Individual Lysine Acetylations on the N Terminus of Saccharomyces cerevisiae H2A.Z Are Highly but Not Differentially Regulated*§

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The multi-functional histone variant Htz1 (Saccharomyces cerevisiae H2A.Z) is acetylated on up to four N-terminal lysines at positions 3, 8, 10, and 14. It has thus been posited that specific acetylated forms of the histone could regulate distinct roles. Antibodies against Htz1-K8Ac, -K10Ac, and -K14Ac show specific acetylated forms of the histone could regulate distinct functions. The basic repeating unit of chromatin is the nucleosome core particle: ~146 bp of DNA wrapped around a core histone octamer composed of a (H3-H4)2 tetramer and two (H2A-H2B) dimers (1). The major histones (H2A, H2B, H3, and H4) are each encoded by multi-copy genes, highly expressed during the S phase, and deposited in chromatin during DNA replication. Histone variants are nonallelic isoforms that are usually (although not exclusively) encoded by single copy genes. In many cases variants can substitute for the major histones in specific nucleosomes through the action of dedicated deposition machineries (3). Both major and variant histones are subject to extensive post-translational modification by the addition of small chemical moieties, including phosphorylation, acetylation, methylation, sumoylation, and ubiquitylation (4). These groups are thought to regulate access to the DNA in the modified nucleosome by various means, including directly modulating the charge on the nucleosome surface or generating sites for the recruitment of regulatory proteins (5, 6).

Histone H2A has one of the largest variant families and includes H2A.Z, a protein that is highly conserved across eukaryotes but differs considerably from the major H2A in each species (7). H2A.Z has been ascribed a large number of roles (8, 9), including most recently suppressing antisense RNAs (10) and stabilizing the association of condensin with mitotic chromosomes (11). We still have a poor understanding of precisely how the variant mediates any specific function, although differential enrichment at specific locations and distinct post-translational modifications are likely to contribute. In all species examined, their respective H2A.Zs are subject to multiple N-terminal tail acetylations (12, 13). This modification is integral to fission yeast H2A.Z function, with completely unacetylatable alleles phenocopying complete deletion of the histone in genome scale transcriptome and genetic analyses (11). Multiple regulatory roles have been assigned to acetylated H2A.Z in Saccharomyces cerevisiae (Sc), e.g. heterochromatin restriction (14), transcription (15), and chromosome stability (16). However, many of these ascribed functions were derived from completely unacetylatable alleles, so the importance of any individual acetylation is unclear. Finally, it is also unknown whether the effects of any of these modifications are direct (e.g. steric hindrance or charge modulation) or indirect (e.g. via the recruitment of regulatory proteins).

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§ The abbreviations used are: Sc, S. cerevisiae; SWR-C, SWR complex; WCE, whole cell extract; TBZ, thiabendazole; CC, correlation coefficient; SS/SL, synthetic sick/lethal.
Differential Analysis of S. cerevisiae H2A.Z Acetylation

In this work we examine the regulation and function of four individual acetylations on the Sc H2A.Z (gene name HTZ1) N terminus: K3Ac, K8Ac, K10Ac, and K14Ac. We have raised antibodies to the latter three acetyl forms and show that each is chromatin-associated and primarily regulated by the NuA4 acetyltransferase and Hdac1 deacetylase complexes. We have identified a range of novel Htz1Ac regulators, all of which have equivalent effects on K8Ac, K10Ac, and K14Ac. This suggests that each acetylation is redundant, a proposal supported by epistasis mapping analyses of a comprehensive panel of unacetylatable HTZ1 alleles. However, N-terminal acetylation is important for Htz1 function, and completely unacetylatable HTZ1 alleles show a large number of genetic interactions in common with and distinct from htz1Δ. Furthermore, each Htz1Ac is actively regulated in response to benomyl, a microtubule destabilizing agent. Thus our results indicate that N-terminal acetylation is important for the Htz1 function, but the cell does not distinguish between individual acetylated lysines on this histone.

EXPERIMENTAL PROCEDURES

Materials—The antibodies and strains used in this study are listed in supplemental Tables S1 and S2.

