Site-specific Loss of Acetylation Upon Phosphorylation of Histone H3

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SUMMARY

Post-translational modification of histones is a central aspect of gene regulation. Emerging data indicate that modification at one site can influence modification of a second site. As one example, histone H3 phosphorylation at serine 10 (S10) facilitates acetylation of lysine 14 (K14) by Gcn5 in vitro(1,2). In vivo, phosphorylation of H3 precedes acetylation at certain promoters. Whether H3 phosphorylation globally affects acetylation, or whether it affects all acetylation sites in H3 equally, is not known. We have taken a genetic approach to this question by mutating S10 in H3 to fix either a negative or a neutral charge at this position, followed by analysis of the acetylation states of the mutant histones using site-specific antibodies. Surprisingly, we find that conversion of S10 to glutamate (S10E) or aspartate (S10D) causes almost complete loss of H3 acetylation at lysine 9 (K9) in vivo. Acetylation of K9 is also significantly reduced in cells bearing mutations in the Glc7 phosphatase that increase H3 phosphorylation levels. Mutation of S10 in H3 and the concomitant loss of K9 acetylation has minimal effects on expression of a Gcn5-dependent reporter gene. However, synergistic growth defects are observed upon loss of GCN5 in cells bearing H3 S10 mutations that are reminiscent of delays in G2/M progression caused by combined loss of GCN5 and acetylation site mutations. Together these results demonstrate that H3 phosphorylation directly causes site-specific and opposite changes in acetylation levels of two residues within this histone, K9 and K14, and they highlight the importance of these histone modifications to normal cell functions.
INTRODUCTION

The packaging of DNA into chromatin impacts every process that uses DNA as substrate. In recent years, the importance of chromatin remodeling to transcriptional regulation has become especially clear due to the discovery and functional characterization of histone modifying enzymes and ATP-dependent chromatin remodeling complexes (3-5). Chromatin is built from nucleosomal subunits, which consist of 147 base pairs of DNA spooled around the exterior of an octamer of the four core histone proteins. Post-translational modifications of the histones may alter histone:DNA, histone:histone, and histone:non-histone protein interactions (6,7). These changes can affect the placement and stability of individual nucleosomes as well as the formation of higher order chromatin structures, thereby affecting accessibility of particular DNA elements to trans-acting factors.

Histones are subject to a variety of post-translational modifications, including acetylation, phosphorylation, ADP-ribosylation, methylation, and ubiquitination (7-9). Modulations in histone acetylation levels in particular accompany transcriptional changes. In the last few years, multiple coactivator complexes that house histone acetyltransferase (HAT) activity and corepressor complexes that recruit histone deacetylase (HDAC) activity have been described (10-12). These HAT and HDAC complexes directly participate in chromatin remodeling at promoter regions. Emerging studies indicate that histone methylases are also involved in both the activation and repression of gene expression (13-17). In addition, specific kinases phosphorylate histone H3 in response to mitogenic signals or for mitotic events (18-20).

The sites for these post-translational modifications are clustered within the first 30 amino acids of the core histones. Histone H3, for example, is acetylated at lysines (K) 9, 14, 18, and 23, phosphorylated at serines (S) 10 and 28, and methylated at K4 and K9 (21). The juxtaposition of these sites provides potential for cross regulation of different modification events. Indeed, deacetylation of K14 in H3 appears to augment methylation of K9 at centromeric heterochromatin in S. pombe (17). Phosphorylation of S10
in H3 enhances acetylation of K14 by Gcn5 in vitro and precedes K14 acetylation at specific promoters in vivo (1,2). S10 phosphorylation also inhibits methylation of K9 in vitro, and K9 methylation limits S10 phosphorylation both in vitro and in vivo (15,22).

This potential for cross regulation of different histone modifications, together with findings that particular non-histone proteins selectively bind to histones in specific modification states, has led to the idea of a ‘histone code’ (23). In this model, not only the levels of modifications, but also the individual types and sites of modification directly facilitate or antagonize association of regulatory proteins with chromatin. These proteins may themselves contribute to further chromatin organization, or they may recruit additional activator or repressor activities.

