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Telomeric heterochromatin boundaries require NuA4-dependent acetylation of histone variant H2A.Z in *Saccharomyces cerevisiae*

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SWR1-Com, which is responsible for depositing H2A.Z into chromatin, shares four subunits with the NuA4 histone acetyltransferase complex. This overlap in composition led us to test whether H2A.Z was a substrate of NuA4 in vitro and in vivo. The N-terminal tail of H2A.Z was acetylated in vivo at multiple sites by a combination of NuA4 and SAGA. H2A.Z acetylation was also dependent on SWR1-Com, causing H2A.Z to be efficiently acetylated only when incorporated in chromatin. Unacetylatable H2A.Z mutants were, like wild-type H2A.Z, enriched at heterochromatin boundaries, but were unable to block spreading of heterochromatin. A mutant version of H2A.Z that could not be acetylated, in combination with a mutation in a nonessential gene in the NuA4 complex, caused a pronounced decrease in growth rate. This H2A.Z mutation was lethal in combination with a mutant version of histone H4 that could not be acetylated by NuA4. Taken together, these results show a role for H2A.Z acetylation in restricting silent chromatin, and reveal that acetylation of H2A.Z and H4 can contribute to a common function essential to life.

**Keywords**: Silencing; SIR proteins; histone code; HTZ1

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Differences in the pattern of histone modifications found at different places in the genome lead to unique properties of the chromatin at these positions. These links have been formalized as the histone code hypothesis (Strahl and Allis 2000; Peterson and Laniel 2004). Histone variants substitute for canonical histones in certain highly specialized chromatin structures, but unlike canonical histones, modification of histone variants is largely unexplored. Not all modifications can be considered unique components of a code. For instance, acetylation of Lys 5, Lys 8, and Lys 12 of histone H4 contribute quantitatively to gene expression across the yeast genome. This is in contrast to acetylation of Lys 16 of histone H4, which has distinct consequences (Dion et al. 2005). Histone H4 Lys 16 acetylation is a hallmark of euchromatin, and hypoacetylation of this position is a hallmark of heterochromatin. H4 Lys 16 is acetylated by the SAS histone acetyltransferase (HAT) complex (Kimura et al. 2002; Suka et al. 2002). Heterochromatin is found in three locations in the *Saccharomyces cerevisiae* genome: the cryptic mating loci (HML and HMR), telomeres and some subtelomeric regions, and ribosomal DNA (Rusche et al. 2003). Silencing at the cryptic mating loci and telomeres is mediated by the Silent-Information Regulator (SIR) proteins, and is caused in part by histone deacetylation. Sir2 protein is a histone deacetylase whose preferred substrates include H4 Lys 16. Sir3p and Sir4p are histone tail-binding proteins, and Sir3p preferentially binds unmodified histone H4 tails in vitro and presumably in vivo as well (Carmen et al. 2002).

Post-translational acetylation and deacetylation of histones are carried out by large, multisubunit complexes. In *S. cerevisiae*, NuA4 is the only essential HAT complex (Smith et al. 1998; Clarke et al. 1999). Deletion of the catalytic subunit of NuA4, encoded by *ESA1*, results in inviability. Temperature-sensitive alleles of *ESA1* cause reduced acetylation of histones H2A and H4, and sensitivity to genotoxic stress by drugs such as methyl methanesulfonate and camptothecin (Bird et al. 2002). In addition, *ESA1* has a role in DNA double-strand break repair. The SAGA complex is another HAT that acetylates histone H3 through its catalytic subunit encoded by *GCN5* (Trievel et al. 1999; Timmers and Tora 2005).

The dynamic structure of chromatin involves more than just reversible covalent modifications. The replacement of canonical histones with minor histone variants at particular positions in the genome also has specific
H2A.Z acetylation and heterochromatin boundaries

H2A.Z was a substrate for NuA4 in vitro

The SWR1-Com deposits H2A.Z into chromatin and shares several subunits with the NuA4 HAT complex [Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004]. This sharing of subunits suggested that H2A.Z may be a substrate for NuA4. To test this hypothesis, S. cerevisiae H2A.Z was tested for whether it could be acetylated by NuA4. In these experiments, H2A.Z was expressed in Escherichia coli and purified from inclusion bodies. H2A.Z was then reconstituted into dimers with H2B, and into octamers with S. cerevisiae histones H2B, H3, and H4 that had also been purified following expression in E. coli. Hence these histones lacked eukaryotic modifications. Native NuA4 complex was purified from S. cerevisiae using a strain that carried a TAP-tagged version of Esa1p, the catalytic subunit of NuA4.

