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An N^{α}-Acetyltransferase Responsible for Acetylation of the N-terminal Residues of Histones H4 and H2A*

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A yeast gene has been identified that encodes a novel, evolutionarily conserved N^{α}-acetyltransferase responsible for acetylation of the N-terminal residues of histones H4 and H2A. The gene has been named *NAT4*. Recombinant Nat4 protein acetylated a peptide corresponding to the N-terminal tail of H4, but not an H3 peptide nor the peptide adrenocorticotropin. H4 and H2A are N-terminally acetylated in all species from yeast to mammals and hence blocked from sequencing by Edman degradation. In contrast, H4 and H2A purified from a *nat4* mutant were unacetylated and could be sequenced. Analysis of yeast histones by acid-urea gel electrophoresis showed that all the H4 and H2A from the mutant migrated more rapidly than the same histones from a wild type strain, consistent with the histones from the mutant having one extra positive charge due to one less acetylated amino group. A comparison of yeast proteins from wild type and a *nat4* mutant by two-dimensional gel electrophoresis showed no evidence that other yeast proteins are substrates of this acetyltransferase. Thus, Nat4 may be dedicated specifically to the N-terminal acetylation of histones H4 and H2A. Surprisingly, *nat4* mutants grow at a normal rate and have no readily observable phenotypes.

Eukaryotic proteins are subject to two cotranslational modifications as the nascent polypeptides emerge from the ribosome, methionine cleavage by methionine aminopeptidase, and acetylation of the α-amino group of the N-terminal amino acid (1). Removal of the methionine occurs only if the second amino acid is a small one such as serine, alanine, or glycine. Acetylation of the α-amino group on either methionine, or on the N-terminal residue resulting from methionine cleavage, occurs in the great majority of (but not all) eukaryotic proteins, and is accomplished by one of several N^{α}-acetyltransferases (NATs)[1] (1). A number of years ago we identified the genes for a yeast NAT, now called NatA, that consists of two subunits, Ard1 and Nat1 (2). Subsequent work showed that NatA was responsible for the acetylation of many, but not all, yeast proteins beginning with small residues such as serine, alanine, or glycine (2–5). Yeast *ard1* and *nat1* mutants are viable and the many proteins that are no longer N-terminally acetylated in the mutant strains are as stable as they are in a wild type strain (2). Thus, there is no evidence that N-terminal acetylation serves to protect proteins from degradation in yeast.

Ard1 protein has an acetyl-CoA binding motif found in members of the GNAT superfamily, enzymes that acetylate amino groups on proteins and other molecules (6). Thus, Ard1 is very likely to be the catalytic subunit of NatA. Two other NATs have been identified in yeast, called NatB and NatC, with catalytic subunits Nat3 and Mak3, respectively (4, 7). NatB and NatC both acetylate proteins with N-terminal methionine residues, although they have different specificities dictated by the nature of the subsequent amino acids (4).

Histone H4 is a highly conserved protein with an N-terminal serine residue that is acetylated in both mammals and yeast (8, 9). In our initial paper on NatA, we noted that H4 from *ard1* or *nat1* mutants had the same mobility on Triton-acid-urea gels as when isolated from wild type strains (2). We therefore concluded that H4 was not acetylated by NatA. But, as noted above, NatB and NatC only acetylate proteins beginning with methionine, not serine, so the question arose as to whether another yeast NAT existed that was responsible for the N-terminal acetylation of H4. We therefore examined uncharacterized yeast genes that are members of the GNAT superfamily of acetyltransferases (6) to see if one of them might be the NAT that acetylates H4. Here we describe an evolutionarily conserved yeast protein, encoded by the gene we call *NAT4*, that N-terminally acetylates not only histone H4, but also H2A.

MATERIALS AND METHODS

Nat4 Expression Plasmid and nat4 Mutant—The *NAT4* coding sequence was amplified from yeast genomic DNA by PCR and cloned into the expression vector, pET28a, as an *EcoR*I-*Xho*I fragment. This led to the expression of full-length Nat4 protein with a His-tag at the N terminus. The protein was expressed well in *Escherichia coli* and purified by Ni^{2+} affinity chromatography. Yeast Δ*nat4* mutants were constructed by replacing the entire *NAT4* open reading frame either with the *kanMX6* gene (strain OS1) or with the *S. pombe his5*$^{+}$ gene (strain OS4), as described previously (10).