Antibody Generation—Rabbit polyclonal affinity-purified anti-Sc Htz1-K8Ac, -K10Ac, and -K14Ac were from Millipore, with αK14Ac described previously (16). For each new target (αK3Ac, αK8Ac, and αK10Ac) five rabbits (prescreened for minimal reactivity to Sc proteins) were immunized (peptides as in Fig. 1A coupled to keyhole limpet hemocyanin), and test bleeds/booster were performed monthly. Initial screening of each bleed was by immunoblotting against whole cell extracts (WCEs) from WT and htz1Δ cells. Attempts to raise αK3Ac failed at this stage because all rabbits failed to raise a detectable immune response (not shown). Higher titer sera (usable at >1/200 dilution) from each rabbit were then tested for specificity against WCEs from unacetylatable htz1 point mutant cells (e.g. htz1-K8R; see Fig. 1B). Specificity was further characterized by Luminex bead assay against a comprehensive peptide panel, including the relative immunogen, its acetylated form, and all other acetyl peptides from the immunization series (supplemental Fig. S1). These analyses confirmed the absence of cross-reaction with an inappropriate Htz1Ac species. To derive preparations for this study, positive bleedings were pooled, affinity-purified on the immunizing acetyl peptide, and subtracted with the relevant unmodified peptide.

Cell Fractionation—Sc cell fractionations were as described (16). The total, cytoplasmic, nuclear, and chromatin fractions were analyzed by SDS-PAGE and immunoblotting.

Creation of htz1 Mutant Strains—Various unacetylatable point mutants at HTZ1 (4KR, 3KR, 3Ac*, etc.) were created by a modified Delitto Perfetto approach (17) in a “magic marker” strain compatible with the synthetic genetic array protocol (18) (KFY1069; see supplemental Table S2). In brief, HTZ1 was first replaced by a [Kan8/URA3] cassette. The htz1 ORF was then PCR-amplified from Sc genomic DNA (with the desired mutation inserted by megapriming (19)), and the product was transformed into exponentially growing cells.

Colonies that replaced [Kan8/URA3] with htz1 by homologous recombination were identified on 5-fluoroorotic acid (which counterselects URA3) and confirmed by sequencing. To construct htz1-NΔ, the loxp-PkanMX-loxp cassette from pOM10 (20) was used to replace the N terminus of htz1 (residues 3–14, including all four acetylatable lysines; see Fig. 1A) in situ. Cre-induced recombination resulted in cassette removal with retention of the 24-residue loxp element. In the final step a Nourseothricin resistance cassette (NAT) was incorporated immediately downstream of each htz1 to facilitate locus selection during synthetic genetic array (21). An HTZ1::NAT locus-tracking strain was also created as a WT control.

Growth Curve Analysis—Growth curves were monitored with a Bioscreen C (Oy Growth Curves). Seed cultures were grown to mid-log in nonselective medium and diluted to A600 ≤ 0.1 in medium with the appropriate agents, e.g. synthetic complete ± 6-azauracil (6AU) or mycophenolic acid (MPA), YPD ± benomyl, TBZ, or camptothecin. All of the analyses were performed in triplicate, and A600 curves (30 °C, constant agitation, 15-min time points) were monitored for >48 h.

Htz1 Stability Analyses—Translational shut-off: Cycloheximide (final concentration, 35 μg/ml (22)) was added to exponentially growing cultures (A600 = ~0.5), and 5-ml aliquots were taken at the indicated intervals for WCE isolation (TCA method; see below). Target abundance was estimated by immunoblotting at each time point (see Fig. 3A).

Transcriptional Shut-off—To create GAL1p-HTZ1.HA3, the GAL1 promoter was integrated by homologous recombination to replace the endogenous promoter upstream of HTZ1.HA3. Immunoblotting (αHA) confirmed that comparable Htz1.HA3 levels are derived from each promoter in YPGR (2% galactose, 1% raffinose) medium (not shown). An exponentially growing GAL1p-HTZ1.HA3, culture (A600 = ~0.5 in YPGR) was collected by centrifugation, washed in double distilled H2O, and resuspended in YPD (containing 2% glucose to repress transcription from GAL1p). Aliquots were taken at the indicated intervals for WCE isolation. Target abundance was estimated by immunoblotting at each time point (see Fig. 3, B and C).