Following purification, the various histone complexes were incubated with purified NuA4 in the presence of 3H Acetyl-CoA. NuA4 efficiently acetylated H4 and H2A. The strong signal from the position in the gel corresponding to H2A.Z either as H2A.Z–H2B dimers or as octamers defined H2A.Z as a new substrate of the NuA4 complex in vitro (Fig. 1A). However, under the salt concentrations of these assays, the octamers fall apart into

Figure 1. H2A.Z was acetylated by NuA4 in vitro and in vivo. [A] 3H-Acetyl-CoA was incorporated into H2A and H2A.Z by NuA4. [B] 3H-Acetyl-CoA was incorporated into H2A.Z-containing nucleosomes by NuA4. [C] H2A.Z was acetylated in vivo primarily by NuA4. The image represents an anti-Flag immunoblotting of an AU gel with times referring to minutes past shift to 37°C.
H3–H4 tetramers and H2A.Z–H2B dimers. To determine if NuA4 could acylate H2A.Z-containing nucleosomes, chromatin was purified from a strain that was deficient for NuA4 [eaf1Δ] and that contained a C-terminal triple-Flag-tagged version of H2A.Z expressed from the HTZ1 locus. This tagged version of H2A.Z complemented all htz1Δ phenotypes [data not shown]. Nucleosomes were released by micrococcal nuclease (MNase) treatment, and H2A.Z nucleosomes were purified using αFlag affinity resin. Native NuA4 complex was added along with 3H Acetyl-CoA. H2A.Z was acetylated when present in nucleosomes [Fig. 1B]. As observed for Drosophila HA2v, NuA4 preferentially acetylated H2A.Z over H4 contained in the same nucleosome [Kusch et al. 2004]. In addition to H2A.Z, a second protein was acetylated whose mobility is consistent with a protein the size of either H2A or H2B. One possibility is H2B, as NuA4 acetylation of H2B is observed in H2A.Z–H2B dimers [Fig. 1A]. A second possibility is H2A. It has been shown recently that H2A.Z can form mixed nucleosomes with H2A in vitro [Chakravarthy et al. 2004]. H2A.Z-containing nucleosomes purified using this method contain H2A, presumably in nucleosomes carrying one H2A molecule and one H2A.Z [data not shown].

In vivo, NuA4 acetylates three lysines on histone H4 and two on H2A [Suka et al. 2001]. To determine the number of acetyl groups that were added to H2A.Z by NuA4 in vitro, we used ion-trap mass spectrometry. The in vitro reaction was performed as described above using free H2A.Z as a substrate. Mass spectrometry analysis identified a peak at the predicted molecular weight of H2A.Z and, only in the presence of acetyl-CoA, a peak corresponding to H2A.Z + 42 Da, the molecular weight of a single acetyl group. No additional peaks suggesting di- or triacetylation were observed [data not shown]. These data established that H2A.Z could be a direct substrate for NuA4. However, the in vivo experiments [below], revealed multiple sites of acetylation.

**H2A.Z was a substrate for NuA4 in vivo**

To determine whether H2A.Z was acetylated by NuA4 in vivo, we used acetic acid–urea gels (AU gels) to separate histone isoforms based on size and charge [Lennox and Cohen 1989]. Since acetylation of a lysine neutralizes its positive charge, acetylated histones migrate more slowly than unmodified versions, with single-acetylation resolution possible. Because H2A.Z is a minor histone variant, with an abundance ~10% that of H2A, we first immunoprecipitated H2A.Z-3Flag such that the amounts of H2A.Z exceeded the binding capacity of the αFlag affinity resin used. This was done so that minor differences in absolute H2A.Z amounts between different mutant strains would not influence our interpretations. The immunoprecipitated H2A.Z was separated by AU gels, transferred to a membrane, subject to αFlag immunoblots, so that the signal from H2A.Z would not be masked by that from H2A [Ren and Gorovsky 2001, 2003].

On AU gels, H2A.Z had at least four modified forms that migrated more slowly than the fastest migrating and presumptively unmodified band, suggesting multiple acetylations, or phosphorylations, or both [Fig. 1C]. This result contrasted with the in vitro data suggesting that H2A.Z was monoacetylated by NuA4. The difference in results from the two assays may reflect differences between in vivo and in vitro HAT specificities, or modification of H2A.Z by multiple HATs in vivo.

To test whether one or more of these modified forms of H2A.Z was due to the function of the NuA4 HAT, we expressed H2A.Z-3Flag in a strain with a severe temperature-sensitive allele of ESA1, the gene encoding the catalytic subunit of NuA4. *esa1-1851* strains have defects in histone H4 acetylation even at the permissive temperature [Bird et al. 2002]. If H2A.Z was acetylated in vivo by NuA4, then the *esa1-1851* allele would cause a reduction in the amount of slowly migrating, and therefore acetylated, forms of H2A.Z [Fig. 1C]. Three of the four modified forms of H2A.Z were eliminated at both the permissive and restrictive temperature, leaving a single NuA4-independent modification. These data identified the NuA4 HAT as being responsible for H2A.Z modification in vivo. The in vitro data above suggested that at least one of these forms resulted from direct acetylation of H2A.Z by NuA4. Whether NuA4 was responsible for the other species, or if other HATs add those modifications in a NuA4-dependent manner, is resolved below.