The functions of H3 S10 phosphorylation are particularly intriguing because this modification is associated with two apparently disparate processes, which perhaps illustrate the potential of the histone code. H3 S10 phosphorylation peaks during mitotic chromosome condensation in all eukaryotes examined, and S10 mutations cause defects both in chromosome condensation and segregation in *Tetrahymena* (18). However, H3 S10 phosphorylation is also linked to chromatin opening and activation of genes in response to certain signals, such as the mitogenic stimulation of *c-fos* (1,20). In both cases, H3 S10 phosphorylation might lead to a transient opening of the chromatin, allowing binding of either condensation factors during mitosis or activation factors during G1. Alternatively, alteration of other modifications upon S10 phosphorylation might create a ‘code’ on the H3 tail that is ‘read’ by binding of different factors at different stages of the cell cycle to aid either chromosome condensation or gene activation. The functions of S10 phosphorylation during mitosis might be redundant with other histone modification states in some organisms, since S10 mutations do not affect chromosome segregation or mitotic progression in yeast (18).

To further probe the function of S10 phosphorylation in yeast and to test the hypothesis that this modification is functionally linked to other H3 modification events, we have examined the effects of S10 mutations on global levels of H3 acetylation. Surprisingly, we find that conversion of S10 to aspartate (D) or glutamate (E) specifically and drastically lowers acetylation of K9. Decreases in K9
acetylation levels are also observed in the presence of \textit{gbc7} phosphatase mutations (19) that increase H3 phosphorylation levels in vivo. All S10 mutations tested confer synergistic growth defects with loss of the Gcn5 histone acetyltransferase, resulting in delayed progression through G2/M. Taken together with previous studies by others that indicate S10 phosphorylation enhances acetylation at K14 (1,2), our data indicate that H3 phosphorylation has opposite effects on two neighboring acetylation sites. Moreover, our results suggest that a proper balance of H3 acetylation and phosphorylation is required for normal cell cycle progression.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**

Strains used for this study are listed in Table III. The \textit{gbc7} strains have been described previously (24, 25). All histone mutations were introduced into the MX1-4C strain (a gift from C. David Allis and M. Mitchell Smith, University of Virginia, Charlottesville, VA) by plasmid shuffle. In brief, MX1-4C contains deletions of both loci that encode histone H3 and H4 (\textit{HHT1-HHF1} and \textit{HHT2-HHF2}). The strain also carries a plasmid with \textit{URA3} and \textit{HHT1-HHF1}. Histone mutations were introduced on a \textit{TRP1} and \textit{HHT2-HHF2} plasmid, pWZ414-F13 (26). The \textit{TRP1} plasmids containing histone mutations were transformed into MX1-4C, and \textit{TRP1} \textsuperscript{+} colonies were selected. These colonies were transferred to 5-FOA media (to select against \textit{URA3}). Plasmids from the 5-FOA resistant colonies were rescued and sequenced to confirm loss of wild type histone alleles and presence of the mutant histone alleles.

**Mutagenesis**

Mutations were introduced into \textit{HHT2-HHF2} by two approaches. Histone H3 S10A (pRS414-59), S10D (pJD112), and S10E (pJD113) were made using the previously described single strand DNA mutagenesis technique (27). The combined mutations H3 S10D K9Q (pBM1), S10D K9R (pBM2), S10D K14Q (pBM3), and S10D K14R (pBM4) were made with the Stratagene QuikChange site-directed mutagenesis kit, using the S10D mutant plasmid pJD112 as the template. The entire coding region of both histone H3
and H4 were sequenced in all constructs to confirm the presence of the desired mutation and the absence of any additional mutations.

**Deletion of GCN5**

A PCR based strategy was used to disrupt *GCN5*. The entire coding region was replaced with the kan^r^ gene as previously described (28). Both PCR and Southern blot analysis were used to confirm the presence of the *gcn5::Kan^r^* allele.

**Immunoblotting**

Equivalent numbers of cells (~5-25 x 10^7^) in exponential growth were harvested from each yeast strain by centrifugation for 5 minutes at 1000 g. Yeast pellets were frozen in a dry ice-methanol bath and stored at −80°C. Frozen pellets were resuspended in 1.5X SDS-PAGE loading buffer, mixed with an equivalent volume of acid-washed glass beads, and vortexed at high speed in microfuge tubes for 10 minutes at 4°C. Extracts were spun through a small hole pierced in the bottom of the microfuge tubes and clarified by centrifugation at high speed in a microcentrifuge for 5 minutes (4°C). Similar results were obtained from extracts prepared from isolated nuclei and from fresh (never frozen) pellets. For TSA experiments, cultures were treated with 25µM TSA for 3 hours prior to harvest. For GLC7 experiments, extracts were prepared as described by Hsu, et al (19).

