Rtt109 Is Required for Proper H3K56 Acetylation

A CHROMATIN MARK ASSOCIATED WITH THE ELONGATING RNA POLYMERASE II

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Jessica Schneider, Pratibha Bajwa, Farley C. Johnson, Sukesh R. Bhauik, and Ali Shilatifard

From the Department of Biochemistry, Saint Louis University School of Medicine, St. Louis, Missouri 63104, and the Department of Biochemistry and Molecular Biology, Southern Illinois University, School of Medicine, Carbondale, Illinois 62901, and the Saint Louis University Cancer Center, Saint Louis University School of Medicine, St. Louis, Missouri 63104.

Histone acetylation has been shown to be required for the proper regulation of many cellular processes including transcription, DNA repair, and chromatin assembly. Acetylation of histone H3 on lysine 56 (H3K56) occurs both during the prereplicative and mitotic S phase and persists throughout DNA damage repair. To learn more about the molecular mechanism of H3K56 acetylation and factors required for this process, we surveyed the genome of the yeast Saccharomyces cerevisiae to identify genes necessary for this process. A comparative global proteomic screen identified several factors required for global H3K56 acetylation, which included histone chaperone Asf1 and a protein of an unknown function Rtt109 but not Spt10. Our results indicate that the loss of Rtt109 results in the loss of H3K56 acetylation, both on bulk histone and on chromatin, similar to that of asf1Δ or the K56Q mutation. RTT109 deletion exhibits sensitivity to DNA damaging agents similar to that of asf1Δ and H3K56Q mutants. Furthermore, Rtt109 and H3K56 acetylation appear to correlate with actively transcribed genes and associate with the elongating form of polymerase II in yeast. This histone modification is also associated with some of the transcriptionally active puff sites in Drosophila. Our results indicate a new role for the Rtt109 protein in the proper regulation of H3K56 acetylation.

Once referred to as an amorphous coating or passive polymeric counterion of DNA, histone proteins are now known to be integral to the structural organization of eukaryotic DNA and are required for its packaging into chromatin (1). Chromatin appears as a series of “beads on a string,” consisting of individual nucleosome “beads” and the linker DNA “string.” Each nucleosome consists of eight core histone proteins (usually two each of H3, H4, H2A, and H2B) (2–6). X-ray studies suggest that histone amino termini tails extend outside the core of the nucleosome and are available for interactions with the DNA and/or other proteins (3). In fact, histone tails are the sites of many potential structure-altering covalent modifications (7, 8). Posttranslational modifications of histones, including acetylation, phosphorylation, methylation and ubiquitination, occur on the unstructured tail of histones protruding from the core of the nucleosome (3, 4, 7, 8). Changes in chromatin architecture may be regarded as a regulatory principle allowing discrimination of transcriptionally active and inactive regions that facilitate the execution of a gene expression program and directs the establishment of specialized structures such as centromeres and telomeres (5, 6). Covalent modifications of histones within the nucleosomes control many aspects of chromatin function. Modification of histones by acetylation is a dynamic process that is associated with the regulation of gene expression. Histones can be acetylated on multiple lysine residues and their steady-state balance is brought about by the opposing catalytic activities of histone acetyltransferases and histone deacetylases (7).

Acetylation and deacetylation of lysine residues on histones are respectively linked to transcriptional activation or repression. Enzymes responsible for acetylation of histones at different residues were initially characterized as transcriptional co-activators and later as enzymes. For the most part, the histone acetylation pattern is associated with transcriptionally active genes. However, there are some exceptions to this rule on some residues (9).

Lysine 56 of histone H3 (H3K56),2 which is located in the globular domain where it extends toward the DNA major groove at the entry exit point of the DNA superhelix as it wraps around the nucleosome, is acetylated (10, 11). Previously, it was reported that Spt10 is a putative acetyltransferase to be required for cell cycle specific acetylation at histone genes (10). To better learn about the molecular mechanism of H3K56 acetylation, we took advantage of our global proteomic screen (GPS) in Saccharomyces cerevisiae (12). Employing GPS, we have demonstrated that several factors including the histone chaperone protein, Asf1 as well as a protein of unknown function, Rtt109, are required for global H3K56 acetylation both from bulk histone and also on chromatin. Although it was demonstrated via chromatin immunoprecipitation (ChIP) studies that Spt10 is required for proper cell cycle-specific H3K56 acetylation at histone genes (10), our data indicate that this putative histone acetyltransferase is dispensable for global acetylation of H3K56. Our biochemical and molecular analyses have demonstrated that H3K56 acetylation is associated with actively transcribed genes. Rtt109 appears to be directly involved in the regulation of H3K56 acetylation as is enriched with elongating form of RNA polymerase II (Pol II) on chromatin. We also show that the association of H3K56 acetylation as a