To determine if the reduction in the number of modified species of H2A.Z was specific to the *Esalp* subunit of NuA4, we evaluated H2A.Z modification in an *eaf1Δ* mutant. *EAF1* encodes a nonessential subunit of NuA4, and *eaf1Δ* mutants, although viable, have histone H4 acetylation defects [Kobor et al. 2004]. H2A.Z-3Flag in an *eaf1Δ* mutant produced a similar modification pattern to *esa1-1851* [Fig. 2A] on AU gels. Therefore, two different
disruptions of the NuA4 complex had similar phenotypes with respect to H2A.Z modifications.

**SAGA acetylated H2A.Z in vivo**

To determine the source of the remaining modifications of H2A.Z in a cell compromised for NuA4 activity, we tested the contribution of other HATs to H2A.Z acetylation. GCN5 and SAS2 were of particular interest, as gcn5Δ htz1Δ double mutants exhibit a striking slow-growth phenotype (Santisteban et al. 2000), and SAS2, like HTZ1, helps to constrain the spread of heterochromatin (Kimura et al. 2002; Suka et al. 2002). GCN5 encodes the catalytic subunit of SAGA. gcn5Δ mutant cells had a modest yet clear reduction of the number of modified forms of H2A.Z [Fig. 2A]. Instead of the four modified forms of H2A.Z in wild type, there were two modified forms, with a third modified form of low abundance in a gcn5Δ mutant. These data suggested that SAGA added a single acetyl group. In contrast, loss of Sas2p had no effect on H2A.Z modifications [Fig. 2A].

Given that both SAGA and NuA4 contributed to H2A.Z acetylation in vivo, we tested whether the residual modification of H2A.Z in an esa1-1851 and eaf1Δ mutant was due to acetylation by SAGA. Unexpectedly, the gcn5Δ and eaf1Δ [SAGA and NuA4] double-mutant combination was lethal [Fig. 2C]. Because gcn5Δ would also likely be lethal in combination with the severe esa1-1851 allele, we introduced the gcn5Δ allele into a strain carrying the milder esa1-L357H mutation. esa1-L357H strains exhibited normal histone H4 acetylation at the permissive temperature [Bird et al. 2002], as well as a normal H2A.Z-3Flag acetylation pattern at the permissive temperature [Fig. 2B]. Upon shifting esa1-L357H to restrictive temperature for 60 or 120 min, the acetylation pattern looked similar to esa1-1851 and eaf1Δ. The esa1-L357H allele was viable in combination with gcn5Δ; however, these cells grew notably more slowly than either single mutant [data not shown]. At permissive temperature, the esa1-L357H gcn5Δ H2A.Z acetylation pattern appeared to be similar to gcn5Δ [Fig. 2, cf. A and B]. Shifting these cells to nonpermissive temperature did not result in the loss of the residual H2A.Z modification. Thus, the remaining modification was not due to SAGA. This epistasis analysis suggested that NuA4 acetylation of H2A.Z was a prerequisite for SAGA acetylation.

**SWR1-Com was necessary for H2A.Z acetylation**

The sharing of subunits between SWR1-Com and NuA4 suggested two different models for the roles of these two complexes in the incorporation of H2A.Z into chromatin. In one model, SWR1-Com would deposit H2A.Z into chromatin, and the shared subunits with NuA4 would then recruit that complex to acetylate H2A.Z. In the alternative model, H2A.Z would require prior acetylation by NuA4 to either interact with SWR1-Com or to be incorporated into chromatin by SWR1-Com. H2A.Z in a swr1Δ mutant showed reduction in H2A.Z acetylation similar to reduction exhibited in the NuA4 mutant strains [Fig. 2A]. Thus acetylation of H2A.Z by NuA4 appeared to require prior incorporation into chromatin by SWR1-Com.

To provide an independent test of the dependency relationships between chromatin association and H2A.Z acetylation, we evaluated the modifications present on both the chromatin-associated and soluble fractions of H2A.Z. Spheroplasted cells were gently lysed with a non-denaturing detergent, and the chromatin was separated from the soluble non-chromatin-bound proteins. The chromatin was subsequently digested with micrococcal nuclease to release individual nucleosomes [Donovan et al. 1997; Liang and Stillman 1997]. As expected from analysis of the swr1Δ mutant [above], soluble H2A.Z was largely unacetylated [Fig. 3], and the soluble fraction contains the majority of the unmodified H2A.Z in the cell. In swr1Δ cells, soluble H2A.Z was similarly unmodified, but the chromatin-associated fraction had significantly reduced acetylation compared with wild type [Fig. 3]. Thus, efficient acetylation of H2A.Z by NuA4 could not occur in the absence of SWR1-Com.