Samples were loaded onto a 22% SDS-PAGE gel (60:0.4 acrylamide:bis-acrylamide) or for GLC7 experiments a 15% SDS-PAGE gel, and blotted to PVDF as described previously (29, 30). Blots were blocked in 2-5% dry milk dissolved in 1X TBST (0.15 M NaCl, 0.1 M Tris-HCl, pH7.5, 0.1%(w/v) Tween-20) for 2 hours at room temperature or overnight at 4°C with agitation. Antisera were diluted in block and blots were incubated with primary antibody overnight at 4°C. Dilution of antisera used were: αH3 Ac 9,14 (Upstate) 1:2000, αH3 Ac 9,18 (31)1:1000, αH4, penta (Upstate) 1:4000, αH3 Ac 9 (Upstate) 1:1000, αH3 Phospho Serine 10 (Cell Signaling) 1:100. Blots were washed four times for ten minutes each in TBST, followed by incubation with secondary antibody (goat anti-rabbit conjugated to HRP; Amersham) diluted 1:10,000 in block. After an incubation of 1 hour to overnight, blots were
washed six times for five minutes each in TBST and developed with SuperSignal (Pierce) as per the manufacturer’s protocol.

**β-Galactosidase Assays**

Indicated yeast strains were transformed with plasmid pPC97-VP16413-470 (a gift from S. Berger, Wistar Inst., Philadelphia, PA) and plasmid pLGSD5 (32), which carries the β-galactosidase gene under the control of a single Gal4 binding site. Multiple colonies of each strain were assayed at least twice. β-galactosidase assays were performed as described (33) except that cells were frozen as a pellet at –80°C prior to extract preparation.

**FACS**

FACS was performed essentially as described in Zhang, et al (34). Briefly, yeast in log phase growth were fixed in 70% ethanol overnight at 4°C. After rehydration in PBS, cells were treated with RNase A (1 mg/ml in 200 mM Tris pH 8.0, 20 mM EDTA) at 37°C overnight. PBS-washed cells (1.5 x 10^6) were stained with 50 mM propidium iodide in a final volume of 0.1 ml of PBS for 2 h at room temperature in the dark. The cells were diluted with 400 µl of PBS, briefly sonicated and then counted on a Coulter EPICS XL-100 MCL flow cytometer.

**RESULTS**

**Mutation of S10 lowers H3 acetylation in vivo.**

Phosphorylation of histone H3 peptides at S10 augments acetylation at K14 by Gcn5 in vitro, and conversion of S10 to alanine (S10A) decreases activation of some Gcn5-regulated promoters in vivo (1,2). These results suggest that prevention of phosphorylation by mutation of S10 to alanine weakens Gcn5-mediated acetylation of H3, which normally facilitates gene activation. To directly test the effects of the S10A mutation on H3 acetylation levels, we probed whole cell protein extracts from cells bearing wild type or mutated H3 on immunoblots using antibodies specific for particular acetylated-H3 isoforms.
In cells bearing H3 mutations, both wild type copies of the H3 gene were deleted so that the plasmid-born, mutated copy provided the sole source for H3 in the cell (34,35).

Two antibodies were predominantly used in our studies. One commercially available antibody that is often used to evaluate H3 acetylation levels was raised against an H3 peptide containing acetyl-lysine at positions 9 and 14 (anti-H3 Ac 9,14). A second antisera was raised against an H3-peptide with acetyl-lysines at positions 9 and 18 (anti-H3 Ac 9,18) (31). We observed a decrease (~60%) in the amount of H3 acetylation detected by the H3 Ac 9,14 antisera in extracts from cells bearing the H3 S10A mutation (Fig. 1 and Table I), as expected from the above reports. Less decrease was observed using the anti-H3 Ac 9,18 antisera (Fig. 1).

If the introduction of a negative charge at S10 by phosphorylation is important for augmentation of Gcn5 (or other HAT) functions, then placement of a constitutive negative charge at this position should increase acetylation. However, in contrast to our expectations, S10D and S10E H3 mutations reduced acetylation of H3 detected by the anti-H3Ac 9,14 antibody by 90% or more (Fig. 1 and Table I). Acetylation detected by anti-H3 Ac9,18 was also reduced, but to a lesser extent (~40%). The persistence in staining with this antibody demonstrates that intact H3 is present in extracts from the mutant cells. Acetylation of H4 was not altered in extracts from the mutant strains, indicating that the effects on acetylation are specific for the mutated H3 histone (Fig. 1).