Synthetic Genetic Array Screening—Genetic interactions were determined by the partially automated synthetic genetic array method (21). htz1 alleles in the magic marker background (supplemental Table S2) were mated in quadruplicate to either a mutant library of 1,286 factors involved in chromatin metabolism (23) or two libraries that together cover >98% of yeast genes (i.e. ~4800 nonessential genes individually deleted with a KanMX cassette (24) or hypomorphic alleles of ~842 essential genes with KanMX disrupting their polyadenylation site (25)). All of the libraries were arrayed at 1536 colony density/12.5 × 8.5-cm plate and replica plated with a Singer RoToR. The growth of all double-mutant haploid daughters was compared with the respective single-mutant parents to identify and quantify positive or negative genetic interactions (26, 27). For epistasis mapping (23), the genetic profile of each htz1 allele was compared within a set of 2,255 profiles to calculate pair-wise Pearson correlation coefficients.
TCA Cell Extracts for Western Blotting—WCEs were isolated by the TCA method, which efficiently extracts chromatin and preserves labile modifications (28). In brief, ∼10-ml cultures were grown to mid-log ($A_{600} = \sim 1.0$), and cells were collected by centrifugation and washed with 20% TCA. All further steps were performed on ice with prechilled solutions. Cell pellets were resuspended in 250 μl of 20% TCA and subjected to glass bead lysis. The suspension minus the glass beads was collected, 1 ml of 5% TCA was added, and the precipitate was collected by centrifugation. The pellets were washed with 750 μl of 100% ethanol, and the proteins were solubilized in 50 μl of 1 m Tris, pH 8.0, 100 μl of 2× SDS-PAGE loading buffer (60 mm Tris, pH 6.8, 2% SDS, 10% glycerol, 100 mm DTT, 0.2% bromphenol blue). After 5 min at 95 °C, insoluble material was removed by centrifugation, and the supernatant was analyzed further by immunoblotting.

RESULTS

Htz1-K8Ac, K10Ac, and K14Ac Are Chromatin-associated and Regulated by Esa1 and Hda1—Sc H2A.Z (Htz1) has four acetylatable lysines on its N terminus at positions 3, 8, 10, and 14 (Fig. 1A) (14, 16, 29). Antibodies to each modification would greatly aid in their analysis. We have previously described anti-Htz1-K14Ac (aK14Ac) in detail (16) and have now raised aK8Ac and aK10Ac (Fig. 1B and supplemental Fig. S1). Attempts to raise aK3Ac were unsuccessful (see “Experimental Procedures”). Each reagent (aK8Ac, aK10Ac, and aK14Ac) shows strong specificity for its respective target in immunoblotting, losing recognition if the appropriate lysine is mutated to arginine (e.g. K10R; Fig. 1B). These antibodies also indicate that individual Htz1 N-terminal acetylations show no interdependence (e.g. K10Ac is not impacted by K3R, K8R, or K14R) or compensation (e.g. K10Ac levels do not increase relative to WT if K10 is the sole acetylatable residue on the Htz1 N terminus) (Fig. 1B and supplemental Fig. S2).

The abundance of Htz1 K8Ac, K10Ac, and K14Ac are strongly reduced on deletion of SWR1, the eponymous ATPase subunit of the SWR complex (SWR-C; Fig. 1C). Because Swr1 is required for the insertion of Htz1 into nucleosomes (30, 31), this indicates that all three lysines are acetylated after chromatin assembly. Cell fractionation confirmed that each Htz1Ac is chromatin-associated (Fig. 1C) and further that its acetylation status does not regulate entry to this cellular compartment (supplemental Fig. S3).

The Esal and Gcn5 acetyltransferases or Hda1 deacetylase are each reported to regulate the acetylation of Htz1 (15, 16, 32). In direct analyses K8Ac, K10Ac, and K14Ac are abolished in cells containing mutants of Nua4, including a temperature-sensitive allele of Esa1 (esa1-L254P) or deletion of various complex subunits (Fig. 1, D and E). In contrast gcn5Δ has no effect on K8Ac, K10Ac, or K14Ac levels (Fig. 1D), although we note that Gcn5 could still target Htz1-K3Ac*; this could not be determined without an antibody to the latter modification. Regarding the deacetylation of Htz1, Hda1 has been shown to target Htz1-K14Ac (32). We have confirmed this and determined that Hda1 also deacetylates K8Ac and K10Ac (Fig. 1F). Thus three distinct Htz1Ac species are chromatin-associated and metabolized by the same enzymes in rapidly growing cells.

