The Saccharomyces cerevisiae histone H2A variant Htz1 is acetylated by NuA4

Michael-Christopher Keogh, Thomas A. Mennella, Chika Sawa, Sharon Berthelet, Nevan J. Krogan, Adam Wolek, Vladimir Podolny, Laura Rocco Carpenter, Jack F. Greenblatt, Kristin Baetz and Stephen Buratowski

Genes & Dev. 2006 20: 660-665
Access the most recent version at doi:10.1101/gad.1388106
The *Saccharomyces cerevisiae* histone H2A variant Htz1 is acetylated by NuA4

Michael-Christopher Keogh,1 Thomas A. Mennella,1 Chika Sawa,1,7 Sharon Berthelet,2 Nevan J. Krogan,3 Adam Wolek,4 Vladimir Podolny,1 Laura Rocco Carpenter,4 Jack F. Greenblatt,5,6 Kristin Baetz,2 and Stephen Buratowski1,8

1Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, USA; 2Ottawa Institute of Systems Biology, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada; 3Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco 94143, California, USA; 4Upstate USA Inc., Lake Placid, New York 12946, USA; 5Banting and Best Department of Medical Research and 6Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario MSG 1L6, Canada.

The histone H2A variant H2A.Z (*Saccharomyces cerevisiae* Htz1) plays roles in transcription, DNA repair, chromosome stability, and limiting telomeric silencing. The Swr1-Complex (SWR-C) inserts Htz1 into chromatin and shares several subunits with the NuA4 histone acetyltransferase. Furthermore, mutants of these two complexes share several phenotypes, suggesting they may work together. Here we show that NuA4 acetylates Htz1 Lys 14 (K14) after the histone is assembled into chromatin by the SWR-C. K14 mutants exhibit specific defects in chromosome transmission without affecting transcription, telomeric silencing, or DNA repair. Function-specific modifications may help explain how the same component of chromatin can function in diverse pathways.

Supplemental material is available at http://www.genesdev.org.

Received October 26, 2005; revised version accepted January 20, 2006.

Two classes of enzymes have been implicated in regulating chromatin structure and access to the underlying DNA template. The ATP-dependent chromatin remodeling enzymes use ATP hydrolysis to induce nucleosome mobility or disrupt histone-DNA interactions. The second class of enzymes covalently modify (e.g., lysine acetylation, serine phosphorylation, lysine and arginine methylation, ubiquitylation, or ADP ribosylation) various histones, usually on their N-terminal tails (Strahl and Allis 2000; Jenuwein and Allis 2001). Acetylation is carried out by histone acetyltransferases (HATs), which in *Saccharomyces cerevisiae* include the Gcn5-containing ADA and SAGA complexes, Hat1, Elongator, NuA3, and NuA4 (for review, see Bottomley 2004). These typically have specificity for distinct lysine residues on certain histone N-terminal tails. The acetylation of lysine residues on the N-terminal tails of histones H3 and H4 neutralizes their positive charge, possibly decreasing their affinity for DNA and facilitating chromatin decompaction and disassembly (Eberharter and Becker 2002). Perhaps more important than simple charge neutralization is the specific pattern of acetylation at individual lysine residues, at least some of which recruit bromodomain-containing proteins (Matangkasombut and Buratowski 2003, and references therein).

Further chromatin specialization can be introduced by incorporation of variant histones. The major histones are assembled during DNA replication, but can be replaced by variants at specific locations (for review, see Malik and Henikoff 2003). The histones with known variants are H3 and H2A, both of which self-interact within a single nucleosome core particle (Malik and Henikoff 2003). Among the H2A variants is H2A.Z (Htz1 in *S. cerevisiae*), which is inserted into chromatin by the Swr1-ATPase complex, SWR-C (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004). Htz1 plays roles in multiple processes, including transcription (Santisteban et al. 2000; Krogan et al. 2003; Meneghini et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004), limiting telomeric silencing (Krogan et al. 2003; Meneghini et al. 2003), and chromosome segregation (Krogan et al. 2004).