S10D and S10E mutations specifically affect acetylation of K9 in H3.

The apparent loss of acetylation detected by the anti-H3 Ac 9,14 antisera might reflect a decrease in HAT recognition of the mutant H3, increased HDAC activity towards this mutant, or a destruction of the epitope recognized by this antibody. To determine whether the S10D or S10E H3 mutations preclude acetylation in vivo, cells bearing these mutations were grown in the presence of trichostatin A (TSA), a specific inhibitor of histone deacetylase activities. Under these conditions, both the S10D and S10E mutant forms of H3 exhibited increased acetylation detected by the anti H3 Ac 9,14 antibody (Fig. 2, upper panel), confirming the ability of these histones to be acetylated in vivo. Importantly, these results
also confirm the ability of the H3 Ac 9,14-specific antisera to recognize these mutant forms of H3, demonstrating that the epitope for the antibody is intact.

To further define the effects of the S10D and S10E mutations on particular acetylation sites, we reinvestigated the specificity of the H3 Ac 9,14 antibody. In previous work, we established that K14 mutations had negligible effects on detection of H3 by this antibody (34). In contrast, mutation of K9 virtually abolished H3 recognition by the anti-H3 Ac 9,14 antisera. These results suggested either that the antibody has a much higher specificity for acetyl-K9 than for acetyl-K14, or that bulk yeast histones are acetylated at K9 much more frequently than at K14. To distinguish these possibilities, we analyzed the ability of the H3 Ac K9,14 antibody to recognize peptides synthesized with acetyl-lysine at position 9, position 14, or other sites using a slot blot assay. Equal amounts of peptides were applied to the membrane, as confirmed by staining with India Ink (Fig. 3). The anti-H3 Ac 9,14 antibody did not recognize the unmodified H3 peptide, but did recognize all H3 peptides containing acetyl-K9. In contrast, a peptide acetylated at K14, K18, and K23, but lacking K9 acetylation, was not recognized. Therefore, the H3 Ac K9,14 antibody appears largely specific for H3 acetylated at K9, although other studies have shown that this antibody can be blocked effectively with a H3-Ac K14 peptide (1). Similar slot blot analysis of the anti-H3 Ac 9,18 antibody confirms its specificity for acetylation events at either K9 or K18 in H3 (Fig. 3).

Together with our above experiments, these results indicate that the H3 S10D and S10E mutations likely inhibit acetylation of K9. We confirmed this effect by probing immunoblots of whole cell extracts with antibodies raised against a peptide acetylated individually at K9. This monospecific H3 Ac-K9 antibody reacted well with wild type yeast H3, but staining was drastically reduced for the H3 S10D or S10E mutants (Fig. 2, bottom panel). Acetylation of K9 as detected by this antibody was regained upon growth of cells in TSA, again confirming the ability of this residue to be acetylated within the context of the S10 mutations and the integrity of the epitope in the mutant histones. The S10D and S10E mutations, therefore, severely and specifically inhibit acetylation of histone H3 at K9.
Glc7 phosphatase mutations that increase H3 phosphorylation also inhibit K9 acetylation.

If conversion of S10 to D or E mimics phosphorylation, then phosphatase mutations that increase S10 phosphorylation in vivo should also decrease acetylation of K9. The Glc7 phosphatase is an essential gene in yeast. Particular mutant alleles of the GLC7 (glc7-127 and glc7-129) increase H3 S10 phosphorylation levels, whereas other alleles of GLC7 (glc7-109) do not (19)(Fig. 4b). Immunoblots of equal loads of extracts from isogenic wild type or glc7 cells reveal that H3 acetylation detected by the anti-H3 Ac 9,14 antibody is decreased in the presence of those alleles that result in increased phosphorylation at S10 (*’s in Fig 4a). Thus, high levels of H3 phosphorylation cause a decrease in K9 acetylation just as did conversion of S10 to D or E. A negative charge at this position in H3 apparently antagonizes acetylation at K9, even though it stimulates acetylation at K14.