Genome Scale Genetic Analyses Indicate That Individual Htz1 Acetylations Are Redundant—To determine whether each Htz1 acetylation could have a specific role, we tested the genetic interactions of a comprehensive range of unacetylatable alleles. A number of synthetic genetic array compatible strains were created, including complete deletion (htz1Δ), N-terminal deletion (ΔN), all four lysines mutated to arginine (4KR) or glutamine (4KQ), each individual lysine mutated to arginine (e.g. K3R) or glutamine (e.g. K3Q), or a single remaining acetylatable residue (e.g. K8R/K10R/K14R = K3Ac*). A locus-marked HTZ1::NAT strain was also created as a WT control. In immunoblotting each mutant was expressed at similar levels to WT with the exception of htz1Δ-N, which is weakly hypomorphic (not shown). Notably this hypomorphism is also seen with a comparable allele of Schizosaccharomyces pombe H2A.Z (pht1-NΔ) (11).

Each strain was individually mated to a mutant library of 1,286 factors involved in chromatin metabolism (23), and double-mutant haploid daughters were isolated. We then derived scores covering each negative (i.e. synthetic sick/lethal (SS/SL)) or positive (i.e. epistasis or suppression) genetic interaction using colony size as a quantitative readout (27, 33). The genetic interaction profile of a particular mutant can be used as a high resolution phenotype, with functionally related factors displaying similar profiles (23, 27). Thus deletions of individual members of the SWR-C are highly correlated with htz1Δ and ΔN (Fig. 2A, green nodes). This is expected because SWR-C is required for the insertion of Htz1 into chromatin (Fig. 1C) (30, 31). The profiles for htz1Δ, ΔN, 4KR, and 4KQ are also highly correlated (Fig. 2A), suggesting that significant functionality of Htz1 resides in its N terminus and furthermore that the primary role of this region is to harbor the acetylations. Indeed the 4KR and 4KQ profiles were most similar to ΔN within those 2,255 profiles collated to date (Fig. 2B) (23, 27, 34). Furthermore the 4KR and 4KQ profiles were themselves very highly correlated (Pearson correlation coefficient (CC) 0.876; Fig. 2C), suggesting that the charge on these residues does not regulate their function (see “Discussion”).

In contrast to the widespread genetic interactions of completely unacetylatable htz1 alleles, single point (e.g. K3R) or singly acetylatable (e.g. K3Ac*) mutants gave few consistent interactions and clustered near the wild-type control (Fig. 2A, white node). Thus the loss of any single Htz1 acetylation does not confer a genotype strong enough to distinguish the allele, suggesting no significant loss-of-function in any case. Because the retention of any single acetylatable lysine has a similar effect, this suggests that the individual Htz1 acetylations are redundant.

Closer inspection of the individual genetic interactions for htz1Δ, ΔN, 4KR, and 4KQ revealed many that are common between these four mutants. This includes SS/SL interactions with components of the PAF complex (RTF1 and CDC73), the replication checkpoint complex (CSM3 and MRC1), and the chromatin regulators BRE1, SET2, and SET3 (Fig. 2D). However, and as expected given their imperfect CCSs, htz1Δ also shows many interactions distinct from the completely unac-
etylatable alleles. This may be related to the loss of the Htz1 core domain or C terminus in the complete deletion (see “Discussion”). For example, we observed SS/SL interactions between htz1Δ and components of the Lsw1, Ino80, and Elongator complexes, as well as factors involved in kinetochore (MCM16, MCM21, and MCM22) and spindle (MAD2, MAD3, and RAD61) function. Finally, we also observed SS/SL interactions common to htz1Δ and NΔ but not shared by 4KR or 4KQ, such as with components of the Rpd3L deacetylase complex (Fig. 2D). These last may be related to the hypomor-
The primary function of the Htz1 N terminus is to harbor lysine acetylations. A, Pearson CC plot compares the genetic interaction profiles of $htz1\Delta$ or $htz1$-NΔ (X or Y-axes) with 2,255 profiles performed to date. Blue and black spots, as labeled; green spots, SWR-C components ($swr1\Delta$, $yar9\Delta$, $vps71\Delta$, $vps72\Delta$, $swc3\Delta$, $swc5\Delta$, and $arp6\Delta$); white spot, WT control; red spots, all other mutants on array. B, distribution plot of the CCs of NΔ against 2,245 mutant profiles. The most highly correlated profiles include those derived from 4KR and 4KQ. C, a correlation plot of CCs for 2,245 individual genetic screens relative to 4KR or 4KQ. D, as expected from their strong but imperfect pairwise CCs $htz1\Delta$, NΔ, 4KR, and 4KQ show both common and unique genetic interactions. Blue and yellow correspond to negative or positive genetic interactions.