**H2A.Z was acetylated in the N-terminal tail**

The four lysines in the N-terminal tail of H2A.Z at positions 3, 8, 10, and 14 provided a potential explanation for the four modified forms of H2A.Z evident in the AU gels. These positions in HTZ1-3Flag were mutated to arginine codons, to mimic the unacetylated state, creating htz1-K3,8,10,14R. This mutant H2A.Z was largely unmodified, as judged by mobility on AU gels [Fig. 4A]. Comparing the H2A.Z mobility in an eaf1Δ and htz1-K3,8,10,14R [Fig. 4A] revealed that H2A.Z was more severely unmodified in the htz1-K3,8,10,14R mutant than in the eaf1Δ mutant. The remaining modified species in the htz1-K3,8,10,14R mutant was in low abundance, and might reflect acetylation at a site other than the N-terminal tail, or perhaps phosphorylation elsewhere in the protein. As expected, this mutant could not be acetylated by NuA4 in vitro [data not shown]. To determine the acetylated sites of H2A.Z, the modification state was monitored in cells with individual N-terminal lysines changed to arginine [Fig. 4B]. All four individual lysine mutants appeared similar, exhibiting loss of a modified form on AU gels, suggesting that no one residue was more important for acetylation than the others.

**Figure 3.** Soluble and chromatin-associated H2A.Z was analyzed for acetylation in wild-type and swr1Δ. Shown is an anti-Flag immunoblot of an AU gel.

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**H2A.Z acetylation and heterochromatin boundaries**
H2A.Z acetylation blocked the spread of silencing

The boundaries that restrict the spread of heterochromatin are compromised in htz1Δ mutants (Meneghini et al. 2003). To test whether H2A.Z acetylation was important for the function of these boundaries, quantitative RT-PCR (qRT–PCR) was used to quantitate mRNA levels of genes potentially susceptible to heterochromatic position effects in wild-type, htz1Δ, and htz1-K3,8,10,14R strains. The expression of four genes was monitored: YPS6 and YIRO42c are near the telomeres of chromosome IX-R; YCR095c and GIT1 are near the HMR left and right boundaries, respectively. ACT1, whose mRNA level does not change in an htz1Δ mutant (Meneghini et al. 2003), was used for normalization. The unacetylatable htz1-K3,8,10,14R mutant was defective in boundary function, as reflected by the reduction in the expression level of YPS6 and YIRO42c in the unacetylatable mutant compared with wild type [Fig. 5A]. This repression was entirely Sir protein-dependent, as a deletion of SIR2 in the htz1-K3,8,10,14R mutant restored wild-type expression levels. In principal, these results could have been due to a mislocalization of H2A.Z, because the silencing defect in htz1-K3,8,10,14R cells was similar to that of htz1Δ cells. However, chromatin immunoprecipitation (ChIP) of HTZ1-3Flag and htz1-K3,8,10,14R-3Flag showed similar enrichment at YPS6 and YIRO42c compared with the negative control, PRP8 [Fig. 5C], establishing that the loss of boundary function was due to the loss of H2A.Z acetylation and not due to mislocalization of H2A.Z. As an independent test of the boundary function of the mutant H2A.Z, we tested whether the SIR proteins were spread inward from the telomeres beyond their position in wild-type cells, as would be predicted from the reduced expression of subtelomeric genes. In this experiment, SIR3 was TAP-tagged in both HTZ1 and htz1-K3,8,10,14R strains. ChIP of Sir3-TAP followed by qPCR showed a gradient of Sir3p at telomere IX-R, such that genes farther from the telomere are less enriched for Sir3p [Fig. 5C]. Comparison of the Sir3-TAP gradient of wild type and htz1-K3,8,10,14R showed that Sir3p was present in greater levels and spread farther in the mutant. These data confirm the qRT–PCR results that indicated an inability of htz1-K3,8,10,14R to block the spread of silent chromatin. These data identify the first specific function for H2A.Z acetylation in vivo. We next assayed the boundaries at HMR by testing the expression levels of YCR095c and GIT1 [Fig. 5A]. In contrast to the telomeres, the boundaries blocking the spread of silencing at HMR were unaffected in the htz1-K3,8,10,14R. The lack of heterochromatic spreading in this context was likely due to the dual boundary mechanisms at work at this locus (Donze et al. 1999; Oki and Kamakaka 2005), as discussed below.

If acetylated H2A.Z were the chief contributor to the block in spreading of heterochromatin at telomeres, then a deficiency in NuA4 should lead to a similar boundary defect at the telomeres. Indeed, esa1-1851 cells were more severely defective in boundary function than either htz1Δ or the unacetylatable form of H2A.Z at telomere IX-R [Fig. 5A]. The more severe phenotype of the esa1-1851 cells was probably due to the combination of a two-fold decrease in H2A.Z enrichment [Fig. 5C] and the decrease in acetylation of the remaining H2A.Z.