H3 S10 mutations do not affect Gcn5-dependent activation by Gal4-VP16

Activation of Gcn5-dependent genes is accompanied by increased acetylation of histones associated with promoter regions (36). Since Gcn5 exhibits an enhanced recognition of phosphorylated H3 peptides in vitro (1,2), phosphorylation of H3 should increase activation in vivo. Accordingly, mutation of H3 S10 to alanine, which prevents phosphorylation, has been reported to limit activation of some Gcn5-dependent genes in yeast (2). We reasoned that the H3 S10D and S10E mutations might enhance expression of Gcn5-dependent genes by facilitating acetylation of K14 by Gcn5. However, since these mutations also abolish acetylation of H3 K9, they might limit activation. Therefore, we examined expression of a Gcn5-dependent reporter gene in isogenic strains containing wild type H3 or our H3 S10 mutations to determine the relative contributions of S10 phosphorylation, K9 acetylation, or K14 acetylation to the activation of this gene.

Activation of reporter genes containing Gal4 binding sites by the Gal4-VP16 fusion protein requires Gcn5. Previously, we demonstrated that mutation of multiple lysines in H3 and H4 to glutamine can bypass the Gcn5 requirement, supporting an important role for histone acetylation in Gal4-VP16 mediated activation (34). Therefore, we chose this same system to test the effects of the S10 mutations on Gal-VP16 mediated activation. As expected, the reporter was activated in cells containing wild type
histones, and this activation was diminished in the absence of Gcn5 (Fig. 5). Surprisingly, none of the S10 mutations affected activation of the reporter in cells containing Gcn5. Reporter activation was decreased in *gcn5* cells carrying the H3 S10 mutations. However, the reporter reproducibly exhibited higher expression levels in these cells relative to *gcn5* cells containing wild type H3. In our previous work, this reporter gene also exhibited slightly higher activity in *gcn5* cells in the presence of H3 K9 mutations (to arginine or to glutamine), although single mutations in H3 at K9 or K14 had little effect on the overall Gcn5-dependence of Gal-Vp16 mediated activation (34). The similar effects we observe in cells carrying the H3 S10A, S10D, S10E, K9R or K9Q mutations indicate that S10 and K9 do contribute to gene activation, but that the charge of these residues is less important than their overall structure.

S10 mutations and loss of Gcn5 synergistically cripple cell cycle progression.

Previous studies from our lab revealed synergistic growth defects upon loss of the Gcn5 acetylase and mutation of predominant acetylation sites in H3 and H4 (34). Mutation of K9, for example, had little effect on yeast doubling time, but when this mutation (or a mutation of K14) was combined with a disruption of *GCN5*, doubling times were significantly increased. Since S10 mutations globally affect K9 acetylation, we reasoned that these mutations might also exhibit synergistic defects when combined with disruptions of *GCN5*.

We first determined if our H3 S10 mutations altered cell growth on their own. Comparison of doubling times indicated no significant difference between strains bearing the S10A, S10D, or S10E mutations or cells bearing wild type H3 (Table II), consistent with previous reports that S10A mutations do not affect the yeast cell cycle (18). Loss of *GCN5* alone lengthened doubling times, as we reported previously (34). However, combination of any of the S10 mutations with a *GCN5* disruption further lengthened cell doubling times (> 250 min). FACS analyses indicate that a majority of the double mutant cells have a 2N content of DNA, consistent with a delay in progression through G2/M (Fig 6).

To determine if the lengthened cell doubling times observed in the *gcn5* strains bearing the S10 mutations are due to specifically to a loss of acetylation of H3 at K9 or K14, we analyzed the growth of *GCN5* strains bearing the S10D mutation together with mutation at either of these lysines (Table III). We
did not expect mutation of K9 to mimic the effect of GCN5 loss, since acetylation of K9 is already abolished in the S10D mutant strain. Indeed, mutation of K9 had little effect upon the growth of the S10D mutant. However, since H3 K14 is a preferred substrate of Gcn5, mutation of this site might lead to an increased doubling time in the presence of the S10D mutation. The S10D K14Q and the S10D K14R double H3 mutants both exhibited slightly slower growth than the S10D mutant (Table III), but these cell doubling times were still significantly shorter than the H3 S10D gcn5 mutant (Table II). These results indicate that loss of acetylation at additional sites and substrates likely contributes to the delayed cell cycle progression observed in the S10D gcn5 strains. Interestingly the similar effects of the K14R and K14Q mutations in the presence of the S10D mutation indicate that the structure of the lysine residue, or acetyllysine, is more important than the charge at this position in H3.