Acetylation Reactions—Histones or peptides were acetylated in 40-μl reactions with 75 mM Tris·Cl (pH 8.8), 135 mM NaCl, 0.2 mg/ml chicken erythrocyte histones or 50 μM peptide, 1 μCi of [^{3}H]acetyl-CoA (Amersham Biosciences, 4.2 Ci/mmol), and Nat4 or Hat1 enzymes. Reactions were incubated at 37 °C for 30 min. After incubation, the reactions were spotted to Whatman P81 cation-exchange paper and air dried. The dried papers were washed for 5 min three times with 1 M sodium carbonate (pH 9.0), followed by a wash in acetone. Papers were air-dried, and radioactivity was quantitated in a liquid scintillation counter.

Histone Purification—Yeast histones were purified for sequencing as described previously (11). The histones were separated by electrophoresis on 15% SDS-polyacrylamide gels. After electrophoresis, proteins were blotted onto polyvinylidene difluoride membranes (0.2 μm; Bio-

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[1] The abbreviations used are: NAT, N^{α}-acetyltransferase; HAT, histone acetyltransferase; ACTH, adrenocorticotropin.

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Ard1 (238 aa) 112 NGHITSLSVMRTYRRMGIAENLMRQALFALREVHQ 146
Nat3 (251 aa) 75  HTHITAVTVAPRFRRISLASKLCN-TLETMTDVMP 108
Mak3 (176 aa) 78  RGYIGMLAVESTYRGHGIAKKLVEIAIDKMQREH- 111
Nat4 (285 aa) 181 VIYLYEVHVASAHRGHGIGRRLLEHALCDGVARHT 215
                    ::  : *   .*  .:. .* . ::
                       Motif A

Ard1  147 ---AE---YVSLHVRQSNRAALHLYRDTLAFEVLSIEKS 179
Nat3  108 ---HEVN-FIDLFVKCNNQLAIKLYEK-LGYSVYRRVVG 142
Mak3  112 ---CD---EIMLETEVENSAALNLYEG-MGFIRMKRMFR 143
Nat4  216 RRMCDNFFGVALTVFSDNTRARRLYEA-LGFYRAPGSPA 253
              :    : * .  .*  * .**.  :.:
                    Motif B
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FIG. 1. **Nat4 shows sequence similarity to three known yeast NATs.** The *numbers* in *parentheses* are the total number of amino acids in each protein. Motifs A and B refer to the acetyl-CoA binding motif present in members of the GNAT superfamily (6). *Asterisks* indicate residues identical in all four proteins; *colons* indicate very similar residues; and *periods* indicate similar residues.

Rad). The membranes were washed with water five times for 5 min and then stained with 0.2% Ponceau S for 1 min to visualize the proteins, followed by a wash in water. Sections of the membrane with specific histone bands were excised, soaked briefly in 50% methanol, and washed with water five times. The membrane fragments were air dried and subjected to protein sequencing. For the acid-urea gels, histones were purified as described previously (12).

In Vivo [^{35}S]Methionine Labeling and Protein Extraction—Yeast cells were grown in synthetic complete medium lacking methionine to 2×10^7/ml. A 1-ml culture was labeled for 15 min with 100 μCi of *trans*-[^{35}S]methionine (ICN) and harvested by centrifugation. The cells were washed with 1 ml of ice-cold water and resuspended in 100 μl of water containing protease inhibitors (Roche Applied Science). Cells were lysed by vortexing with the same volume of glass beads. A 0.1-ml solution with 0.6% SDS, 2% mercaptoethanol, 0.1 M Tris·Cl (pH 8.0), which has been kept at 100 °C, was immediately added to the disrupted cell mixture and vortexed for 1 min. After a brief centrifugation 100 μl of the supernatant was mixed with 10 μl of a solution containing 0.5 mg/ml RNase, 1 mg/ml DNase, 500 mM Tris·Cl (pH 7.0), and 50 mM $MgCl_2$. Protein extracts were frozen in dry ice/ethanol and later analyzed by two-dimensional gel electrophoresis at Cold Spring Harbor Laboratory.

