Effects of Oncohistone Mutations and PTM

Crosstalk on the N-terminal Acetylation Activities

of NatD

*Yi-Hsun Ho and Rong Huang**

Department of Medicinal Chemistry and Molecular Pharmacology, Purdue Institute for Drug Discovery, Purdue University Center for Cancer Research, Purdue University, West Lafayette, Indiana 47907, United States

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ABSTRACT. Acetylation at the α -N-terminus (N α) is one of the most abundant modifications detected on histone H4 and H2A, which is catalyzed by N-terminal acetyltransferase D (NatD). NatD substrate recognition motif, N-terminal SGRGK, is enriched with frequent oncohistone mutations and post-translational modifications (PTMs). However, there is no information on how oncohistone mutations and other PTMs affect NatD-catalyzed acetylation. Herein, we determined how changes of local chemical environment on the N-terminal SGRGK sequence regulate NatD-catalyzed N α acetylation on histone H4/H2A. Our studies indicate that all oncohistone mutations at SGRG suppressed the catalytic efficiency of NatD. Meanwhile, H4 serine 1 phosphorylation and arginine 3 methylation also negatively affect the NatD activity, but the lysine 5 acetylation has a marginal effect on NatD. This work reveals the impacts of oncohistone mutations on NatD

activity and unravels the crosstalk between NatD and PTMs. To our knowledge, this is the first report on the potential regulatory mechanism of NatD, highlighting different revenues to interrogate the NatD-mediated pathway in the future.

Introduction. Histone modifications alter the chromatin structures and DNA accessibility, serving as a critical mechanism in the regulation of gene expression, DNA replication, and DNA repair.^{1,2} Concerning the modification type and site, at least 450 modifications have been identified on histones, such as phosphorylation, methylation, and acetylation.³ Crosstalk among these modifications adds another dimension to the dynamical regulation of transcription.² The α -N-terminal ($N\alpha$) acetylation catalyzed by N-terminal acetyltransferases (NATs) is a predominantly modification for approximate 80% of proteins in humans.^{4–6} NATs have been classified into NatA–H based on their subunit compositions and substrate specificity profiles.⁶ Among NATs, NatD specifically acetylates an SGRG sequence at the protein N-terminus (Figure 1A). Until now, H4 and H2A are the only two confirmed protein substrates for NatD.^{7–10} Notably, $N\alpha$ -acetylation of H4 ($N\alpha$ -acH4) and H2A ($N\alpha$ -acH2A) were the most abundant marks of histone proteins through several proteomic investigations.^{11–16} For instance, over 80% of H4 and H2A were found $N\alpha$ -acetylated in mouse brains.¹⁴ In addition, 97–98% of $N\alpha$ -acH4 was reported in breast cancer cell lines.¹¹

Recently, oncohistones have drawn significant attention because of their oncogenic features and recurrence at the epigenetic modification-rich histone tails.^{17–21} Interestingly, multiple mutations have been identified in all four amino acids of the SGRG recognition motif of NatD (Figure 1B).^{21–24} Notably, Ser-1 and Arg-3 are the top two frequently mutated residues of H4, and Ser-1 is the most frequently mutated residue of H2A.²³ Although the detailed mechanism of these

mutations remains enigmatic, it raises the question of how those oncohistone mutations at the N-terminal regions affect NatD-catalyzed N α -acetylation.