Differential Analysis of S. cerevisiae H2A.Z Acetylation

Two approaches were used: general inhibition of the translational machinery by cycloheximide (Fig. 3A) or the specific transcriptional repression of GAL1p-HTZ1.HA₃ by glucose (Fig. 3B). After each shut-off, the abundance of Htz1 and Htz1-K8Ac, -K10Ac, and -K14Ac were monitored for up to 6 h. In each case total Htz1 had a t₁/₂ greater than 3 h or approximately two complete cell cycles, strongly suggesting reuse after nucleosome eviction. Each Htz1Ac decayed significantly faster than total Htz1 on cycloheximide (Fig. 3A). However, this is likely due to the translational inhibition of an acetylation regulator (e.g. a subunit of NuA4; Fig. 1, D and E), because each Htz1Ac tracked with total Htz1 when transcription of the histone was repressed (Fig. 3B). Furthermore the observation that Htz1-K8Ac, -K10Ac, and -K14Ac decayed at similar rates on transcriptional shut-off also argues against their differential usage at distinct locations.

To examine whether its acetylation could regulate Htz1 turnover, we compared GAL1p-HTZ1.HA₃ to two additional strains: GAL1p-hzt1-4KR.HA₃ and [GAL1p-HTZ1.HA₃/$htz1\Delta$]. The former contains an unacetylatable form of Htz1, the latter is increased for all acetylated species of the histone (Fig. 1F). On transcriptional repression, total Htz1 levels decayed with similar rates in all three backgrounds (Fig. 3C), indicating that its acetylation status has no impact on the turnover of this histone variant.

Each Htz1Ac Is Similarly Regulated in a Range of Mutant Backgrounds—To further investigate the possibility of their differential regulation, we used a proteomic screening approach (36) to identify modifiers of each Htz1Ac. Viable mu-
tants of ~50 genes, primarily factors related to the gene ontology terms “histone acetylation” or “histone deacetylation,” were isolated from the Sc haploid deletion collection and immunoblotted for Htz1-K8Ac, -K10Ac, and -K14Ac (see supplemental Table S3). This confirmed that all three Htz1Ac forms are dependent on individual subunits of NuA4 (YAF9, YNG2, and EAF1) but independent of SAGA components (see also Fig. 1, D and E). The approach identified many additional regulators of Htz1Ac (e.g. Asf1, Rpd3L, and Elongator) but no differential regulators of any individual acetylation, i.e. K8Ac, K10Ac, and K14Ac tracked together in every background (supplemental Table S3 and data not shown). This includes Bud14 and Clb2, whose deletions were previously identified as reducing K14Ac levels (34); these deletions impact K8Ac and K10Ac to a similar degree (not shown). In many of these deletion backgrounds, the effect on Htz1Ac may be indirect, almost certainly in Rpd3L complex mutants where reduced K8Ac, K10Ac, and K14Ac is an unlikely outcome when inhibiting a deacetylase.

We chose Asf1 as a novel Htz1Ac regulator to examine further. Deletion of this H3-H4 chaperone reduces the level of each Htz1Ac without affecting the abundance of the histone (Fig. 4A). Each acetylation takes place after Htz1 is assembled into chromatin (Fig. 1C), so we tested whether asf1Δ impacts...
the deposition and/or acetylation step(s). In fractionation analyses, the chromatin of \textit{asf1}/H9004 cells shows WT levels of total Htz1 but a reduction in each Htz1Ac (Fig. 4B). Thus Asf1 appears to regulate the Htz1 acetylation step. Asf1 is multifunctional and among other roles regulates various acetylations on histones H3 and H4 (H3-K9Ac, K27Ac, K56Ac; H4-K5Ac, K8Ac, K12Ac; see Fig. 3C (37)). We thus tested whether Htz1Ac was dependent on the prior modification of any of these residues using a range of mutant strains with unacetylable alanine at each position (\textit{i.e.} H3-K9A, K27A, K56A; H4-K5A, K8A, K12A (38)). In each mutant the abundance of each Htz1Ac was comparable with WT (supplemental Fig. S5).