RESULTS

The sequence of the protein encoded by yeast ORF, *YMR069w*, has the acetyl-CoA binding motifs characteristic of members of the GNAT superfamily (6). Furthermore, the protein has sequence similarity to the catalytic subunits of the three known NATs, Ard1, Nat3, and Mak3, and is of similar size (Fig. 1). We expressed and purified the protein encoded by *YMR069w* in *E. coli*. The recombinant protein (that we call Nat4 based on the results to be presented below) was able to acetylate a peptide with a sequence corresponding to the N-terminal 28 amino acids of H4, but not a corresponding H3 peptide or an unrelated peptide, adrenocorticotropin (ACTH; Fig. 2*A*). We chose ACTH because NatA is able to acetylate this peptide *in vitro* (13). Notably, Nat4 protein could not acetylate chicken histones even though a known histone acetyltransferase (HAT), Hat1, could acetylate both the H4 peptide and histones (Fig. 2*A*). The observation that Nat4 could acetylate a synthetic H4 peptide but not chicken histones suggested that the protein might be a NAT for H4, because chicken H4 is already fully acetylated on its N-terminal serine. Further support for this idea came from studies comparing *in vitro* acetylation of a completely unacetylated H4 peptide with one internally acetylated on all four acetylatable lysines, namely residues 5, 8, 12, and 16. As can be seen in Fig. 2*B*, Nat4 could acetylate the internally tetraacetylated peptide almost as well as the unacetylated peptide, whereas Hat1 could only acetylate the peptide with internal unacetylated lysines.

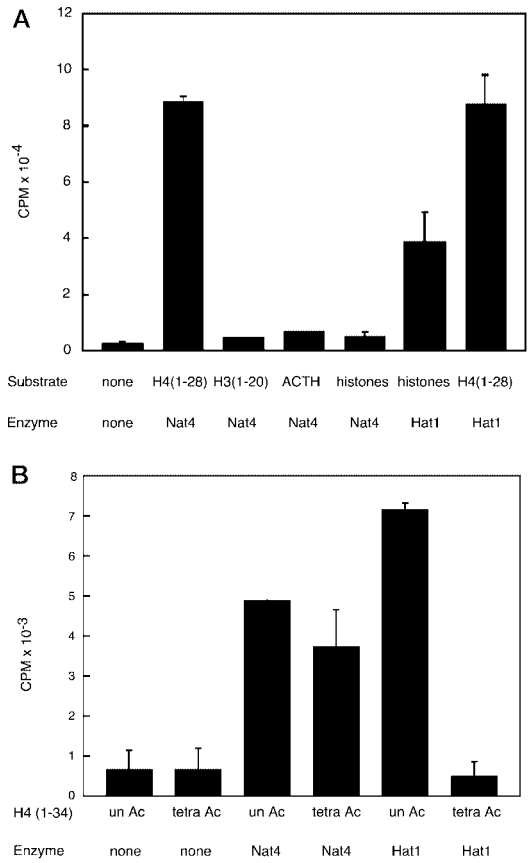

FIG. 2. ***In vitro* assays with Nat4 and Hat1.** Assays were done in duplicate, and the *error bars* indicate the spread in the data. *A*, recombinant Nat4 acetylates an unacetylated H4 peptide, but not H3 or ACTH peptides or chicken histones. Recombinant Hat1 acetylates both the H4 peptide and chicken histones. *B*, recombinant Nat4 acetylates both completely unacetylated (*un Ac*) and internally tetraacetylated (*tetra Ac*) H4 peptides, whereas Hat1 acetylates only the unacetylated peptide.

Next we constructed a mutant with a complete deletion of the ORF for Nat4, *YMR069w*. The mutant was viable and grew at a normal rate. Histones were purified from the mutant, as well as from a wild type and an *ard1* strain (Fig. 3). The proteins were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and the bands at the positions of H4 and H3 excised and subjected to Edman degradation and protein sequencing. Histone H3, which is known to be unblocked (*i.e.* not acetylated) at its N terminus, gave the expected N-terminal sequence, Ala-Arg-Thr-Lys. Histone H4 gave no readable sequence from the wild type and the *ard1* strains, as expected if the protein was blocked by acetylation. However, a sequence corresponding to the first four amino acids of H4, Ser-Gly-Arg-Gly, was obtained for H4 from the strain with a deletion of ORF *YMR069w* (Fig. 3 and Table I). This strongly suggested that this gene encodes a NAT that acetylates the N terminus of H4, and thus the gene was named *NAT4*.