Meanwhile, the H4 tail is abundant with post-translational modifications (PTMs), including phosphorylation, methylation, and acetylation. N α -acH4 has been found to affect other PTMs at Ser-1, Arg-3, and Lys-5 on H4 to influence biological outcomes.^{25–30} For instance, N α -acH4 interferes with asymmetrical dimethylation on H4 Arg-3 residue (H4R3me2a) in yeast.^{25,26} Caloric restriction in yeast suppresses NatD expression and increases the ratio of H4R3me2a to N α -acH4, promoting the expression of metabolic and stress-response genes.²⁶ Consistent with the findings in yeast, a biochemical study demonstrated that N α -acetylation on H4 peptide reduced the rate of H4R3me2a catalyzed by protein arginine methyltransferase 1 (PRMT1) and PRMT3 but had a marginal effect on the Arg-3 dimethylation by PRMT5 and PRMT8.^{28,30} In colorectal cancer, loss of N-acH4 decreased symmetric dimethylation on H4 Arg-3 (H4R3me2s) levels by downregulating PRMT5, but has marginal effect on monomethylation (H4R3me1) or H4R3me2a.²⁹ N α -acH4 has been demonstrated to block casein kinase 2 α -mediated phosphorylation at the Ser-1 (H4pS1) in lung cancer cells, inducing Slug expression and metastasis.²⁷ Additionally, loss of NatD alters the levels of H4R3me2s, H4R3me1, H4R3me2a, and acetylation of H4 Lys-5 (H4K5ac).²⁹ Although N α -acetylation on H4 has exhibited to impact Arg-3 methylation, Ser-1 phosphorylation, and Lys-5 acetylation, it remains elusive if the communication is unidirectional or mutual. Because the first four residues SGRG are critical for substrate recognition, we hypothesize that modifications on SGRG may affect NatD-catalyzed N α -acetylation. Herein, we examine how the oncohistone mutations and post-translational modifications of H4S1, H4R3, and H4K5 affect N α -acetylation by NatD (Figure 1B).

Figure 1. (A) N α -acetylation catalyzed by NatD. (B) Mutations of oncohistone H4 and H2A and chemical modifications at residues Ser-1, Arg-3, and Lys-5 on the H4 substrate. Modifications include phosphorylation (p), methylation (me), and acetylation (ac). Mutations detected on H4 and H2A: yellow circle; mutations detected only on H4: green circle; mutations detected only on H2A: blue circle.

Results and Discussion.

Effect of histone H4 length on NatD activity. Previous studies suggested that yeast NatD requires a more extended substrate peptide sequence for N α -acetylation than human NatD (hNatD).⁷ Specifically, complete acetylation was detected in yeast when the first 50 residues of H4 were present but incomplete in the presence of the first 30 residues.⁷ In contrast, hNatD displayed a comparable catalytic efficiency for H4 synthetic peptides consisting of the first 5, 8, and 19 residues in a biochemical radioactive study.¹⁰ To systematically understand how substrate length affects hNatD catalysis, we determined the steady-state kinetic parameters for both full-length H4 protein and a panel of truncated H4 synthetic peptides using recombinant hNatD and a continuous fluorescence assay.³¹ The Michaelis-Menten parameters are comparable for H4 protein and the cofactor acetyl coenzyme A (AcCoA) (Table 1). Indeed, hNatD exhibited comparable

catalytic activity (k_{cat}) for its substrates with variable lengths ranging from full-length protein to a tripeptide (Figure 2). However, the catalytic efficiency (k_{cat}/K_m) of hNatD altered for different lengths of substrates, which is mainly due to decreased binding affinity as reflected by K_m values.

Notably, hNatD demonstrated equivalent activity and efficiency for both H4-21 and full-length H4 protein, implying that the C-terminal sequence after Val-21 does not contribute to hNatD recognition. When peptide length decreased from H4-21 to H4-12, no changes were observed for the catalytic activity of hNatD bound with substrate molecules as reflected by k_{cat} values ($11 \pm 0.37 \text{ min}^{-1}$ and $11 \pm 0.35 \text{ min}^{-1}$, respectively). However, there was about a 2.5-fold decrease for H4-12 in catalytic efficiency due to increased K_m values. Similar trends were observed when peptide length reduced from H4-12 to H4-8 residues, while the kinetic parameters were comparable for H4-8 and H4-5 peptides. When the peptide length decreased from H4-5 to H4-4 and H4-4 to H4-3, the K_m values increased about 5-fold and 8-fold, respectively. This result agrees with the previous NatD crystal structure, certifying that the first four N-terminal residues are crucial for NatD-specific binding and acetylation.^{9,10}

In summary, the K_m values of six investigated peptide substrates were inversely proportional to their length. The k_{cat} values were comparable for peptide substrates with varying lengths from 3–21 residues. These results suggest that the C-terminal truncation (5–20 residues) of H4 substrate has modest effects on the binding towards hNatD but a negligible effect on the catalytic rate of hNatD. Thus, the specificity constant of hNatD is proportional to the peptide length when peptide length is shorter than 21-mer.