The SWR-C shares several subunits with the NuA4 HAT complex (Kobor et al. 2004; Krogan et al. 2004; Mizuguchi et al. 2004), and expression microarray analysis shows the two complexes have common regulatory targets (Krogan et al. 2004). NuA4 is required for the majority of histone H4 acetylation on Lys 5 (K5), K8, and K12 and some on histone H2A K7 (Smith et al. 1998; Allard et al. 1999). Htz1, SWR-C, and NuA4 have each been implicated in the maintenance of chromosome stability (Krogan et al. 2004). This function for H2A.Z is conserved in the fission yeast *Schizosaccharomyces pombe* (Carr et al. 1994) and metazoans (Rangasamy et al. 2004).

How NuA4 and SWR-C are functionally connected remains unclear. Htz1 incorporation into chromatin is dependent on SWR-C, but independent of NuA4 (Krogan et al. 2004). Therefore, acetylation of histone H4 by NuA4 is not required to recruit Htz1. Another possibility is that the HAT acetylates Htz1 after its incorporation into chromatin. H2A.Z N-terminal tails are acetylated in mammals (Pantazis and Bonner 1981, 1982; Bruce et al. 2005), chicken (Bruce et al. 2005), and *Tetrahymena* (Ren and Gorovsky 2001), although the mediating HATs and biological relevance of these modifications is unknown. Here we show that NuA4 acetylates *S. cerevisiae* Htz1 on K14 after it is assembled into chromatin and that this modification plays a role in maintaining stable propagation of chromosomes.

Keywords: H2A.Z; NuA4; acetylation; chromosome segregation

*Present address: Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501, Japan.*

*Corresponding author.*

E-MAIL steveb@hms.harvard.edu; FAX (617) 738-0516.

Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.188106.
**Results and Discussion**

*S. cerevisiae* Htz1 has four lysine sites on its N-terminal tail: K3, K8, K10, and K14 [Fig. 1A]. These were mutated individually to arginine (R) and expressed as the sole source of Htz1 [Fig. 1B]. Mutants were as sensitive to benomyl (75 µg/mL) in YPD. Htz1-K14R, K3R, K8R, and K10R were individually examined for phenotypes associated with silencing in the *htz1-H9004* strain. None of the other point mutants exhibited any sensitivity in these assays. Htz1 levels and acetylation of K14 in vivo. Strains containing *htz1-K14Q* or *htz1-K14R* cells were examined after 2 days at room temperature. **Figure 1.** The Htz1-K14R mutant is selectively sensitive to benomyl. (A) Cross-species alignment of the H2A.Z N-terminal tail; upper set is multiple *Saccharomyces* species (Cliften et al. 2003), lower set is metazoans. Residues different from *S. cerevisiae* peptide used to raise the Htz1 K14 Ac antibody is boxed and shaded. Htz1 (K3, K8, K10, and K14) are indicated with asterisks. The peptide used to raise the Htz1 K14Ac antibody is boxed and shaded. (B) Htz1 K3, K8, K10, and K14 were individually mutated to arginine (R). Mutants were expressed as the sole source of Htz1 and 10-fold serial dilutions of each strain were spotted onto the indicated plates (HU [100 mM], benomyl [15 µg/mL] in YPD). Plates were incubated for 3 days at 30°C. (C) As in B, except the sensitivity of the indicated strains to 6AU (75 µg/mL) or MPA (15 µg/mL) was examined after 72 h.