2 The abbreviations used are: H3K56, lysine 56 of histone H3; GPS, global proteomic screen; ChIP, chromatin immunoprecipitation; Pol II, polymerase II; HU, hydroxyurea; CPT, camptothecin; ORF, open reading frame.
mark of the elongating form of Pol II is conserved in *Drosophila melanogaster*. All together, our report demonstrates that Rtt109 associates with transcriptionally active genes and is required for proper H3K56 acetylation.

**EXPERIMENTAL PROCEDURES**

**GPS for the Identification of Factors Required for Proper H3K56 Acetylation**—GPS analyses were performed as described previously (12). GPS was carried out using antibodies specific for H3K56 acetylation (Upstate Biotechnology).

**Growth Sensitivity Assays**—Cells were grown in a 5-ml YPD (yeast extract containing peptone plus 2% dextrose) culture and then diluted to a starting $A_{600}$ of 0.1. 10-fold serial dilutions of each culture were made and spotted onto YPD plates containing or lacking either 0.1M hydroxyurea (HU) or 20 μM CPT. Cells were allowed to grow for 48–72 h at 30 °C. The H3K56Q strain was a generous gift from Dr. Paul D. Kaufman, which was used in their previous study (15).

**ChIP Assays**—Formaldehyde-based *in vivo* cross-linking and ChIP assay was performed as described previously (13). For the analysis of Rtt109 recruitment at the *PHO84*, *ADH1*, *GAL1*, and *RPS5* genes, we modified the above ChIP protocol as follows. 800 μl of lysate was prepared from 100 ml of yeast culture. 200 μl of lysate was used for each immunoprecipitation (using 50 μl of IgG-Sepharose or Sepharose beads from GE Healthcare), and

**FIGURE 1. Global proteomic analyses in defining factors required for proper H3K56 acetylation.** A–C, total cellular extracts were prepared from each of the nonessential yeast gene deletion mutants of the *S. cerevisiae* genome. Each extract was subjected to SDS-PAGE, Western blotted, and probed with anti-acetyl-H3 Lys56 antibody. Blue arrows indicate the positions of *spt10Δ* in which H3K56 acetylation is present (A) and *asf1Δ* (B) and *rtt109Δ* (C) where H3K56 acetylation is absent. Red arrows indicate empty wells as plate markers. D, extracts from the *rtt109Δ* strain were titrated and analyzed by SDS-PAGE to determine the level of H3K56 loss in the absence of Rtt109. Western blots probed with the appropriate antibodies demonstrated a loss in H3K56 acetylation. H3 Lys4 trimethylation remained unaffected and anti-acetyl-histone H3 was used as a loading control.

**FIGURE 2. Deletion of RTT109 results in a loss of H3K56 acetylation.** A, a plasmid containing the RTT109 coding sequence, controlled under the GAL1 promoter, was introduced into a RTT109-deficient strain. The addition of this plasmid and not empty vector restored H3K56 acetylation. Extracts were analyzed by SDS-PAGE, Western blotted, and probed using the appropriate antibodies. Antiserum toward multiply acetylated histone H3 was used as our loading control. B, strain deficient of RTT109 is sensitive to HU. Small spots of each strain were plated as 10-fold serial dilutions on YPD plates containing or lacking 0.1 M HU or 20 μM CPT.
the immunoprecipitated DNA sample was dissolved in 10 μl of Tris-EDTA of which 1 μl was used in PCR analysis. In parallel, the PCR for “input” DNA was performed using 1 μl of DNA that was prepared by dissolving purified DNA from 5 μl of lysate in 100 μl of Tris-EDTA.

**Immunostaining of Drosophila Polytenic Chromosomes—** Chromosomes were dissected from third instar wild-type larvae in 1× phosphate-buffered saline (130 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄) and fixed in fixing buffer before squashing following methods described by Silver and Elgin (14). Chromosomal squashes were stained using H3K56 polyclonal antibodies (Upstate Biotechnology) at 1:100 dilution. Chromosome images were obtained using a Leica DM 5000 microscope operated with FW 4000 software.