Table 1. Effect of C-terminal truncations of hNatD recognition.

ID	Sequence	K_m (μM)	k_{cat} (min^{-1})	$k_{\text{cat}}/K_m (\text{M}^{-1} \text{min}^{-1})$
Full length Histone H4		0.80 ± 0.10	12 ± 0.42	1.5×10^7

H4-21	SGRGKGGKGLGKGGAHRHRKV	0.83 ± 0.095	11 ± 0.37	1.3×10^7
H4-12	SGRGKGGKGLGK	2.1 ± 0.22	11 ± 0.35	5.2×10^6
H4-8	SGRGKGGK	3.8 ± 0.38	10 ± 0.26	2.6×10^6
H4/H2A-5	SGRGK	5.9 ± 0.59	11 ± 0.46	1.9×10^6
H4/H2A-4	SGRG	28 ± 2.4	11 ± 0.39	3.9×10^5
H4/H2A-3	SGR	211 ± 21	9.5 ± 0.48	4.5×10^4
AcCoA	AcCoA	0.63 ± 0.055	9.2 ± 0.17	1.5×10^7

Figure 2. $N\alpha$ -acetylation of histone H4 peptides with variable length by hNatD. Fold change of (A) K_m , (B) k_{cat} , and (C) k_{cat}/K_m normalized to full-length histone H4 (FL H4).

$N\alpha$ -acetylation of Oncohistone H4 and H2A. The H4 and H2A are the only two validated substrates of hNatD, containing an identical SGRGK motif at their N-termini. The high-frequency mutations observed in the first four residues of H4/H2A prompted us to investigate how $N\alpha$ -acetylation is affected by H4/H2A oncohistone mutations. Hence, we synthesized pentapeptides derived from the N-terminus of oncohistones H4/H2A and determined their steady-state kinetic parameters using a continuous fluorescence assay (Table 2). Since the fluorescence assay monitors the acetylation progression by detecting the thiol group of CoA products, the existence of cysteine residue in S1C and R3C mutant pentapeptides would interfere with this fluorescence assay. Therefore, we utilized a previously developed MALDI-MS assay to directly detect acetylated products to monitor the acetylation progress of S1C and R3C mutant.³² Because the cysteine is

prone to form dimers, a reducing agent dithiothreitol (DTT) was added to the reaction mixture to prevent dimer formation. For 1 mM of cysteine-containing peptides, 5 mM DTT was selected to completely suppress the formation of the dimer. To compare the performance of two assays and the effect of DTT, we used H4-5 as control and performed kinetic analysis for H4-5 using the MALDI-MS assay in the presence or absence of 5 mM DTT (Figure S1). The Michaelis-Menten constants obtained from the MALDI-MS assay are comparable to those from the fluorescence assay, validating the feasibility of comparing results from two different assays for those mutant peptides containing a cysteine residue.

Ser-1 and Gly-2 are two critical residues for substrate-binding with hNatD because the small hydroxyl side chain of Ser-1 and a specific angle of Gly-2 enable the substrate to enter into the substrate-binding pocket of hNatD.¹⁰ In H4 and H2A, S1C is one of the high-frequency mutations detected in several cancers such as bladder, colorectal, lung, and liver cancers.²¹ We determined the steady-state kinetic parameters of S1C with the highest concentration at 1 mM in the presence of 5 mM of DTT to completely repress its dimer form. We also employed the MALDI-MS assay to determine the kinetics of S1F because of unknown interference from S1F with the fluorescence assay. As shown in Table 2 and Figure 2, hNatD displayed an approximate k_{cat} of 7 min^{-1} and 80-fold reduction in K_m ($>500 \mu\text{M}$) for S1C mutant that only replaces the hydroxyl side chain of Ser-1 with a thiol group. When the bulky phenylalanine (F) is at the first position, hNatD exhibited a k_{cat} of 6.8 min^{-1} and an 800-fold decrease in K_m . For S1L and G2E mutants, the catalytic efficiency was dramatically reduced by over 10,000-fold ($k_{cat}/K_m = 2.5 \times 10^2$ and $1.4 \times 10^2 \text{ M}^{-1} \text{ min}^{-1}$, respectively). These losses are mainly caused by a 1,400 to 3,800-fold reduction in K_m (S1L $K_m = 8.8 \text{ mM}$ and G2E $K_m = 23 \text{ mM}$, respectively), while their catalytic rates k_{cat} are moderately affected by 3- to 5-fold compared with H4-5.