Htz1-K14Ac antibody for the acetylated (K14Ac) relative to the unmodified (K14) Htz1 peptide. Indicated amounts (in picomoles) of each peptide were spotted onto nitrocellulose before immunoblotting. (B) Htz1-K14 is acetylated in vivo. HA-tagged forms of the indicated Htz1 proteins were individually expressed as the sole source of the histone and strains were analyzed by immunoblotting. Deletion of Htz1 or mutation of K14 to arginine (K14R) or glutamine (K14Q) abolishes recognition by the anti-K14Ac antibody. In contrast, mutation of residues K3, K8, or K10 has no effect on K14 Ac levels. Total Htz1 was detected with anti-HA. Anti-Rpt1 and anti-Rpn8 were used as loading controls. (C) Esal is required for acetylation of Htz1-K14 in vivo. Strains containing esalΔ or the temperature-sensitive mutants esal-L327S or esal-L325P were grown at room temperature (RT) or for 4 h at the nonpermissive temperature (37°C). Growth was assayed after 2 days at room temperature (RT) or 37°C. Cell extracts were assayed by immunoblotting with the indicated antibodies. (D) Htz1-K14 acetylation is differentially dependent on subunits of NuA4. Extracts from indicated deletion strains were immunoblotted with the antibodies shown to the right of each panel. (E) NuA4 efficiently acetylates Htz1-K14 in vitro. Partial TAP purifications of indicated HATs were monitored by reactivity of the protein A tag. (Top) An untagged strain (NT) was used as a control. IgG-purified HATs were added to reactions containing recombinant Htz1/H2B dimer and acetyl CoA. Htz1-K14Ac and Htz1 were detected by immunoblotting. **Figure 2.** Htz1-K14 is acetylated by NuA4. (A) Specificity of the Htz1-K14Ac antibody for the acetylated (K14Ac) relative to the unmodified (K14) Htz1 peptide. Indicated amounts (in picomoles) of each peptide were spotted onto nitrocellulose before immunoblotting. (B) Htz1-K14 is acetylated in vivo. HA-tagged forms of the indicated Htz1 proteins were individually expressed as the sole source of the histone and strains were analyzed by immunoblotting. Deletion of Htz1 or mutation of K14 to arginine (K14R) or glutamine (K14Q) abolishes recognition by the anti-K14Ac antibody. In contrast, mutation of residues K3, K8, or K10 has no effect on K14Ac levels. Total Htz1 was detected with anti-HA. Anti-Rpt1 and anti-Rpn8 were used as loading controls. (C) Esal is required for acetylation of Htz1-K14 in vivo. Strains containing esalΔ or the temperature-sensitive mutants esal-L327S or esal-L325P were grown at room temperature (RT) or for 4 h at the nonpermissive temperature (37°C). Growth was assayed after 2 days at room temperature (RT) or 37°C. Cell extracts were assayed by immunoblotting with the indicated antibodies. (D) Htz1-K14 acetylation is differentially dependent on subunits of NuA4. Extracts from indicated deletion strains were immunoblotted with the antibodies shown to the right of each panel. (E) NuA4 efficiently acetylates Htz1-K14 in vitro. Partial TAP purifications of indicated HATs were monitored by reactivity of the protein A tag. (Top) An untagged strain (NT) was used as a control. IgG-purified HATs were added to reactions containing recombinant Htz1/H2B dimer and acetyl CoA. Htz1-K14Ac and Htz1 were detected by immunoblotting.

---

*S. cerevisiae* H2A.Z is acetylated on K14 by NuA4

reactivity was not seen in extracts from *htz1Δ*, *htz1-K14R*, or *htz1-K14Q* cells [Fig. 2B]. Htz1 levels and K14 acetylation were unaffected when K3, K8, or K10 were individually mutated to arginine [Fig. 2B]. Of multiple HATs tested, Htz1-K14 acetylation was exclusively dependent on NuA4 [Fig. 2C; Supplementary Fig. 2]. Htz1-K14Ac levels were strongly reduced in strains with conditional alleles of the NuA4 catalytic subunit Esa1 [Fig. 2C] or deletions of other NuA4 subunits required for acetylation of H4 [Fig. 2D; Supplementary Fig. 2A]. To determine if NuA4 acts directly on Htz1, in vitro acetylation was tested. Esa1-TAP complexes display robust HAT activity toward Htz1-K14. In contrast, Elp3-TAP- or Gen5-TAP-containing complexes do not acetylate the substrate under the same conditions [Fig. 2E].