H2A.Z acetylation was essential in stress conditions when NuA4 is compromised

An htz1Δ mutation is lethal in combination with null alleles of many genes involved in chromatin structure. These lethal combinations include mutations in genes involved in H2B ubiquitination [rad6Δ and bre1Δ], H3 methylation [set2Δ] [Krogan et al. 2003], and H4 acetylation [esa1Δ] [Kobor et al. 2004]. This trend was extended here by the lethality of the gcn5Δ esa1Δ double-mutant combination, deficient in both SAGA and NuA4. Taken together, the various synthetic lethal interactions suggested that cells lacking certain chromatin modifications require H2A.Z for viability, and that nucleosomes require a minimum amount of modification to support viability. We tested whether an unacetylatable H2A.Z would be lethal in combination with mutations in genes for known histone modifiers. Indeed, htz1-K3,8,10,14R in combination with esa1Δ, a nonessential subunit of NuA4, caused severe growth retardation, evident in the dilution series of a tetra-type tetrad from the cross [Fig. 6A]. The double mutant was more impaired than either single mutant, especially at 37°C. Although esa1Δ cells are somewhat compromised compared with wild type or htz1-K3,8,10,14R, at this temperature the double mutants were not viable. The simplest interpretation of the double-mutant phenotype was that acetylation of either H2A.Z or some other NuA4 target, but not both, was essential to maintain cell viability. These data also suggest that the presence of H2A.Z is not sufficient to pro-

Figure 4. H2A.Z was acetylated in the N-terminal tail. (A,B) Shown are anti-Flag immunoblots of an AU gel. (A) H2A.Z is acetylated at the N-terminal Lys 3, Lys 8, Lys 10, and Lys 14. Lanes 1 and 2 are chromosomal HTZ1-3Flag. Lanes 3 and 4 are plasmid-borne HTZ1-3Flag alleles. (B) Lys 3, Lys 8, Lys 10, and Lys 14 can all be acetylated in vivo. All HTZ1-3Flag alleles are plasmid-borne.
vide full function; rather, modified forms of H2A.Z must be present.

H2A.Z or H4 acetylation provided an essential function

To explore the inviability of cells that could not acetylate H2A.Z, we tested double mutants containing an unacetylatable form of H2A.Z with unacetylatable forms of NuA4 targets, H2A and H4. To test whether acetylated H4 provided the essential function in cells lacking acetylated H2A.Z, a strain was constructed that carried a wild-type version of an H4 gene (HHF2) on a plasmid whose loss could be selected for on medium containing 5-fluoro-orotic acid (FOA). This strain contained an allele of HTZ1 that encoded an unacetylatable H2A.Z (htz1-K3,8,10,14R) as well as a mutant version of H4 that could not be acetylated on the positions recognized by NuA4 (hhf2-K5,8,12R). The second copy of the histone H4 gene (HHF1) was deleted. This strain could not grow in the absence of the wild-type H4 gene as seen by the lack of growth on FOA-containing medium [Fig. 6B]. Hence, growth required acetylated versions of either H2A.Z or H4. Moreover, since H2A was wild type in these cells, it could not provide an equivalent essential function of H4. This was confirmed by combining the unacetylatable H2A.Z (htz1-K3,8,10,14R) and unacetylatable H2A (hta1-K4,7R) and finding that the double mutants were fully viable [data not shown]. Likewise, acetylation of H4 K16 by the SAS HAT complex was unable to provide this function, as sas2Δ htz1-K3,8,10,14R cells had no discernable growth defect [data not shown].

Acetylation was unimportant for some roles of H2A.Z

The htz1-K3,8,10,14R mutant allowed us to determine if H2A.Z had roles other than serving as a boundary to silent chromatin. One striking property of htz1Δ mutants is their sensitivity to a variety of chemicals that cause genotoxic and perhaps other stresses to cells. We assayed the influence of acetylation on the resistance of cells to these chemicals. htz1-K3,8,10,14R, as well as individual point mutants, grew as well as wild type on media containing hydroxyurea, benomyl, formamide, and caffeine [data not shown]. esal-1851 cells are sensitive to methyl methanesulfonate and camptothecin (Bird et al. 2002), yet the unacetylatable H2A.Z mutant also grew as well as wild type under these stresses. The similarity in sequence between canonical H2A and H2A.Z, as well as NuA4’s ability to acetylate both, led to the obvious possibility that acetylation of H2A and of H2A.Z had overlapping roles. To test whether the lack of chemical sensitivity of the htz1-K3,8,10,14R mutant was due...
to acetylation of H2A by NuA4, the \( htz1-K3,8,10,14R \) mutation was combined with a version of H2A that cannot be acetylated [htz1-K4,7R]. This created a double mutant with no version of either H2A or H2A.Z that could be acetylated by NuA4. However, on media containing all of the drugs described above, there was no discernable growth difference between the individual or the double mutant from wild type [data not shown]. Therefore, it was H2A.Z itself, and not its acetylated forms, that conferred resistance to these chemical agents.

**Discussion**

This work focused on whether the H2A variant H2A.Z was subjected to covalent modification, and if so, by which enzymes, and for what purpose. The results revealed that acetylated H2A.Z contributed to the boundaries between euchromatin and heterochromatin, and played an unanticipated role essential for viability.