Arg-3 contributes to hNatD substrate-specific binding through several interactions with the side chains of Asp-127, Glu-129, Tyr-136, and Tyr-138 of hNatD.¹⁰ R3H mutant of H2A has been observed in uterine and ovarian carcinomas.²² In comparison to H4-5, the k_{cat} values were decreased by 3-fold to $3.7 \pm 0.12 \text{ min}^{-1}$ for R3Q and 1.5-fold to $7.5 \pm 0.13 \text{ min}^{-1}$ for R3H. Interestingly, the R3H mutant displayed a moderate change in catalytic efficiency by 6.5-fold ($k_{\text{cat}}/K_{\text{m}} = 2.9 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$). Conversely, a 200-fold reduction in $k_{\text{cat}}/K_{\text{m}}$ ($9.4 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$) was observed in the R3Q mutant. Similarly, hNatD displayed a 17-fold reduction in catalytic efficiency for the R3C mutant ($k_{\text{cat}}/K_{\text{m}} = 1.1 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$). This difference in catalytic efficiency between R3Q, R3H, and R3C is consistent with the hNatD mutagenesis study that demonstrated the importance of the positive charge at the third residue for substrate binding.¹⁰

Like Gly-2, Gly-4 resides in a narrow groove that is tailored for glycine. Mutation of Gly-4 to aspartic acid (G4D) or valine (G4V) resulted in slightly decreased catalytic activities by less than 2-fold (G4D $k_{\text{cat}} = 8.0 \pm 0.66$ and G4V $k_{\text{cat}} = 7.7 \pm 0.13 \text{ min}^{-1}$, respectively). Despite the similar catalytic activity, hNatD displayed a 7-fold higher catalytic efficiency for G4V ($2.1 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$) than G4D ($2.9 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$). When Gly-4 was mutated to serine (G4S), hNatD exhibited a 17- and 7-fold reduction in $k_{\text{cat}}/K_{\text{m}}$ and k_{cat} values, respectively. The co-crystal structure of hNatD in complex with its substrate peptide (PDB: 4U9W) indicated that Gly-4 interacts with Trp-90, Thr-174, and Tyr-211.¹⁰ However, it is unclear why G4S resulted in a salient reduction in $k_{\text{cat}}/K_{\text{m}}$ and k_{cat} values among three mutations.

Table 2. Effects of oncohistone mutations on N-terminal acetylation of H4/H2A peptides

Peptide ID	H4 Frequency	H2A Frequency	Sequence	K_{m} μM	k_{cat} min^{-1}	$k_{\text{cat}}/K_{\text{m}}$ $\text{M}^{-1} \text{ min}^{-1}$
H4/H2A-5			SGRGK	5.9 ± 0.59	11 ± 0.46	1.9×10^6
H4/H2A S1C	High	High	CGRGK	>500	> 7	-

H4/H2A S1F	Moderate	Low	FGRGK	>5 mM	> 6.8	-
H2A S1L	No	Moderate	LGRGK	8.8 ± 1.9 mM	2.2 ± 0.29	2.5 × 10 ²
H2A G2E	No	Moderate	SERGK	23 ± 2.8 mM	3.3 ± 0.31	1.4 × 10 ²
H4/H2A R3Q	High	High	SGQGK	393 ± 35	3.7 ± 0.12	9.4 × 10 ³
H2A R3H	Moderate	Low	SGHGK	26 ± 1.4	7.5 ± 0.13	2.9 × 10 ⁵
H4/H2A R3C	High	Moderate	SGCGK	17	1.8	1.1 × 10 ⁵
H4 G4D	High	Low	SGRDK	272 ± 40	8.0 ± 0.66	2.9 × 10 ⁴
H4/H2A G4V	Moderate	Low	SGRVK	37 ± 1.6	7.7 ± 0.13	2.1 × 10 ⁵
H4/H2A G4S	High	Moderate	SGRSK	14 ± 3.1	1.6 ± 0.16	1.1 × 10 ⁵

Figure 2. $\text{N}\alpha$ -acetylation of oncohistone H4/H2A peptides by hNatD. Fold change of (A) K_m (B) k_{cat} , and (C) k_{cat}/K_m normalized to wild-type H4/H2A-5.