Together, these results demonstrate that H3 S10 is important to normal cell cycle progression in yeast and that the functions of this residue, and its modification, are redundant with multiple acetylation events mediated by Gcn5. Overall, our data suggest that a critical balance in histone acetylation and H3 phosphorylation is required for normal cell division.

DISCUSSION

The histone code hypothesis predicts that specific combinations of histone modifications provide regulatory information through changes in the structure of chromatin and in the association of non-histone proteins with particular nucleosomes (7,23). Inherent in this hypothesis is the idea that modification of one residue in a histone may affect the type and frequency of modifications at other sites. One of the first examples of such cross-regulation was the discovery that phosphorylation of S10 in histone H3 augments recognition of the H3 amino-terminal tail by Gcn5, leading to increased acetylation of K14 (1,2). Here we show that phosphorylation or mutation of S10 has an opposite effect on acetylation of K9. Our results indicate that acetylation of each lysine within a histone tail is independently regulated, supporting the idea
that each lysine has unique functions. These studies provide further in vivo support for the histone code hypothesis.

A particular arginine in Gcn5 (R164 in yeast Gcn5) provides a pocket for binding phosphorylated S10 in H3 (1,2), based on the crystal structure of a ternary Gcn5-H3 peptide-coenzyme A complex (37). These interactions appear to facilitate acetylation of K14 by stabilizing Gcn5-H3 interactions and by aligning K14 in the HAT catalytic center. In support of this model, mutation of R164 in Gcn5 impedes activation of the same subset of Gcn5-dependent genes that are affected by S10A mutations in H3. The inhibition of K9 acetylation that we observe in vivo upon S10 mutation or phosphorylation could reflect a misalignment of this residue within the HAT active site. Unfortunately, K9 (the first residue of the H3 peptide used for crystallization) is highly disordered within the co-crystal, so its location relative to specific active site residues in Gcn5 cannot be discerned from the structures in hand (37).

Although our data indicate that S10 phosphorylation antagonizes K9 acetylation in yeast, they do not rule out the possibility that these modifications might coexist at certain promoters, under specific conditions. Indeed, S10 phosphorylation and K9 acetylation do coexist on H3 molecules associated with the mitogen-stimulated c-fos promoter (38). However, these dually modified H3 molecules occur very rarely in mammalian cells, perhaps reflecting the antagonism we observe here.

We previously reported that mutation of GCN5 causes a delay in G2/M progression that is exacerbated upon mutation of specific lysines in H3 or H4 (34). These studies indicated that a certain threshold of acetylation events is required for normal cell cycle progression. Consistent with this idea, concomitant loss of the genes encoding the Gcn5 and Sas3 H3 HAT activities is lethal in yeast (39). The Esa1 H4 HAT is also essential for yeast cell viability (40,41). The cell cycle delays we observe here upon mutation of H3 S10 and loss of GCN5 are highly reminiscent of those observed in our previous studies (21,34) and those observed by Howe et al upon loss of GCN5 and SAS3 (39). One simple explanation is that the lowered acetylation of K9 that occurs upon S10 mutation becomes critical upon loss of other acetylation events mediated by Gcn5. However, the synergistic phenotype may reflect a more complicated relationship between H3 S10 and Gcn5 functions since all three S10 mutations tested, S10A,
S10D, and S10E, exhibit the same degree of synergy with GCN5 loss, but the S10A mutation causes only a slight (2 fold) change in K9 acetylation levels. The structure of serine or of phosphoserine, not just the charge, at position 10 is apparently important for H3 functions. The G2/M delay that we observe could reflect abnormal chromatin folding in the presence of the H3 S10 and GCN5 mutations due to loss of both phosphorylation and acetylation. Alternatively, these changes in H3 modification might result in abnormal expression of a gene(s) required for proper progression through this phase of the cell cycle. Interestingly, mutations in ESA1 or mutations in multiple H4 acetylation sites trigger a RAD9 dependent G2/M checkpoint response, likely reflecting abnormal chromatin structures and decreased genome integrity in the face of these mutations (35,41,42).