**RESULTS AND DISCUSSIONS**

**Loss of Yeast ASF1 and RTT109 Alters Global Histone H3 Lysine 56 Acetylation Levels—** GPS surveying the entire nonessential yeast gene deletion consortium was used to seek out genes affecting global H3K56 acetylation (12). Using antibodies directed against acetylated H3K56, both Asf1 and Rtt109 were identified to be required for H3K56 acetylation (Fig. 1, A–C). Indeed, when H3K56 acetylation from bulk histone is measured in ASF1 and RTT109 deletion strains, it appears that global H3K56 acetylation levels are reduced similar to that of K56Q point mutants (Fig. 1D). Although Spt10 was reported to be required for cell cycle-specific H3K56 acetylation at histone genes (10), our data indicate that the loss of Spt10 does not alter global levels of H3K56 acetylation. This indicates that perhaps other histone acetyltransferases may function in proper global H3K56 acetylation.
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**FIGURE 4.** Rtt109 and H3K56 acetylation are associated with actively transcribed genes. A, analysis of Rtt109p recruitment to the GAL1, ADH1, PHO84, and RPS5 genes. A yeast strain expressing TAP-tagged Rtt109 was grown and cross-linked as in Fig. 3A. Immunoprecipitations were performed following modified ChIP protocol (see “Experimental Procedures”). Immunoprecipitated DNAs were analyzed by PCR using primer pairs targeted to the upstream activating sequences, core promoters, and ORFs of the GAL1, ADH1, PHO84, and RPS1 genes. B, H3K56 acetylation is associated with some of the transcriptionally active puff sites on Drosophila polytene chromosomes. Fixed polytene chromosome squashes were prepared from third instar larvae and were immunostained with antibody specific for H3K56. The corresponding phase-contrast image from the same chromosome was also taken to determine active transcriptional puff sites (data not shown).

To support a role for Rtt109 in H3K56 acetylation, our data indicate that RTT109 and K56Q mutant behave similarly in the loss of acetylated H3K56 (Fig. 1D). Indeed, to determine that the loss of Rtt109 is the cause for the defect in H3K56 acetylation in our rtt109 deletion strain, we demonstrated that the introduction of a RTT109 gene in this strain can complement the loss of H3K56 acetylation (Fig. 2A).

**Cells Deleted for RTT109 Exhibit Sensitivity to HU and CPT Similar to That of asf1Δ or K56Q Mutants**—Recently, it was demonstrated that strains deleted for ASF1 or strains bearing a mutation in H3K56 demonstrated an extended doubling time in liquid YPD and presented growth sensitivity to drugs that most significantly effect the S phase such as HU and CPT (10, 11, 15). We therefore tested a role of Rtt109 for growth on such agents. Our data indicate that strains deleted for asf1, rtt109, or the K56Q mutation are defective for growth on HU and CPT (Fig. 2B). This information suggests that similar to Asf1, Rtt109 may play a role in either the modification or deposition of Lys56-acetylated H3 onto chromatin during the S phase, and that Rtt109 maybe crucial for genome stability during replication. However, a role for this modification during the process of transcription has not been clarified.

**H3K56 Acetylation Is Associated with Actively Transcribed Genes in S. cerevisiae**—We next seek to determine whether Lys56 acetylation or Rtt109 are associated with actively transcribed genes and/or required for transcription. Using ChIP, we analyzed the upstream activating sequences, core promoter (Core), open reading frames (ORF1 and ORF2) of two housekeeping genes ADH1 and PHO84, and one regulated gene GAL1 for the presence of acetylated H3K56. We first determined the specificity of our antibody in ChIP by using both wild-type and a strain bearing H3K56Q mutation (Fig. 3A). These studies indicate that a single point mutation in H3K56 results in the loss of ChIP signal demonstrating the specificity of the antibody in ChIP. Furthermore, it appears that not only is H3K56 acetylation associated with the promoter regions of the genes tested, it is also found on the body of genes indicating that it might be associated with the elongating form of RNA polymerase II.

Since our GPS studies indicated a role for both Asf1 and Rtt109 in proper H3K56 acetylation from bulk histone, we wanted to determine whether these factors are also required for proper H3K56 acetylation associated on chromatin.

Indeed, it appears that the loss of either Asf1 or Rtt109 results in the loss of H3K56 acetylation associated with chromatin on all the genes tested in this study (Fig. 3, B and C).