Crosstalk between other PTMs and $\text{N}\alpha$ acetylation. Several PTMs have been demonstrated to be affected by $\text{N}\alpha$ -acetylation at the H4 to induce different gene expression patterns.^{25,27,29} $\text{N}\alpha$ -acH4 in lung cancer activate Slug gene expression through the reducing H4pS1 and enhancing H4R3me2a and H4K5ac.²⁷ On the other hand, $\text{N}\alpha$ -acH4 transcriptionally induces PRMT5 expression and thus increases the H4R3me2s in colorectal cancer cells.²⁹ To determine how chemical modifications with charged and steric properties on the substrate peptides affect hNatD activity, we synthesized and determined the steady-state kinetics of several synthetic H4 peptides with common modifications (Table 3). Upon phosphorylation of H4S1, hNatD showed a 2.3-fold

drop in k_{cat} ($4.6 \pm 0.090 \text{ min}^{-1}$) and a 50-fold increase in K_{m} ($303 \pm 21 \text{ }\mu\text{M}$), leading to an over 100-fold reduction in catalytic efficiency. As hNatD has a restrictive size pocket that favors a small side chain, phosphorylation on Ser-1 may not be tolerable in the pocket. In addition, the negatively charged phosphate group may also clash with the Glu-139 of hNatD.¹⁰ Thus, it is not surprising that phosphorylation at Ser-1 inhibits N α -acetylation.

The guanidino group of Arg-3 side chain interacts with Asp-127, Glu-129, and Tyr-138 of NatD through hydrogen bonds.¹⁰ Thus, we set to investigate the effects of Arg-3 methylation on N α -acetylation. Our biochemical studies showed that hNatD had a similar catalytic activity for methylation of H4R3 despite different methylation states (me1, me2a, or me2s) compared to unmodified H4-5 (Table 3, Figure 3). However, the impacts of methylation states on catalytic efficiency are different. Specifically, hNatD displayed 3.6-fold decreased catalytic efficiency for H4R3me1 with a $k_{\text{cat}}/K_{\text{m}}$ value of $5.2 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$, while an 8- and 17-fold reduction were observed for H4R3me2a and H4R3me2s, respectively. As previous biochemical studies reported that N α -acetylation of H4 slightly reduced PRMT1, 3, 5, and 8 activity on Arg-3 dimethylation, our findings suggest the effects between N α -acetylation and Arg-3 dimethylation are mutually inhibitory.^{28,30} On the occasion of Lys-5 acetylation (H4K5ac), hNatD exhibited comparable kinetic parameters for H4K5ac as H4-5. One possible explanation is that the side chain of Lys-5 has less contribution to NatD substrate binding because of its exposure to solvent.¹⁰

Table 3. Impact of histone H4S1, H4R3, and H4K5 modification on N α -acetylation.

ID	Sequence	K_{m} (μM)	k_{cat} (min^{-1})	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1} \text{ min}^{-1}$)
H4-5	SGRGK	5.9 ± 0.59	11 ± 0.46	1.9×10^6
H4pS1	(p)SGRGK	303 ± 21	4.6 ± 0.090	1.5×10^4
H4R3me1	SGR(me)GK	18 ± 0.91	9.3 ± 0.13	5.2×10^5

H4R3me2a	SGR(me2a)GK	47 ± 3.6	11 ± 0.23	2.3×10^5
H4R3me2s	SGR(me2s)GK	73 ± 12	7.8 ± 0.71	1.1×10^5
H4K5ac	SGRGK(ac)	6.1 ± 0.54	10 ± 0.25	1.6×10^6

Figure 3. Impact of chemically modified H4 pentapeptides on hNatD-mediated acetylation. Fold change of (A) K_m (B) k_{cat} , and (C) k_{cat}/K_m normalized to wild-type H4-5.