At least three different kinases phosphorylate H3 S10 in response to specific signals. Ipl1 appears to phosphorylate H3 specifically during mitosis, whereas pp90Rsk-2 and Snf1 phosphorylate H3 in response to mitogenic and nutritional responses, respectively (19,43,44). The involvement of these kinases highlights the potential for H3 as an integrator of multiple signal transduction pathways. Given our data and other recent findings, phosphorylation of H3 S10 may be a nexus for regulation of H3 modifications. In addition to affecting acetylation of both K14 (1,2) and K9 (in opposite directions), S10 phosphorylation inhibits methylation of K9 in S. pombe and Drosophila (15,17,22). H3 K9 methylation in these organisms inhibits S10 phosphorylation, providing potential for feedback regulation (45). Moreover, since S10 augments K14 acetylation, which inhibits K9 methylation, it is easy to envision a cascade of modification events on the H3 tail that might be modulated in response to specific signals to deliver different messages via the histone code.

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**FIGURE LEGENDS**

Figure 1. Diminished acetylation of histone H3 K9 in S10D and S10E mutants.

Yeast protein extracts were resolved by SDS-PAGE electrophoresis, transferred to PVDF membrane and probed with the indicated antibodies. The lower panel shows a Coomassie stain of a parallel gel to illustrate equivalent protein load.

Figure 2. Histone H3 can be acetylated at K9 in S10D and S10E mutants.

Protein extracts were prepared from yeast either treated or not treated with TSA. Histone acetylation was analyzed by immunoblot with the indicated antibodies. Equivalent protein loads were confirmed by Coomassie staining.
Figure 3. Specificity of H3 Ac 9,14 and H3 Ac 9,18 antibodies.
Slot blots of peptides corresponding to the amino terminus of H3 were probed with the indicated antibody or stained with India ink. The acetylation state of the lysine residues in the indicated position is designated by - (no acetylation) or + (acetylated).

Figure 4. H3 acetylation is reduced in GLC7 mutants.
Protein extracts were prepared from yeast of the indicated strain. The asterisk (*) indicates GLC7 mutants reported to display increased H3 phosphorylation. Histone acetylation (A) and phosphorylation (B) were analyzed by immunoblot with the indicated antibodies. Signal from the acetylation specific antibody was quantitated using the Apha Innotech Imaging System and normalized to protein load (relative signal). Equal protein loads for the αH3 Phospho Serine 10 immunoblot were determined by Bio-rad protein assay of the yeast whole cell extracts (19).

Figure 5. Transcriptional activation by Gal4-VP16 in wild type and histone mutant cells in GCN5+ and gcn5- backgrounds.
β-Galactosidase activity resulting from transcriptional activation by Gal4-VP16 of a Gcn5-dependent reporter was measured in extracts from the indicated yeast strains.

Figure 6. FACS analysis of wild type and histone mutant cells in a GCN5+ and gcn5- background.
Yeast cultures of the indicated strain were harvested during log phase growth and stained with propidium iodide and subjected to FACS analysis. The positions of cells with a G1 or G2 content of DNA are marked.
TABLE I. Relative levels of Histone H3 detected by Ac H3 antibodies

| H3 Allele | α H3 Ac 9,14* | α H3 Ac 9,18* |
|-----------|--------------|--------------|
| Wild Type | 1.0          | 1.0          |
| S10A      | 0.4          | 0.8          |
| S10D      | 0.1          | 0.6          |
| S10E      | 0.1          | 0.6          |

*normalized to protein load and average of 2 experiments (difference less than 1% between experiments for αAcH3 9,14 and less than 14% for αAc H3 9,18)
**TABLE II.** Doubling times of H3 S10 mutants with and without Gcn5

| H3 Allele | GCN5 Status | Doubling time (minutes)a | % cells in G1b |
|-----------|-------------|--------------------------|----------------|
| Wild Type | +           | 125                      | 24 ± 9         |
| S10A      | +           | 127                      | 26 ± 3         |
| S10D      | +           | 131                      | 24 ± 2         |
| S10E      | +           | 123                      | 25 ± 3         |
| Wild Type | ∆           | 239                      | 16 ± 5         |
| S10A      | ∆           | 252                      | 13 ± 3         |
| S10D      | ∆           | 307                      | 11 ± 0         |
| S10E      | ∆           | 291                      | 13 ± 2         |

a representative experiment
b average of 3 experiments
**TABLE III.** Doubling times of H3 S10, K9 and K14 mutants

| Histone H3 Allele | Doubling Time (minutes)* |
|-------------------|--------------------------|
| Wild Type         | 151 ± 6                  |
| S10D              | 159 ± 11                 |
| S10D, K9R         | 162 ± 13                 |
| S10D, K9Q         | 165 ± 12                 |
| S10D, K14R        | 177 ± 16                 |
| S10D, K14Q        | 181 ± 18                 |