SWR-C is required for efficient insertion of Htz1 into chromatin [Krogan et al. 2003; Kobor et al. 2004; Mizu-
guchi et al. 2004). In cells lacking SWR-C, total Htz1 levels are unaffected while Htz1-K14Ac levels are significantly reduced [Fig. 3A]. Fractionation experiments show reduced total and acetylated Htz1 in chromatin from swr1Δ cells [Supplementary Fig. 2C]. Therefore, Htz1 acetylation is likely to occur after assembly into chromatin. Unacetylatable Htz1-K14 mutants [htz1-K14R or htz1-K14Q] are expressed at levels similar to wild type [Fig. 2B] and efficiently assemble into chromatin [Fig. 3B], so acetylation is not required for Htz1 incorporation. Furthermore, the distribution of K14 mutants throughout the genome is similar to wild type at all positions tested by chromatin immunoprecipitation (ChIP), suggesting that acetylation is not specifically correlated with insertion at a particular location [Fig. 3C].

Unfortunately, the Htz1-K14Ac-specific antibody did not work in ChIP (data not shown), so we were unable to compare the genomic distribution of Htz1-K14Ac to total Htz1. To determine whether acetylation affects the stability of Htz1 in the nucleosome, chromatin preparations from wild-type, htz1-K14R, and htz1-K14Q cells were subjected to washes of increasing ionic concentrations [Raisner et al. 2005]. No differences were observed, suggesting that K14 acetylation does not dramatically affect interactions between Htz1 and other histones [Fig. 3D].

Acetylation is a reversible modification (Eberharter and Becker 2002), but a screen of several known histone deacetylase (HDAC) mutants failed to find any that affected levels of Htz1-K14Ac [Supplementary Fig. 2B]. In addition, total Htz1-K14Ac levels are unchanged throughout the cell cycle [Supplementary Fig. 3B] or in response to MMS-induced DNA damage [Supplementary Fig. 3C]. It should be noted, however, that all these studies examined the total cellular pool of Htz1-K14Ac and thus could miss the removal of the modification at a specific location or time.

Accurate chromosome transmission requires the coordination of many events. During S phase the chromosomes are duplicated and the resultant sister chromatids held together by the cohesin complex [for review, see Uhlmann 2003]. The centromere [CEN] is the assembly site of a multiprotein kinetochore complex that links the chromosomes with spindle MTs [Fig. 4B; McAinsh et al. 2003; Measday and Hieter 2004]. Once all chromosomes have attached to the spindle, the metaphase-to-anaphase transition proceeds by degradation of the cohesin complex and chromosome segregation. If kinetochores do not attach properly to spindle MTs, spindle checkpoint proteins halt cell cycle progression at the metaphase-to-anaphase transition (Cleveland et al. 2003). Defects in any of these processes can result in chromosome imbalance, or aneuploidy. Although chromatin impacts many aspects of chromosome transmission, including CEN function, the specific regulators that impact chromosome transmission fidelity [CTF] have not been comprehensively identified, nor are the mechanisms understood at the molecular level.

Sensitivity to benomyl is a common phenotype of kinetochores and spindle checkpoint mutants. Like deletions of SWR1 or HTZ1, the htz1-K14R mutant is benomyl sensitive [Fig. 1B]. To further characterize the role of Htz1 acetylation in genome stability, we quantified chromosome missegregation in htz1-K14R and htz1-K14Q diploid strains by colony half-sector analysis [Koshland and Hieter 1987; Krogan et al. 2004]. The htz1-K14R strain shows an increase in the rate of chromosome loss comparable to an htz1Δ strain [6.6- vs. 7.7-fold greater than wild type] [Fig. 4A]. Interestingly, the htz1-K14Q strain, which might mimic constitutive acetylation of Htz1, has normal segregation.

Comprehensive synthetic genetic array [SGA] screening of spindle checkpoint mutants [mad1, mad2, mad3, and bub3] identified genetic interactors, one of which was HTZ1, that may have roles in regulating MTs, kinetochores, or sister chromatid cohesion [SCC] [Daniel et al. 2006]. We also observed genetic interactions between htz1Δ and components of the kinetochore and spindle checkpoint machinery [Krogan et al. 2004]. Like other chromatin components [Sharp and Kaufman 2003], K14 acetylation might contribute to proper centromere.
include transcription and chromosome stability. Our work suggests that this latter function of NuA4 also involves the acetylation of Htz1.