Conclusion. In this study, we examined how the length, oncohistone mutations, and PTMs at the substrate recognition motif affect $N\alpha$ acetylation. Based on our observation, the oncohistone mutations at Ser-1 and Gly-2 of H4 and H2A strongly inhibit hNatD catalysis and hampers substrate recognition by over 100-fold (Figure 4). Compared with mutations at Ser-1 and Gly-2, hNatD was moderately inhibited by most mutations at Arg-3 and Gly-4 but slightly affected by the R3H mutant, which supports the importance of the positive charge status at Arg-3. Meanwhile, our studies provide the first evidence of the negative impact of phosphorylation of Ser-1 and methylation of Arg-3 on $N\alpha$ -acetylation, uncovering the crosstalk between $N\alpha$ -acetylation and other PTMs. Specifically, phosphorylation of H4S1 is over 100-fold inhibitory to $N\alpha$ -acetylation. hNatD displayed a 10–100 fold decreased activity for dimethylation of H4R3 and less than 10-fold reduction in monomethylation of H4R3. The proposed model establishes a relationship between $N\alpha$ -acetylation and local PTMs of histones and oncohistones, which helps to elucidate the complex crosstalk in histones (Figure 4). This work has provided the mechanistic

understanding of how single mutation of oncohistone and adjacent PTMs affect N α -acetylation by NatD, outlining the basis for future investigation on the functions of the NatD-mediated pathway.

Figure 4. Model for summarizing the impact of oncohistone H4/H2A and PTMs of histone H4 on NatD activity. PTMs include phosphorylation (p), monomethylation (me), asymmetric dimethylation (me2a), symmetric dimethylation (me2s), and acetylation (ac). Mutations were detected on H4 and H2A (yellow), only on H4 (green), only on H2A (blue). The charge status contains neutral (0 within in a gray circle), positive (+ within in a blue circle), and negative (- within in a red circle). Solid red line with blunt end = over 100-fold inhibition, red dotted line = 10–100-fold inhibition, and black dotted line = less than 10-fold inhibition.

Experimental section.

Chemical Reagents. The standard Fmoc-protected amino acids were purchased from CEM Corporation. Acetyl coenzyme A lithium salt was purchased from Sigma-Aldrich. High-performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific. Matrix-assisted laser desorption/ionization (MALDI) spectra were acquired using positive-ion mode on a 4800 MALDI TOF/TOF mass spectrometry (Sciex). Peptides were synthesized on a CEM Liberty Blue peptide synthesizer. The purity of the peptides was confirmed by Agilent 1260 Series HPLC system, eluting with a 0–40% acetonitrile/water gradient with 0.1% TFA. All the purity of final peptides showed >95%. The spectra of MALDI and HPLC are available in the Supporting information (Figure S3).

Peptide synthesis. The peptides were synthesized using Rink Amide MBHA resin (0.05 mmol) and followed a standard Fmoc protocol. Fmoc protected amino acids were coupled at 0.2 M in DMF using 0.5 M of DIC and 1.0 M of Oxyma. Double coupling of Fmoc-Ser(HPO₃Bzl)-OH (ChemPep), Fmoc-Arg(me)(Pbf)-OH (AnaSpec), Fmoc-Arg(me)₂-OH (asymmetrical and symmetrical) (AnaSpec), Fmoc-Lys(ac)-OH (Creosalus) were performed. Fmoc deprotection was carried out by 20% piperidine in DMF. The resin was washed with CH₂Cl₂ (5 mL) and MeOH (5 mL) alternatively three times, and the peptide was cleaved by a cleavage cocktail (TFA/TIPS/water/DODT = 94/1/2.5/2.5 v/v) (5 mL) for 0.05 mmol scale of resin for 4 h. The peptide suspension was filtered, and the filtrate was dried under N₂ and precipitated with cold ether (10 mL). The peptide precipitation was pelleted by centrifugation at 4,200 rpm for 10 minutes, and the resulting supernatant was discarded. The crude peptide pellet was dissolved in deionized water (10 mL), passed through a 0.45 µm filter, and purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on an Agilent 1260 Series system with a C18 column (5 µm, 10 mm × 250 mm) at a flow rate of 4.0 mL/min. Peptides were purified by two mobile phases consisting of 0.1% TFA in water and acetonitrile in a linear gradient.

Protein Expression and Purification. Expression and purification of hNatD were carried out as previously described.³¹ Histone H4 protein was a gift from Dr. Chongli Yuan from Purdue University.