Given the multi-functionality of Asf1, it could prove difficult to identify whether a specific role is required for efficient Htz1Ac. We thus took an approach where Asf1 was rapidly removed from cells, and each Htz1Ac was monitored over a succeeding time course. We used two shut-off strains, namely \textit{GAL1p.ASF1.HA3} and \textit{GAL1p.ASF1.HA3.PEST} as the only source of the factor, with \textit{ASF1} transcription in each repressed by glucose addition (39). The Cln2 C-terminal PEST domain confers a rapid, cell cycle-independent turnover on heterologous proteins (40), and its addition to Asf1.HA3 significantly shortens protein half-life ($t_\text{1/2}$ Asf1.HA3 > 60 min; $t_\text{1/2}$ Asf1.HA3.PEST < 15 min; Fig. 4D) (39). However, the abundance of each Htz1Ac (K8Ac, K10Ac, or K14Ac) remained unchanged over each 6-h time course (\textit{i.e.} more than three complete cell cycles under experimental conditions) rather than reducing as expected (Fig. 4D). This is not a strain background issue because their prolonged (> 24 h) incubation in glucose-containing medium recapitulates the original observation of reduced Htz1Ac (Fig. 4E). Thus the reduced Htz1Ac in \textit{asf1} cells may not be due to a direct regulation by Asf1 of Htz1 acetylation but rather may involve some adaptation to the chronic absence of the histone chaperone. This also raises a cautionary note about overinterpreting the relationship of those additional Htz1 regulators identified by the proteome screening approach (supplemental Table S3).

**Htz1 Acetylation Regulates the Response to Microtubule Depolymerizing Agents**—The deletion of H2A.Z in \textit{S. cerevisiae} or \textit{S. pombe} leads to genome instability, synthetic genetic interactions with components of the kinetochore and spindle checkpoint machineries, and sensitivity to benomyl, a microtubule-depolymerizing agent (Figs. 2D and 5A) (11, 16, 41). H2A.Z acetylation contributes to the genome stability role in each species, with strains containing unacetylable alleles showing increased rates of chromosome loss (11, 16).

To further examine how individual acetylations on Htz1 may regulate benomyl resistance, we tested how the full range
of unacetylatable alleles \((htz1\Delta, N\Delta, 4KR, K3R, K3Ac^*, \text{etc.})\) responded to this agent. Rather than spotting onto plates, growth curves were monitored in liquid culture because the latter approach can more easily distinguish the reduced fitness of \(htz1\Delta\) relative to WT on rich media (YPD; Fig. 5, compare \(A\) and \(B\)). In this manner completely unacetylatable \(htz1\) alleles \((N\Delta, 4KR, \text{or } 4KQ)\) show a dose-dependent sensitivity to benomyl, although not to the same degree as \(htz1\Delta\) (Fig. 5B). We (and others) had previously reported an \(htz1-K14R\) strain as sensitive to benomyl (16, 32). However, we have been unable to consistently repeat this phenotype in current analyses using any single unacetylatable \((\text{e.g. } K14R)\) or acetylatable \((\text{e.g. } K14Ac^*)\) \(htz1\) allele (not shown). This may be related to strain differences or some other as yet uncharacterized factor.

Because \(htz1Ac^*\) contributes to benomyl resistance, we next asked whether the acetylations are themselves regulated by the agent. In parallel analyses, cells were treated with thiabendazole (TBZ, another microtubule depolymerizer) or camptothecin (a topoisomerase I inhibitor). These were chosen for comparison because \(htz1\Delta\) but not \(N\Delta\) cells are sensitive to camptothecin \((CPT, 20 \mu\text{M})\). Spots are 10-fold serial dilutions on YPD plates \(\pm\) each agent after 2 days at 30 °C. \(B\), unacetylatable \(htz1\) strains \((N\Delta, 4KR, \text{or } 4KQ)\) showed a dose-dependent sensitivity to benomyl, although not to the same degree as \(htz1\Delta\) (Fig. 5B). We (and others) had previously reported an \(htz1-K14R\) strain as sensitive to benomyl (16, 32). However, we have been unable to consistently repeat this phenotype in current analyses using any single unacetylatable \((\text{e.g. } K14R)\) or acetylatable \((\text{e.g. } K14Ac^*)\) \(htz1\) allele (not shown). This may be related to strain differences or some other as yet uncharacterized factor.