Materials and methods

Materials

Yeast strains used in this study are listed in Supplementary Table 2 and oligonucleotides are listed in Supplementary Table 3. Htz1 point mutants were created by site-directed mutagenesis method (Keogh et al. 2003) and confirmed by sequencing. Peroxidase anti-peroxidase (nPAP) from Sigma was used in immunoblotting to recognize the TAP tag. Monoclonal antibody 12CA5 recognizing the HA-epitope tag was from Covance. Polyclonal rabbit anti-Cib2 was from A. Rudner (Harvard Medical School, Boston, MA), rabbit anti-Rpt1 and anti-Rpn8 from D. Finley (Harvard Medical School, Boston, MA), and rabbit anti-eIF-5A (YELO34W) from R.S. Zitomer (State University of New York at Albany, Albany, NY). Polyclonal rabbit anti-H3 (ab1791) was from Abcam. Polyclonal affinity purified anti-Htz1 antibodies were produced in rabbits by Upstate. The C-terminal antibody [7F7.18] was the standard antibody for amino acids 116–133 of Htz1 (C-PHINKALLKVEKCGSKK), the anti-K14ac antibody [07-719] against amino acids 11–20 [SGAKK/GDSGSLR-KC] (Fig. 1A). The terminal C residue on each peptide was used to couple to KLH for immunization or a sulfo linker resin for affinity purification.

Phenotypic analyses

For spot assays, cells were resuspended at 10^7/mL, subjected to 10-fold serial dilutions and 10 µL of each dilution spotted per plate. Growth was assayed at 48 or 72 h as indicated. Camptothecin (20 µM), HU (100 mM), MMS (0.05%), or benomyl (15 µg/mL) plates were in yeast extract–peptone–dextrose (YPD). Sensitivity to 6AU (75 µg/mL) or MPA (15 µg/mL) was assayed on minimal media lacking uracil–methionine when needed. Strains containing a URA3 plasmid were transformed with a CEN/ARS URA3 plasmid. The telomeric silencing assay (Supplementary Fig. 1) employed cultures of cells expressing the indicated plasmid and grown to OD_600 of 0.2 before plating at 10-fold serial dilutions on YPD or SC medium with 5-Fluoroorotic acid (5-FOA) as indicated. Growth on YPD plates was analyzed after 48 h and 5-FOA after 96 h.

ChIP

ChIPs were performed as described (Keogh et al. 2003; Krogan et al. 2003). Two-hundred-fifty milliliters of each strain were grown to OD_{600} = 0.6 in minimal medium before formaldehyde cross-linking and further processing.
Keogh et al.

to 8 mL SP-Sepharose 4 Fast flow [Pharmacia] as above. The resin was placed in an Econo-Pak column and successively washed with 20 mL U-200 (7 M urea, 100 mM NaCl) and 20 mL U-400. Histones were eluted by U-600, with positive fractions pooled and dialyzed overnight against dH2O (with 5 mM JMPE, 0.2 mM PMSF). Precipitates were removed by centrifugation and the histones were lyophilized and stored at −80 °C. For each sample purity was estimated by 15% SDS-PAGE with colloidal Coomassie staining, and the absence of DNA contamination confirmed by analysis on agarose gels containing ethidium bromide.

In vitro HAT assays

WCEs (untagged, Esal-TAP, Gen5-TAP, or Elp3-TAP) were prepared as described (Keogh et al. 2003). Each HAT complex was affinity purified from 10 mg WCE with IgG agarose (Sigma) [5 µL resin/mg WCE]. After overnight incubation at 4 °C the bead complexes were extensively washed with Lysis Buffer [LB: 20 mM Tris.Cl at pH 7.4, 10% glycerol, 200 mM KAc, 1 mM EDTA, 1 mM DTT + protease inhibitors], resuspended to 200 µL total volume in TEV buffer [TR: 50 mM Tris.HCl at pH 8.0, 1 mM DTT, 0.5 mM EDTA] and complexes cleaved from the beads with recombinant TEV protease [4 h, 4 °C]. The supernatant was collected, dialyzed for 1 h against HAT buffer [HB: 50 mM Tris.HCl at pH 8.0, 10% glycerol, 10 mM butyric acid, 1 mM DTT, 1 mM PMSE], and stored in aliquots at −80 °C. In vitro HAT reactions were performed for 1 h at 30 °C (5 µL reactions containing 100 ng histone, 2 mM acetyl CoA, and 2 or 5 µL of immunopurified HAT complexes; conditions derived from Mizzen et al. 1999), resolved by 15% SDS-PAGE, and specific acetylation of Htz1-K14 determined by immunoreactivity with anti-K14Ac.

