found that the sequence homolog of scRtt109 in S. pombe also exhibits Asf1-stimulated H3-K56 HAT activity in vitro. Finally, we have shown that H3-K56 acetylation mediated by Rtt109 is required for the stable association of several proteins at stalled replication forks and suppression of recombination at forks stalled at the rDNA locus. Thus, H3-K56 acetylation catalyzed by Rtt109 and regulated by Asf1 appears to maintain genome stability through its effect on DNA replication forks.

Several labs have shown that Asf1 is required for H3-K56 acetylation in yeast cells (14, 15, 22, 43). However, Rtt109 acetylates H3-K56 in vitro in the absence of Asf1 (14, 15). Moreover, it is not clear from these studies whether Asf1 functions as a component of Rtt109 or mainly as a component of substrate H3/H4. Thus, the exact role of Asf1 in H3-K56 acetylation is unclear. Here we present three lines of evidence supporting the idea that H3/H4 dimers in a complex with Asf1 serve as the ideal substrate for H3-K56 acetylation by Rtt109-Vps75 complexes. First, we have shown that Rtt109-Vps75 HAT complexes, when present at low enzyme concentrations, acetylate H3-K56 only when H3/H4 is in complex with Asf1, recapitulating the in vivo requirement of Asf1 for H3-K56 acetylation using recombinant proteins. Second, using GST pulldown

3 J. Han and Z. Zhang, unpublished observations.
assays, we found that Asf1, either translated in vitro or recombinant, associates with recombinant Rtt109-Vps75 complexes only in the presence of histones. Third, we have shown that Asf1 does not form a stable complex with either Rtt109 or Rtt109-Vps75 complexes in yeast cells. Instead, the interaction between Asf1 and Rtt109-Vps75 complex could only be detected in the presence of the cross-linker DSP and is dependent on the ability of Asf1 to interact with histones. These results suggest that Asf1 is not likely to directly affect the HAT activity of Rtt109, but instead mediates the effective presentation of H3/H4 to the Rtt109-Vps75 complex and thereby regulates H3-K56 acetylation. The formation of complexes containing H3/H4 dimers and Asf1 could allow for an increased binding affinity between Rtt109-Vps75 complexes and H3/H4 complexes. Alternatively, Asf1 may induce H3 to undergo conformational changes so that H3-K56 is more readily accessible for acetylation by Rtt109-Vps75 complexes. In this regard, in contrast to the H3/H4 tetramers in solution (44), H3/H4 are present as dimers in the Asf1-H3/H4 complexes (23, 37). Moreover, H3/H4 dimers adopt distinct conformations when in a complex with Asf1, as compared with the conformation of H3/H4 in nucleosomes (37, 45). However, the exact location of lysine 56 on H3 in the Asf1-H3/H4 complexes has not been revealed by the crystal structures. Thus, future studies are needed to understand why Asf1-H3/H4s are better substrates for the Rtt109-Vps75 complex than H3/H4 tetramers.

Mutations in the RTT109 exhibit synthetic lethal/slow growth phenotypes with mutations in several genes involved in either DNA replication or homologous recombination (14, 15, 18, 28), suggesting that either DNA replication or homologous recombination (14, 15, 18, 28), suggesting that the reduction of proteins at stalled replication forks does not mean that replication forks are collapsed in the mutant cells. Instead, the reduction of proteins at stalled replication forks may lead to breakdown in some special chromosome locations such as natural pause sites. Indeed, we observe hyper-recombination at these locations downstream of Rtt109 and is required for replication through natural pause sites and damaged DNA templates (29).

Future studies are needed to understand how H3-K56 acetylation is involved in the regulation of genome stability.

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