**H2A.Z acetylation: the interplay of SWR1-Com, NuA4, and SAGA**

The results presented here established that H2A.Z was a substrate for the HAT NuA4 in vitro and in vivo. Moreover, H2A.Z purified from yeast cells was acetylated in vivo at multiple sites. The majority of H2A.Z modifications depended on the HAT NuA4, and at least one depended on SAGA (GCN5). NuA4 acetylation of H2A.Z was a prerequisite for SAGA acetylation, since H2A.Z

**Figure 6.** Genetic interactions between \( HTZ1 \) alleles and mutations affecting histone H4 acetylation. (A) \( htz1-K3,8,10,14R-3\text{Flag} \): KanMX was crossed to \( eaf1\Delta: \text{His}3\text{MX} \), and tetrads were analyzed. Tenfold serial dilutions were spotted on minimal media and grown for 3 d at the indicated temperatures. (B) Wild-type \( HHF2 \) was carried on a \( URA3 \) plasmid in a background with either wild-type \( HTZ1 \) or \( htz1-K3,8,10,14R-3\text{Flag} \). These strains were transformed with either wild-type \( HHF2 \), \( hhf2-K5,8,12R \), or \( hhf2-K5,8,12,16R \) on a \( TRP1 \) plasmid. Cells were grown in medium containing tryptophan. Tenfold serial dilutions were spotted on either minimal medium lacking tryptophan and lacking uracil or minimal medium lacking tryptophan and containing FOA, to select against the \( URA3 \) plasmid. Cells were grown for 3 d at 23°C prior to imaging.

Figure 7. A model linking SWR1-Com and NuA4 in H2A.Z function. Following deposition into chromatin, H2A.Z is first acetylated by NuA4 and subsequently by NuA4 and SAGA.
stantial change in free energy, one would expect the reverse reaction to also be catalyzed by SWR1-Com. Perhaps acetylation of H2A.Z- containing nucleosomes by NuA4 disfavors the reverse reaction. If so, the lack of H2A.Z and/or H4 acetylation in these nucleosomes may allow SWR1-Com to remove H2A.Z–H2B dimers, resulting in a reduced level of H2A.Z in chromatin. A decisive test of this model will require kinetic analysis of H2A.Z deposition in wild-type and NuA4 mutant strains.

H2A.Z acetylation and chromatin boundary function

A heterochromatin boundary function for H2A.Z acetylation was suggested by the esa1-L254P mutation causing a decrease in the expression of telomere-proximal genes (Zhang et al. 2004) combined with the discovery here that H2A.Z is a substrate of NuA4. Indeed, an unacetylatable H2A.Z was defective in boundary function at the telomeres, even though localization of the mutant H2A.Z was indistinguishable from wild type. In addition, expression levels of genes that were repressed in the htz1-K3,8,10,14R mutant were restored in the absence of Sir2p. This inference was confirmed by directly assaying Sir3p levels at these chromosomal locations in wild-type and htz1-K3,8,10,14R mutants and finding that Sir3p spread beyond its normal boundaries in the mutant. Thus, acetylation of H2A.Z was important in restricting the spread of Sir proteins from telomeres into subtelomeric positions. However, boundaries of silenced chromatin at HMR did not depend on acetylation of H2A.Z. One boundary at HMR corresponds to a flanking tRNA gene whose promoter seems to play a critical role in the function of the boundary (Donze et al. 1999; Oki and Kamakaka 2005). Mutations in HAT genes do not result in heterochromatin spreading beyond this boundary. Only when the tRNA gene is deleted in combination with a HAT mutant do the Sir proteins spread. Presumably the htz1-K3,8,10,14R mutation in combination with deletion of the tRNA gene would result in Sir protein spreading beyond this boundary.

The fact that H2A.Z acetylation prevents spreading of silencing raises the intriguing possibility that acetylated H2A.Z modulates a chromatin structure that is incompatible with Sir protein binding and hence with silencing. Indeed, nucleosomal arrays containing Drosophila H2A.Z prevent the formation of highly condensed heterochromatic structures that are incompatible with transcription (Fan et al. 2002). If this model is correct, then acetylated tails of H2A.Z rather than interactions between octamer cores would be the critical limitation to forming these specialized structures. In a similar spirit, a protein could specifically bind acetylated H2A.Z and block the spread of Sir proteins. Such a protein would be unable to bind the htz1-K3,8,10,14R mutant, leading to the ectopic spread of the Sir proteins. Mutations in this protein should result in similar defects in the boundary of heterochromatin. Finally, acetylated tails of H2A.Z may be molecular decoys that can be recognized by and inhibit the Sir2p deacetylase.