S. cerevisiae fractionation

Cells from 50-mL cultures (OD 600 < 1.0) were collected by centrifugation, successively washed with ddH2O, PSB (20 mM Tris.Cl at pH 7.4, 2 mM EDTA, 100 mM NaCl, 10 mM β-ME, 0.2 mM PMSF). Precipitates were removed by centrifugation (2K, 5 min, 4°C), washed twice with SB, and suspended in 500 µL EBX (20 mM Tris.Cl at pH 7.4, 100 mM NaCl, 0.25% Triton X-100, 15 mM β-ME, 50 mM Na-butyrate + protease inhibitors). Triton X-100 was added to 0.5% final to lyse the outer cell membrane, and the samples kept on ice for 10 min with gentle mixing. An aliquot was taken for immunoblotting [Total], and the remainder of the lysate layered over 1 mL NIB (20 mM Tris.Cl at pH 7.4,100 mM NaCl, 1.2 M sucrose, 15 mM β-ME, 50 mM Na-butyrate + protease inhibitors) and centrifuged (13,000 g, 10 min, 4°C). A sample of the upper layer cytoplasmic fraction was taken [Cyto] and the rest of the supernatant discarded. The glassy white nuclear pellet was suspended in 500 µL EBX and Triton X-100 added to 1% final to lyse the nuclear membrane. Samples were kept on ice for 10 min with gentle mixing and an aliquot taken [Nuclear] and chromatin and nuclear debris collected by centrifugation (16,000 g, 10 min, 4°C). Chromatin was washed three times with EBX and suspended in 50 µL 1 M Tris (pH 8.0) [Chromatin]. To each fraction an equal volume of 2× SDS-PAGE loading buffer [60 mM Tris at pH 6.8, 2% SDS, 10% glycerol, 0.2% bromophenol blue, 200 mM DTT] was added, samples were incubated at 95°C for 5 min, centrifuged (16,000 g, 5 min, room temperature) and the supernatant collected. Samples were analyzed by SDS-PAGE and immunoblot analyses.

Chromatin salt stability analyses

EBX-100-isolated chromatin from above was split into aliquots and each washed three times with EBX-100 to EBX-750 (all millimoles NaCl) [Rogowsky et al. 2005]. Pellets were then resuspended and analyzed by immunoblotting.

Chromosomal salt stability analyses

Quantitative half-sector analysis was performed as previously described (Koshland and Hieter 1987; Krogan et al. 2004). In brief, homozygous diploid strains were created containing an ade2Δ::HIS3 allele at the endogenous locus and the SUM11 ochre suppressor on a single chromosomal fragment [CFIII [CEN3.LI] URA3 SUP11]. Strains were plated to single colonies on solid SC-HIS and two-fifths adenine and grown at 25°C for 3 d before the plates were placed at 4°C for optimal red pigment development. Efficient chromosome stability/ transmission results in pink color.

Acknowledgments

We thank J. Babiarz and J. Rine for communicating results prior to publication, and D. Finley, A. Rudner, C. Wu, and R.S. Zitomer for the generous supply of materials. This research was supported by a Doctoral Fellowship from the Canadian Institutes of Health Research (CIHR) to N.J.K., by The Terry Fox Foundation through a grant from the National Cancer Institute of Canada to K.B., and NIH grant GM46498 to S.B. K.B. is a Canada Research Chair in Functional and Chemical Genomics.

References