To determine a direct role for Rtt109 in H3K56 acetylation on chromatin, we next determined whether this protein is associated on the chromatin of these genes upon transcription. We therefore used a tandem affinity purification Tagged::Rtt109 strain to perform our ChIP studies. As shown in Fig. 4A, Rtt109 is associated with all three genes tested and most importantly, its ChIP signal appears to increase toward the body of the actively transcribed gene.

To define whether H3K56 acetylation or Asf1 is required for proper pre-initiation complex formation and initiation of transcription, we analyzed for recruitment of TBP and Pol II to the core promoters and coding sequences of the GAL1, ADH1, and PHO84 genes in the ASF1 wild-type and deletion mutant strains. Our ChIP data demonstrate that recruitment of TBP and Pol II to the core promoters of GAL1, ADH1, and PHO84 was not altered in asf1Δ (data not shown). Thus, H3K56 acetylation does not seem to regulate formation of the preinitiation complex assembly (and hence transcriptional initiation) at the core promoters. Similarly, recruitment of Pol II to the coding
sequences of these genes was not changed in the *ASF1* deletion mutant strain (data not shown), thus suggesting H3K56 is not required for overall association of Pol II with the coding regions; however, it is not clear at this time whether the overall rate of transcription is altered in the absence of H3K56 acetylation. However, it is clear that H3K56 acetylation is associated on the ORFs of actively transcribed genes in *S. cerevisiae*.

**H3K56 Acetylation Is Associated with Some of the Transcriptionally Active Puff Sites on Polytene Chromosome of *Drosophila***—Our studies in yeast *S. cerevisiae* indicate that H3K56 acetylation appears to be a mark associated with actively transcribed genes, and it co-localizes with the elongating form of Pol II. Therefore, we wanted to determine whether this mark is also found on actively transcribed genes in metazoans. Using H3K56 antisera, we performed immunolocalization studies to determine the distribution pattern of this histone modification on the third instar larval polytene chromosomes of *Drosophila*. H3K56 acetylation appears to be widely distributed throughout polytene chromosomes and is concentrated at some of the loci that are transcriptionally active, i.e. transcriptional puff sites (Fig. 4B). Our previous studies have demonstrated that elongating form of Pol II, which is phosphorylated on its conserved COOH-terminal domain, is also associated at such transcriptionally active puff sites (16–18).

The dynamic process of the addition and removal of acetyl moiety on histone tails are linked to transcriptional activation or repression. Recently, Workman and colleagues (19) purified and analyzed subunits of the histone deacetylase Rpd3 with the ultimate goal of determining its functions and mechanisms of recruitment beyond targeted promoters. They identified two Rpd3 complexes, Rpd3L (1.2 MDa) and Rpd3S (0.6 MDa). While the subunits of RpdL were consistent with the promoter-rerecruited co-repressor model previously shown, Rpd3S revealed two unique subunits, Rco1 and Eaf3. Their studies demonstrated that histone methylation within the body of actively transcribed genes, by Set2, can be used as a mark for the recruitment of the RpdS complex via Eaf3 to erase transcription elongation-associated acetylation and to suppress intragenic transcription initiation (19, 20). This study has demonstrated the biological significance of elongation-associated histone acetylation and deacetylation in proper regulation of gene expression. In this manuscript, we have demonstrated that: 1) Rtt109 and Asf1 are required for a proper pattern of H3K56 acetylation, analyzed from bulk histone; 2) *RTT109* deletion exhibits sensitivity to DNA damaging agents similar to that of *asf1Δ* and the H3K56Q mutants; 3) H3K56 acetylation and Rtt109 appears to be associated on the body of actively transcribed genes in yeast; 4) H3K56 acetylation also appears to be associated on active transcriptional puff sites in *Drosophila*. Together, these observations suggest a direct role for Rtt109 in proper H3K56 acetylation and its association on the body of actively transcribed genes.

Given the above information several mechanisms for the role of Rtt109 in H3K56 acetylation can be proposed. First, similar to Asf1, Rtt109 may play a role as a histone chaperone and be required for deposition of already Lys56-acetylated H3 into chromatin in the wake of the transcribing polymerase. A second probable mechanism for the role of Rtt109 in H3K56 acetylation could be explained by the ability of this protein to directly modify H3K56. In support of this model, we do observe association of Rtt109 with the transcribing Pol II. As mentioned above, histone acetylation/deacetylation associated with elongating form of RNA polymerase II can play a major role in the regulation of transcriptional initiation from intragenic transcription initiation sites. It is not clear at this time whether H3K56 acetylation and its deacetylation are also required for intragenic transcription initiation. However, future studies in this regard should be very informative.

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