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Because \(htz1Ac^*\) contributes to benomyl resistance, we next asked whether the acetylations are themselves regulated by the agent. In parallel analyses, cells were treated with thiabendazole (TBZ, another microtubule depolymerizer) or camptothecin (a topoisomerase I inhibitor). These were chosen for comparison because \(htz1\Delta\) but not \(N\Delta\) cells are sensitive to camptothecin \((CPT, 20 \mu\text{M})\). Spots are 10-fold serial dilutions on YPD plates \(\pm\) each agent after 2 days at 30 °C. \(B\), unacetylatable \(htz1\) strains \((N\Delta, 4KR, \text{or } 4KQ)\) showed a dose-dependent sensitivity to benomyl, although not to the same degree as \(htz1\Delta\) (Fig. 5B). We (and others) had previously reported an \(htz1-K14R\) strain as sensitive to benomyl (16, 32). However, we have been unable to consistently repeat this phenotype in current analyses using any single unacetylatable \((\text{e.g. } K14R)\) or acetylatable \((\text{e.g. } K14Ac^*)\) \(htz1\) allele (not shown). This may be related to strain differences or some other as yet uncharacterized factor.
is multiply acetylated and multi-functional. It has thus been posited that specific acetylated forms could regulate distinct roles (14–16). We find that this is not the case: rather the modifications are redundant, with any single lysine on the Htz1 tail sufficient to mediate function. However, we also show that the acetylation of Htz1 is highly regulated and required for full functionality of this histone variant.

N-terminal Acetylation Is a Widespread Modification of H2A.Z Orthologs—The H2A.Z N terminus is acetylated in S. cerevisiae (14–16), S. pombe (11), Tetrahymena thermophila (43, 44), and metazoans (13, 45, 46). In each organism multiple acetyl-lysines are possible: up to four in budding and fission yeasts, five in metazoans, and six in Tetrahymena. However, these acetylations are unequally distributed across all potential lysine substrates in each species. For example, in Sc K3Ac, K8Ac, K10Ac, and K14Ac are ~3, 7, 14, and 38% of total Htz1, respectively (15). In contrast the histone is ~90% unmodified in chicken erythrocytes, with the remainder primarily mono-acetylated on the more N-terminal lysines (K4Ac ~ K7Ac > K11Ac > K13Ac >> K15Ac) (2, 13). Thus the yeast and chicken H2A.Z N-terminal tails differ in both the abundance and precise location of their acetylations. This, coupled with the fact that the N terminus is one of the most variable regions across H2A.Z orthologs, would tend to suggest differences in how these modifications are regulated. Despite this, the respective Kat5 family acetyltransferase appears to be the primary H2A.Z modifier in all tested species, i.e. S. pombe Mst1 (11), Drosophila Tip60 (47), or Caenorhabditis elegans MYS-1 (48). This is certainly the case in Sc, with Htz1-K8Ac, -K10Ac, and -K14Ac each dependent on Esa1 (the budding yeast Kat5 homolog) and multiple members of the Esa1-containing NuA4 complex (Fig. 1, D and E) (14, 16). Esa1 thus shows great promiscuity in its ability to acetylate each lysine on the Htz1 N terminus (MSGKAHGGKGKSGAKD). However, the acetyltransferase is not infinitely capable, with an N-terminal HA3-tagged form of Htz1 being very poorly acetylated (not shown). N-terminal GFP tagging of Drosophila H2A.Z also disrupts its acetylation and compromises the ability of the variant to regulate the heat shock response (45). Alternatively, because the Htz1 N terminus (residues 1–53) regulates nuclear localization (49), N-terminal tagging of the histone may simply interfere with nuclear import and thus availability for nucleosome assembly and subsequent acetylation.

Coregulation and Genetics Indicate Minimal Specialization of Each Htz1Ac—Disparate analyses demonstrate that the individual N-terminal acetylations on Htz1 are coregulated. Thus K8Ac, K10Ac, and K14Ac are added by NuA4 acetyltransferase after Htz1 is inserted into nucleosomes by the SWR-C (Fig. 1, C–E) and subsequently removed by the Hda1 deacetylase complex (Fig. 1 F and supplemental Fig. S7). All three acetylations decay with similar kinetics after Htz1 transcription is repressed (Fig. 3). All three are similarly affected in every mutant background tested (Fig. 4 and supplemental Table S3). Finally, all three acetylations are similarly affected by a diverse range of agents, including 6-azauracil, benomyl, camptothecin, mycophenolic acid, and TBZ (Fig. 5C and supplemental Fig. S8).
Differential Analysis of S. cerevisiae H2A.Z Acetylation