Continuous fluorescence-based acetylation assay. A fluorescence assay was adapted to study the kinetics of NatD, which monitors the formation of a ThioGlo4-thiol adduct that exhibits a strong fluorescence at 465 nm.^{33,34} NatD activity was measured at 25 °C under the following

conditions in a final reaction volume of 40 μ L: 50 nM NatD, 15 μ M ThioGlo4, and 50 μ M AcCoA in reaction buffer (25 mM HEPES, 150 mM NaCl, 0.01% Triton, pH 7.5). For AcCoA characterization, H4-8 was fixed at 50 μ M. All components except for peptide substrate were mixed in a volume of 36 μ L and incubated at 25 °C for 10 min. The reaction was initiated by adding 4 μ L of varying concentrations of peptide substrates. Fluorescence was monitored on a BMG CLARIOstar microplate reader with excitation of 400–415 nm and 460–485 nm. For quantifying the fluorescent signal, a previously reported standard curve ($y = 5176x + 344.6$) was applied to convert arbitrary fluorescence units to the concentration of CoA.³¹ GraphPad Prism 7 was used to fit the initial velocity to Michaelis-Menten or Substrate inhibition model. The experiments were performed in at least duplicate.

MALDI-MS acetylation assay. Kinetics of CGRGK and SGCGK were characterized with a MALDI-MS assay at 25 °C under the following conditions in a final reaction volume of 40 μ L: 50 nM of NatD and 50 μ M of AcCoA in reaction buffer (25 mM HEPES, 150 mM NaCl, pH 7.5). The 10 mM cysteine peptides (CGRGK and SGCGK) were allowed to reduce under 50 mM of DTT at room temperature for 30 min. The peptides were then 2-fold diluted from 1 mM (CGRGK) and 400 μ M (SGCGK) with dilution buffer (25 mM HEPES, 150 mM NaCl, 10 mM DTT, pH 7.5). All components except for peptide substrate were mixed in a volume of 36 μ L, and the reaction was initiated with 4 μ L of varying concentrations of peptide substrates. At desired time points, aliquots were quenched in a 1:1 ratio with the 0.1% of TFA aqueous solution and spotted with matrix solution (10 mg/ml of CHCA in water/acetonitrile/TFA = 1:1:0.1% v/v/v) on the MALDI plate. The initial velocity was determined by calculating the slope from aliquots taken at

differing time points. GraphPad Prism 7 was used to fit the initial velocity to Michaelis-Menten or Substrate inhibition model. The experiments were performed in duplicate.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Kinetic profiles, MALDI-MS, and HPLC spectra of all peptides.

AUTHOR INFORMATION

Corresponding Author

*Rong Huang, Department of Medicinal Chemistry and Molecular Pharmacology, Purdue Institute for Drug Discovery, Purdue University Center for Cancer Research, Purdue University, West Lafayette, Indiana 47907, United States. Email: huang-r@purdue.edu

ORCID

Rong Huang: 0000-0002-1477-3165

Author Contributions

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ABBREVIATIONS

NATs, N-terminal acetyltransferase; AcCoA, acetyl-coenzyme A; H4pS1, histone H4 serine 1 phosphorylation; H4R3me2a, H4R3me1, histone arginine 3 monomethylation; histone H4 arginine 3 asymmetric dimethylation; H4R3me2a, histone H4 arginine 3 symmetric dimethylation; H4K5ac, histone H4 lysine 5 acetylation;

REFERENCES

- (1) Jenuwein, T.; Allis, C. D. Translating the Histone Code. *Science* **2001**, *293*, 1074–1080.
- (2) Lee, J. S.; Smith, E.; Shilatifard, A. The Language of Histone Crosstalk. *Cell* **2010**, *142*, 682–685.
- (3) Huang, H.; Sabari, B. R.; Garcia, B. A.; Allis, C. D.; Zhao, Y. SnapShot: Histone Modifications. *Cell* **2014**, *159*, 458–458.
- (4) Arnesen, T.; Van Damme, P.; Polevoda, B.; Helsens, K.; Evjenth, R.; Colaert, N.; Varhaug, J. E.; Vandekerckhove, J.; Lillehaug, J. R.; Sherman, F.; et al. Proteomics Analyses Reveal the Evolutionary Conservation and Divergence of N-Terminal Acetyltransferases from Yeast and Humans. *Proc. Natl. Acad. Sci.* **2009**, *106*, 8157–8162.