Next, yeast histones were purified to homogeneity by high pressure liquid chromatography from four strains, wild type, an *ard1* mutant, a *nat4* mutant, and an *ard1-nat4* double mutant. The histones were analyzed on an acid-urea gel, which separates them by size and by charge. As shown in Fig. 4, H4 showed the same pattern of isoforms in wild type and in the *ard1* strain, thus confirming our previous conclusion that NatA does not acetylate H4. On the other hand, the H4 isoforms from the *nat4* and *nat4-ard1* strains migrated more rapidly than from the *NAT4* strains, consistent with one extra positive charge due to one less acetylated amino group. Taken together

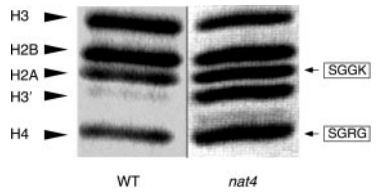

FIG. 3. **Purified yeast histones separated by SDS-PAGE and stained with Coomassie Blue.** In a parallel gel the H4 and H2A bands from the wild type, and the *nat4* strains were transferred to membranes and sequenced by Edman degradation. The indicated N-terminal sequences were obtained only from the *nat4* mutant. H3′ is an H3 degradation product that lacks the first 21 amino acids and is commonly seen in yeast histone preparations.

TABLE I
N-terminal sequences of yeast histones H4, H2A, and H2A.Z

Gene	Protein	No. of amino acids	N-terminal sequence
HHF1	H4	102	SGRGKGGKG
HHF2	H4	102	SGRGKGGKG
HTA1	H2A	131	SGGKGGKAG
HTA2	H2A	131	SGGKGGKAG
HTZ1	H2A.Z	133	SGKAHGGKG

with the H4 sequencing data described above, this experiment convincingly demonstrates that the H4 N-terminal serine is acetylated by Nat4.

H3 exhibited the same electrophoretic mobility in all four strains, as expected, given that H3 is not N-terminally acetylated (Fig. 4). In addition, the electrophoretogram confirmed our previous result that histone H2B is a substrate for Nat1-Ard1 (2). Note that some, but not all, of the H2B isoforms migrate more rapidly in both *ard1* mutant strains. This is discussed below.

To our surprise, H2A isoforms also migrated more rapidly when isolated from both *nat4* mutant strains as compared with the *NAT4* strains (Fig. 4). This suggested that H2A, which has an N-terminal serine residue, just as H4 does, also is a substrate for Nat4. We therefore sequenced purified yeast H2A from wild type and the *nat4* mutant. H2A from the wild type strain gave no readable sequence, while H2A from the mutant gave the sequence Ser-Gly-Gly-Lys, which corresponds exactly to the first four amino acids of H2A (Fig. 3 and Table I). Thus, H2A also is N-terminally acetylated by Nat4.

To examine whether other proteins were substrates for Nat4, we labeled yeast proteins with [^{35}S]methionine in both a wild type strain and a *nat4* mutant. A protein extract from each strain was then analyzed by two-dimensional gel electrophoresis followed by autoradiography. We used this method previously to show that many yeast proteins gain an extra positive charge, and thus shift to the basic side, in *ard1* mutants (2). However, a careful comparison showed that the pattern of protein spots on the gels from the *NAT4* and the Δ*nat4* strains were almost identical (data not shown). The one visible difference was that the Δ*nat4* mutant had a missing spot at a molecular weight consistent with the predicted size of Nat4 protein. Thus, we saw no evidence that other proteins were substrates for Nat4. Histones H4 and H2A do not contain methionine and thus would not have been seen in the autoradiogram. Also, highly basic proteins like histones are not well resolved by the usual two-dimensional gel methods (14).

As mentioned above, the Δ*nat4* mutant was viable. The mutant grew normally at all temperatures tested on both rich medium and synthetic medium. The strain grew normally on galactose or glycerol medium. A *nat4*/*nat4* homozygous diploid underwent normal sporulation and meiosis, leading to four viable haploids per spore. Three experiments failed to uncover any transcription defects for *nat4* mutants. First, the strain