Their coregulation strongly suggests that individual Htz1 N-terminal acetylations are redundant, a proposal supported by comparing the genetic profiles of a comprehensive range of htz1 alleles (Fig. 2). If the cell utilizes each individual acetylation for a specific role, we would expect to see distinct defects (and thus unique genetic interactions) associated with the mutation of these residues. However, completely unacetylable mutants (NA, 4KR, and 4KQ) are highly correlated with htz1Δ, whereas singly mutated (e.g. K3R) or singly acetylable (e.g. K3Ac*) alleles cluster closer to HTZ1 (Fig. 2A). This suggests that a significant degree of the function of Htz1 resides in its N terminus or more specifically in the acetylable lysines of this region. Furthermore it is strong evidence against an individual function for each N-terminal acetylation.

How Does Acetylation Regulate Htz1 Function?—Previous analyses have identified some role for Htz1 acetylation in the maintenance of euchromatin-heterochromatin boundaries (14), transcription (15), and chromosome transmission (16). Gene ontology categories covering these functions are among the most highly represented when the genetic interactions of completely unacetylable htz1 alleles are examined (supplemental Fig. S4). In addition, and consistent with a role in chromosome stability, unacetylable htz1 alleles are sensitive to the microtubule destabilizing agents benomyl and TBZ (Fig. 5, A and B, and supplemental Fig. S6, D and E). One possibility is that Htz1Ac somehow regulates the response to spindle stress; this is supported by the observation that each acetylation is regulated by the HDA complex on exposure to benomyl (Fig. 5C). This could in turn suggest how this deacetylase complex regulates normal chromosome segregation (50).

Approximately 30% of the genetic interactions of htz1Δ are conserved with NA or 4KR (Fig. 2 and supplemental Fig. S4). However, the list of interactors with unacetylable htz1 alleles are not simply a subset of those with the complete deletion. The htz1Δ-specific group may be due to a loss of the Htz1 core domain or C terminus. Alternatively it may be caused by the inappropriate activity of SWR-C in the absence of Htz1 (51, 52). However, the genetic interactors unique to unacetylable htz1 alleles are more difficult to explain.

H2A.Z acetylation does not regulate the incorporation of the histone into chromatin in either Sc (supplemental Fig. S3A) or S. pombe (11). In addition the acetylation status of Htz1 does not impact nucleosome turnover (Fig. 3C). It is possible that nucleosomes containing unacetylable htz1 are unable to use an H2AAc-dependent compensation pathway. This might indicate that cells differentiate certain histone tails as one of two states, acetylated or not, and make little distinction between the particular modified residue. This would appear to be a retrograde step in our understanding of chromatin function. However, we note that NuA4 acetylates Htz1 in a nucleosome-dependent manner (Fig. 1, D and E), H2A, and H4 (53), and mutant combinations that reduce the total number of NuA4-dependent acetylations (e.g. [htz1–4KR, eaf1Δ] or [htz1–4KR, hfh2-K5/812KR]) are invariably synthetic (14). In addition the Esa1-targeted H4 N terminus also shows significant redundancy, such that the loss of all four acetylable lysines leads to slow growth and camptothecin sensitivity, but the retention of any single lysine complements both phenotypes (54). Furthermore an ectopic lysine in an otherwise unacetylable H4 N terminus is acetylated (likely by Esa1) and also repairs the slow growth and camptothecin sensitivity (54).

As noted above, the simultaneous loss of all N-terminal acetylation on Htz1 has widespread effects. Thus the modifications regulate the functions of this histone variant. However, is this by charge modulation or effector recruitment? Arginine and glutamine are respectively considered charge mimics for unacetylated and acetylated lysine (13), although there is no evidence that they mimic the unmodified/modified state as it relates to recognition by acetyl-binding proteins. We note that the htz1–4KR and 4KQ mutants are indistinguishable throughout this work, an observation also made with comparable mutants in fission yeast (11). This strongly argues against the acetylation of Htz1 acting via charge modulation. However, no chromatin effector has yet been identified that is so selectively promiscuous in its binding (i.e. only to KAc’s on a H2A.Z tail). One possibility is that the Htz1 tail functions by dynamic charge modulation; i.e. the acetylations must be added and then removed (constitutively one or the other will not work). If this is indeed the case, our current belief that the simple presence of many histone marks is enough may need some revision.

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