Essential and nonessential roles for H2A.Z in Saccharomyces

A common genetic misconception is that genes in which null alleles are viable do not encode essential functions. Essential functions may be shared by overlapping proteins. The latter case seems to apply broadly to H2A.Z, as revealed by many synthetic lethal double-mutant combinations (A. Allison and M. Smith, pers. commun.). This study uncovered an essential function specific to the acetylated form of H2A.Z that in combination with eaf1Δ was severely growth-impaired at 23°C and lethal at 37°C. The essential function provided by acetylated H2A.Z could also be fulfilled by acetylated H4 but not by acetylated H2A. At present, the difference between the ability of H2A and H4 to carry out an essential function shared with H2A.Z could reflect either qualitative or quantitative differences between the two proteins. There are only two possible sites for acetylation of H2A, and four on H2A.Z and H4. Hence, cells might need a minimum amount of nucleosome acetylation for viability. However, since H2A.Z is only 10% as abundant as H2A, qualitative differences between H2A and H4 seem more likely. Interestingly, no NuA4 mutant has been recovered that completely abolishes H2A.Z acetylation. Presumably the last remaining acetyl group is important for robust growth when H4 acetylation is also reduced.

Materials and methods

Yeast strains and plasmids

All yeast strains are in the W303 background and are listed in Table 1. One-step integration of knockout cassettes (Longtine et al. 1998; Goldstein and McCusker 1999) and the C-terminal Flag-tag (Gelbart et al. 2001) have been previously described (see Supplementary Table 1 for primer sequences). HTZ1 was cloned into pRS316 using gap-repair, resulting in plasmid pJR2658. HTZ1 was amplified from genomic DNA using Pfu-Ultra (Stratagene) (the primers are listed in Supplementary Table 1). The purified PCR product was cotransformed into JRY7754 (htz1Δ::His5MX) with pRS316 digested with HindIII and BamHI. Plasmids were rescued from Ura+ transformants, and the integrity of the insert was verified by DNA sequencing. pJR2658 was transformed into JRY7754 (htz1Δ::His5MX). A C-terminal 3Flag-tag was integrated on the plasmid in frame with the HTZ1 open reading frame and verified by DNA sequencing, resulting in pJR2959. Site-directed mutagenesis was carried out using Quikchange XL [Stratagene] on pJR2969 mutating K3 (pJR2970), K8 (pJR2971), K10 (pJR2972), K14 (pJR2973), and K3,8,10,14R (pJR2974) [see Supplementary Table 1 for primers]. htz1-K3,8,10,14R-3Flag:: KanMX was integrated at its chromosomal locus by amplifying the gene from the plasmid using Pfu-Ultra and transforming a strain htz1Δ::URA3MX [JRY9790] and selecting for G418 resistance. G418-resistant cells were then screened for FOA resistance, to identify cells that had gained the htz1 multiple site mutant at the HTZ1 locus and lost the URA3 insertion at that locus. The HTZ1 locus from strains that were resistant to both drugs was PCR-amplified using Pfu-Ultra and sequenced.

Protein purifications

S. cerevisiae canonical histones were expressed individually in E. coli and purified from inclusion bodies as described (Luger et
Table 1. Yeast strains

| Strain        | Genotype   | Source                  |
|--------------|------------|-------------------------|
| W303-1a      | MATa ade2-1 leu2-3, 112 his3-1 ura3-52 trpl-100 can1-100 | R. Rothstein |
| JRY7721      | MATa ESA1-TAP::TRP1 | Kobor et al. 2004 |
| JRY7754      | MATa htz1Δ::HIS3MX | Kobor et al. 2004 |
| JRY7763      | MATa eaf1Δ::HIS3MX | Kobor et al. 2004 |
| JRY968       | MATa esa1-1851::URA3 | Bird et al. 2002 |
| JRY969       | MATa esa1-L357H::URA3 | Bird et al. 2002 |
| JRY970       | MATa htz1Δ::URA3MX | This study |
| JRY971       | MATa HTZ1-3Flag::KanMX | This study |
| JRY972       | MATa HTZ1-3Flag::KanMX | This study |
| JRY973       | MATa esa1-1851::URA3 HTZ1-3Flag::KanMX | This study |
| JRY974       | MATa esa1-1851::URA3 HTZ1-3Flag::KanMX | This study |
| JRY975       | MATa eaf1Δ::HIS3MX HTZ1-3Flag::KanMX | This study |
| JRY976       | MATa swf1Δ::HIS3MX HTZ1-3Flag::KanMX | This study |
| JRY977       | MATa gcn5Δ::HIS3MX HTZ1-3Flag::KanMX | This study |
| JRY978       | MATa sas2Δ::HIS3MX HTZ1-3Flag::KanMX | This study |
| JRY979       | MATa esa1-L357H::URA3 HTZ1-3Flag::KanMX | This study |
| JRY980       | MATa esa1-L357H::URA3 gcn5Δ::HIS3MX HTZ1-3Flag::KanMX | This study |
| JRY981       | MATa htz1Δ::HIS3MX [pRS314 HTZ1-3Flag::KanMX] | This study |
| JRY982       | MATa htz1Δ::HIS3MX [pRS314 htz1K3,8,10,14R-3Flag::KanMX] | This study |
| JRY983       | MATa htz1::K3,8,10,14R-3Flag::KanMX | This study |
| JRY984       | MATa htz1::K3,8,10,14R-3Flag::KanMX sir2Δ::His3MX | This study |
| JRY985       | MATa htz1Δ::HIS3MX [pRS314 htz1K3,8,10,14R-3Flag::KanMX] | This study |
| JRY986       | MATa htz1Δ::HIS3MX [pRS314 htz1K8R-3Flag::KanMX] | This study |
| JRY987       | MATa htz1Δ::HIS3MX [pRS314 htz1K10R-3Flag::KanMX] | This study |
| JRY988       | MATa htz1Δ::HIS3MX [pRS314 htz1K14R-3Flag::KanMX] | This study |
| JRY989       | MATa [hh1-ht1Δ::HphMX[hh2-bht2Δ::NatMX [pRS316 HHF2-HHT2]] | This study |
| JRY990       | MATa [hh1-ht1Δ::HphMX[hh2-bht2Δ::NatMX htz1-K3,8,10,14R-3Flag::KanMX] | This study |
| JRY8121      | MATa HTZ1-3Flag::KanMX SIR3-TAP::TRP1 | This study |
| JRY8122      | MATa htz1-K3,8,10,14R-3Flag::KanMX SIR3-TAP::TRP1 | This study |