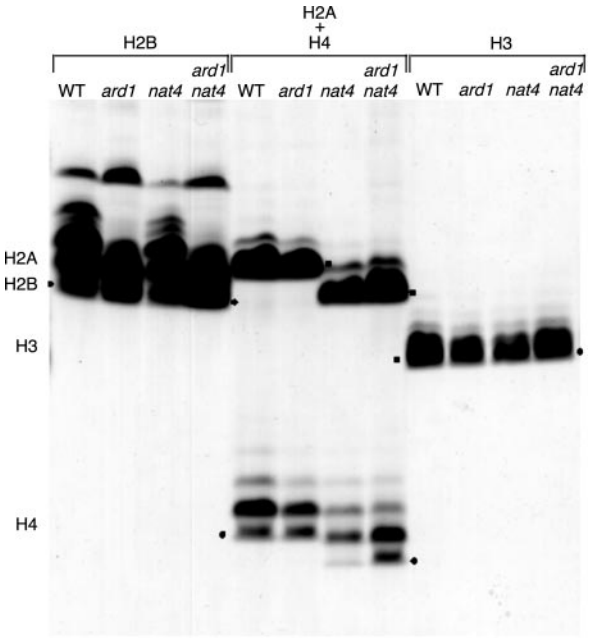

FIG. 4. **Purified yeast histones separated by electrophoresis on acid-urea gels.** Histones were isolated and purified from four strains: wild type (*WT*, W303-1a), and *ard1* (JRM5), *nat4* (OS1), and *ard1-nat4* (OS4) mutants. High pressure liquid chromatography was used to obtain three separate fractions containing purified H2B, a mixture of H2A and H4, and purified H3, respectively (12). These fractions were electrophoresed and the gel stained with Coomassie blue to visualize the histone isoforms. The *black dots* are used to indicate the fastest moving isoform for each histone and the shift in mobility in *nat4* mutants.

could grow on a medium lacking inositol. This is a sensitive test, because many viable mutants in genes for HATs or other transcriptional coactivators are defective in transcription of the *INO1* gene, leading to an inositol auxotrophy (15). Second, a *nat*4 mutation was introduced into a strain with an *HO-lacZ* reporter gene. No defect in *lacZ* expression was seen. Many mutations affecting the Gcn5 HAT complexes or the Swi/Snf chromatin remodeling complex have a defect in transcription of the *HO* gene (16, 17). Finally, the *nat4* mutation was moved into a strain with a *his4*δ mutation to check for an Spt^{-} phenotype (18, 19). The strain remained His^{-}, indicating the lack of an Spt phenotype.

DISCUSSION

Three experiments demonstrated that Nat4 protein acetylates the N terminus of H4. First, the recombinant protein acetylated an H4 peptide *in vitro*, even one already acetylated on the four acetylatable lysines (Fig. 2). Second, and most convincing, H4 isolated from a *nat4* mutant could be sequenced by Edman degradation and yielded the expected N-terminal sequence, whereas H4 from a wild type yeast strain was blocked and could not be sequenced (Fig. 3). Third, H4 from the mutant had an altered mobility on acid-urea gels, consistent with an extra positive charge (Fig. 4). The fact that all the H4 shifted position in the *nat4* mutant suggested that all the H4 was acetylated by Nat4 in wild type yeast and that no other NAT could acetylate it.

All of the H2A from the *nat4* mutant also shifted position in acid-urea gels (Fig. 4). This prompted us to sequence H2A. As with H4, H2A gave the expected sequence when isolated from the *nat4* mutant and gave no sequence from wild type (Fig. 3). Interestingly, H2A has an N-terminal sequence that is quite similar to that of H4 (Table I). Only one other yeast protein is similar to H4 and H2A, comparing the first 9 amino acids, and that is the H2A variant, H2A.Z (Table I). It is quite possible