* average of 3 experiments
| Name     | Genotype                              | Histone H3 mutation |
|----------|---------------------------------------|---------------------|
| MX1-4C   | $MAT\alpha, ura3-52, his3A1, leu2-3,112, trp1-289,$  
          | $\Delta(hht1-hhf1), \Delta(hht2-hhf2),$ pms327(CEN ARS URA3 HHT1 HHF1) | WT                  |
| JDY16    | as MX1-4C, except plus pWZ414-F13 instead of pms327 | WT                  |
| JDY20    | as JDY16, except $\Delta gcN5::Kan^R$       | WT                  |
| JDY17    | as MX1-4C, except plus pRS414-59 instead of pms327 | S10A                |
| JDY21    | as JDY17, except $\Delta gcN5::Kan^R$       | S10A                |
| JDY18    | as MX1-4C, except plus pJD112 instead of pms327 | S10D                |
| JDY22    | as JDY18, except $\Delta gcN5::Kan^R$       | S10D                |
| JDY19    | as MX1-4C, except plus pJD113 instead of pms327 | S10E                |
| JDY23    | as JDY19, except $\Delta gcN5::Kan^R$       | S10E                |
| BMY1     | as MX1-4C, except plus pBM1 instead of pms327 | S10D, K9Q           |
| BMY2     | as MX1-4C, except plus pBM2 instead of pms327 | S10D, K9R           |
| BMY3     | as MX1-4C, except plus pBM3 instead of pms327 | S10D, K14Q          |
| BMY4     | as MX1-4C, except plus pBM4 instead of pms327 | S10D, K14R          |
| KT1112   | $MAT\alpha leu2 ura3-52 his3$              |                     |
| KT1667-a | as KT1112, except $gbc7-129$               |                     |
| KT1638   | as KT1112, except $gbc7-109$               |                     |
| KT1640   | as KT1112, except $gbc7-127$               |                     |
Figure 1

Wild type | S10A | S10D | S10E | Wild type

\(\alpha\text{ AcH3 9,14}\)

\(\alpha\text{ AcH3 9,1 \ 8}\)

\(\alpha\text{ AcH4  penta}\)

Coomassie
Figure 2

| TSA | wt | S10A | S10D | S10E |
|-----|----|------|------|------|
|     | -  | +    | -    | +    |
|     | -  | +    | -    | +    |
|     | -  | +    | -    | +    |

αH3 Ac9,14

αH3 AcK9
Figure 3

αH3Ac9,14

αH3Ac9,18

India Ink

H3 peptides

| 9 | 14 | 18 | 23 |
|---|----|----|----|
| - | - | - | - |
| + | + | + | + |
| - | + | + | + |
| + | . | + | + |
| + | + | . | + |
| + | + | + | . |

2.0 nmol 0.4 nmol

2.0 nmol 0.4 nmol

2.0 nmol 0.4 nmol
Figure 4

A

|       | wild type | glc7-109 | glc7-127* | glc7-129* | wild type |
|-------|-----------|----------|-----------|-----------|-----------|

α H3 Ac9,14

1.0 0.7 0.3 0.3 1.0 relative signal

B

|       | wild type | glc7-109 | glc7-127* | glc7-129* |
|-------|-----------|----------|-----------|-----------|

α H3 Phos10
Figure 5

![Graph showing the expression levels of H3 WT, H3 S10A, H3 S10D, and H3 S10E in GCN5+ and gcn5- strains. The y-axis represents expression levels ranging from 0 to 4000. Each bar represents the mean expression level with error bars indicating the standard deviation. The graph compares the expression levels between the two strains, highlighting the differences in expression patterns.](http://www.jbc.org/)

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Figure 6

H3 allele + GCN5 - GCN5

Wild type

S10A

S10D

S10E
Site-specific loss of acetylation upon phosphorylation of histone H3
Diane G. Edmondson, Judith K. Davie, Jenny Zhou, Banafsheh Mirnikjoo, Kelly Tatchell
and Sharon Y.R. Dent

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