Histone acetylation assays

HAT assays were performed as described (Allard et al. 1999). 3H-acetyl-CoA (0.125 µCi) was added to 10 µL of NuA4 along with varying amounts of wild-type dimers or octamers, and H2A.Z dimers or octamers in HAT buffer [50 mM Tris-Cl at pH 8.0, 50 mM KCl, 0.1 mM EDTA, 5% glycerol, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 mM sodium butyrate] for 30 min at 30°C. N-terminal tagged Htz1p with a six-histidine epitope was expressed in E. coli, and inclusion bodies containing recombinant proteins were purified as described. After denaturing the inclusion bodies, His,–Htz1p was purified using Talon Cobalt Affinity Resin [BD Biosciences] and eluted with 300 mM imidazole. Reconstitution of octamers containing canonical H2A, or H2A.Z, was done as described (Luger et al. 1999). Untagged H2A.Z for mass spectrometry was purified as described for canonical H2A (Luger et al. 1999; Dyer et al. 2004). Untagged H2A.Z containing recombinant proteins were purified as described. Native NuA4 complex was purified from 6Lo f. Complete Protease Inhibitor Cocktail (Roche), 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 0.05 M 2-mercaptoethanol, and 1x Complete Protease Inhibitor Cocktail (Roche), and the TEV cleavage was performed overnight at 4°C (Kobor et al. 2004).

AU gels

Cells were grown to mid-log phase, and 100 OD600 units were harvested by centrifugation. Cells were resuspended in Buffer HIP [50 mM Tris-Cl at pH 7.8, 200 mM NaCl, 1.5 mM MgAc, 100 mM Na Butyrate, 5 mM Nicotinamide, 10 mM NaPPi, 0.1 mM Na3VO4, 5 mM NaF, Complete Protease inhibitor cocktail (Roche)]. Acid-washed glass beads were added, and the cells were disrupted mechanically using a bead beater [BioSpec Products] for 5 min. CaCl2 was added to a final concentration of 1 mM, and chromosomal DNA was digested using 10 U of micrococcal nuclease [Sigma] for 10 min at 37°C. Insoluble material was removed by centrifugation. The supernatant was removed and incubated with 25 µL of α-Flag M2 beads [Sigma] for 90 min at 4°C. Beads were then pelleted and washed three times with 0.6 mL of Buffer HIP. After washing, the supernatant was completely removed by aspiration with a 30-gauge needle. The dry beads were resuspended in AU gel loading buffer (6 M urea, 5% acetic acid, 0.02% Pyronin Y). AU gels were set up and run as described, except Triton X-100 was omitted (Lennox and Cohen 1989). The gel was transferred to a PVDF membrane [Millipore] in 0.9% acetic acid toward the cathode [Rogakou et al. 2000] and immunoblotted using Rabbit α-Flag [Sigma], followed by secondary anti-rabbit IgG conjugated to Alexa Fluor 680 [Invitrogen]. The immunoblot was scanned using a LiCor Odyssey Infrared Imaging System.
Chromatin association assay

The chromatin association assay was carried out essentially as described, except 100 mM Na butyrate and 5 mM nicotinamide were added to inhibit histone deacetylases (Donovan et al. 1997; Liang and Stillman 1997). The soluble fractions and MNease-digested fractions were affinity-purified using αFlag M2 beads (Sigma) and subjected to AU gel analysis as described above.

Quantitative PCR

RNA was purified from S. cerevisiae using the hot-phenol method (Schmitt et al. 1990). DNA was digested with RNase-free DNase I (Roche and Qiagen). cDNA was synthesized using a cDNA synthesis method (Schmitt et al. 1990). RNA was digested with RNase-in Supplementary Table 2.

Sham). Input DNA and ChIP DNA were analyzed using an

and IPs of Sir3p-TAP were performed using IgG resin (Amer-

somes)/H9251

Samples were analyzed in triplicate for two independent RNA

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