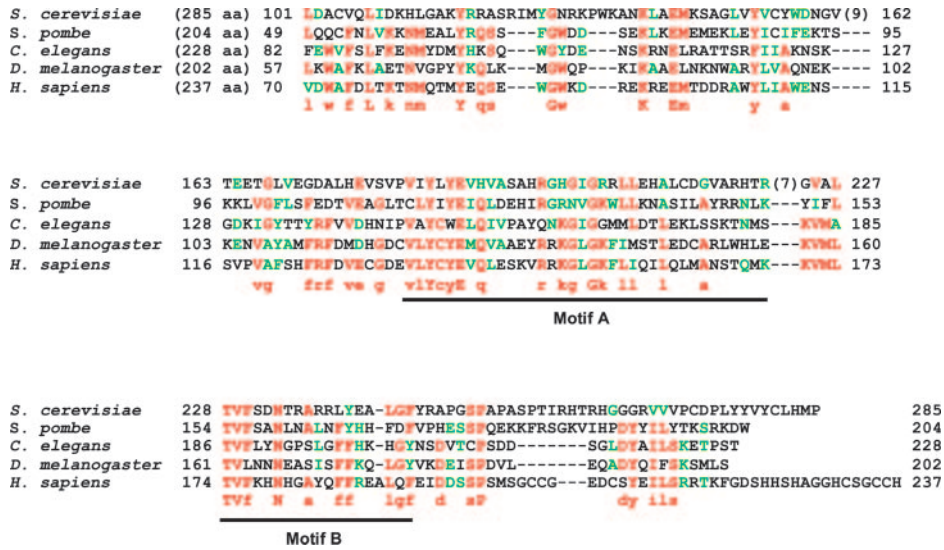

FIG. 5. **Nat4 homologs in other species.** A sequence alignment between *Saccharomyces cerevisiae* Nat4 and proteins from four other species is shown. Residues in *red* are highly conserved among the five species, and residues in *green* are moderately conserved. A consensus sequence is shown below the alignment, with *capital letters* signifying identity among all five proteins. Motifs A and B refer to the acetyl-CoA binding motif present in members of the GNAT superfamily (6).

that H2A.Z is also a substrate for Nat4, but, like H4 and H2A, H2A.Z has no methionine residues and thus could not have been seen in the two-dimensional gel analysis. Interestingly, recombinant H2A.Z purified from *E. coli* (as an H2A.Z-H2B dimer) was an excellent substrate for *in vitro* acetylation by Nat4, while H2B was not acetylated (data not shown). In addition to H4, H2A, and H2A.Z, there are 61 other yeast proteins predicted to begin with (Met)-Ser-Gly. As noted above, we did not observe any mobility shifts comparing proteins from wild type and *nat4* mutant strains by two-dimensional gel electrophoresis analysis. Therefore, we tentatively conclude that Nat4 is dedicated to the acetylation of histones H4 and H2A (and perhaps H2A.Z) and does not act on other proteins.

Since all the H4 and H2A isoforms had an altered mobility on the acid-urea gel, we conclude that all of the H4 and H2A in yeast are acetylated by Nat4 (Fig. 4). But, as noted, some, but not all, of the H2B isoforms had an altered mobility on acid-urea gels when isolated from *ard1* mutants (Ref. 2 and Fig. 4). One explanation for this result is that NatA (with the catalytic subunit, Ard1) acetylates H2B inefficiently. Another explanation takes note of the fact that the two yeast H2B genes code for slightly different proteins, one starting with Ser-Ser-Ala and the other starting with Ser-Ala-Lys. Perhaps NatA can only acetylate one of these two H2Bs, and the other one either is not acetylated or is acetylated by another, as yet unidentified, NAT.

Nat4 is well conserved from yeast to mammals, not just in the acetyl CoA binding domain, but throughout the length of the protein (Fig. 5). We predict that those homologs from larger eukaryotes also will acetylate the N terminus of H4 and H2A, and possibly H2A.Z. Both H4 and H2A are known to be blocked from N-terminal sequencing in all species in which they have been examined, and in the case of the bovine proteins, N-terminal acetylation has been directly demonstrated by elegant biochemistry (9).

It is surprising that *nat4* mutants did not exhibit readily observable phenotypes, especially, since every nucleosome in the mutant cell will have four extra positive charges, one for each H4 and H2A present in the octamer. And yet the mutant grew normally under all conditions tested. Furthermore, the mutant had no observable transcription defects using tests that do exhibit phenotypes for mutants affecting the Swi/Snf chromatin remodeling complex, the Gcn5 HAT complexes and the various Spt proteins involved in transcription.

To try to uncover phenotypes, we constructed *nat4-ard1* and *nat4-hat1* double mutants. We also constructed a strain with the *nat4* mutation plus only one copy of the duplicate H3-H4 genes (either *HHT1-HHF1* or *HHT2-HHF2*). None of those strains had phenotypes over and above that of the single mutants (data not shown). Nevertheless, since acetylation of the N-terminal serine on H4 and H2A is conserved, the modification must be important under some environmental conditions. Additional phenotypic studies or a synthetic lethal screen may uncover the role(s) for N-terminal acetylation of H4 and H2A.

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