- (5) Aksnes, H.; Drazic, A.; Marie, M.; Arnesen, T. First Things First: Vital Protein Marks by N-Terminal Acetyltransferases. *Trends Biochem. Sci.* **2016**, *41*, 746–760.
- (6) Aksnes, H.; Ree, R.; Arnesen, T. Co-Translational, Post-Translational, and Non-Catalytic Roles of N-Terminal Acetyltransferases. *Mol. Cell* **2019**, *73*, 1097–1114.
- (7) Polevoda, B.; Hoskins, J.; Sherman, F. Properties of Nat4 , an N-Acetyltransferase of *Saccharomyces Cerevisiae* That Modifies N Termini of Histones H2A and H4. *Mol. Cell. Biol.* **2009**, *29*, 2913–2924.
- (8) Song, O.; Wang, X.; Waterborg, J. H.; Sternglanz, R. An N α -Acetyltransferase Responsible for Acetylation of the N-Terminal Residues of Histones H4 and H2A. *J. Biol. Chem.* **2003**, *278*, 38109–38113.
- (9) Hole, K.; Van Damme, P.; Dalva, M.; Aksnes, H.; Glomnes, N.; Varhaug, J. E.; Lillehaug, J. R.; Gevaert, K.; Arnesen, T. The Human N-Alpha-Acetyltransferase 40 (HNaa40p/HNatD) Is Conserved from Yeast and N-Terminally Acetylates Histones H2A and H4. *PLoS One* **2011**, *6*, e24713.
- (10) Magin, R. S.; Liszczak, G. P.; Marmorstein, R. The Molecular Basis for Histone H4- and H2A-Specific Amino-Terminal Acetylation by NatD. *Structure* **2015**, *23*, 332–341.
- (11) Wang, T.; Holt, M. V.; Young, N. L. Early Butyrate Induced Acetylation of Histone H4 Is Proteoform Specific and Linked to Methylation State. *Epigenetics* **2018**, *13*, 519–535.
- (12) Phanstiel, D.; Brumbaugh, J.; Berggren, W. T.; Conard, K.; Feng, X.; Levenstein, M. E.; McAlister, G. C.; Thomson, J. A.; Coon, J. J. Mass Spectrometry Identifies and Quantifies 74 Unique Histone H4 Isoforms in Differentiating Human Embryonic Stem Cells. *Proc.*

- Natl. Acad. Sci.* **2008**, *105*, 4093–4098.
- (13) Pesavento, J. J.; Bullock, C. R.; LeDuc, R. D.; Mizzen, C. A.; Kelleher, N. L. Combinatorial Modification of Human Histone H4 Quantitated by Two-Dimensional Liquid Chromatography Coupled with Top Down Mass Spectrometry. *J. Biol. Chem.* **2008**, *283*, 14927–14937.
 - (14) Tweedie-Cullen, R. Y.; Brunner, A. M.; Grossmann, J.; Mohanna, S.; Sichau, D.; Nanni, P.; Panse, C.; Mansuy, I. M. Identification of Combinatorial Patterns of Post-Translational Modifications on Individual Histones in the Mouse Brain. *PLoS One* **2012**, *7*, e36980.
 - (15) Yamamoto, K.; Chikaoka, Y.; Hayashi, G.; Sakamoto, R.; Yamamoto, R.; Sugiyama, A.; Kodama, T.; Okamoto, A.; Kawamura, T. Middle-Down and Chemical Proteomic Approaches to Reveal Histone H4 Modification Dynamics in Cell Cycle. *Mass Spectrom.* **2015**, *4*, A0039.
 - (16) Jiang, T.; Hoover, M. E.; Holt, M. V.; Freitas, M. A.; Marshall, A. G.; Young, N. L. Middle-Down Characterization of the Cell Cycle Dependence of Histone H4 Posttranslational Modifications and Proteoforms. *Proteomics* **2018**, *18*, 1–11.
 - (17) Funato, K.; Tabar, V. Histone Mutations in Cancer. *Annu. Rev. Cancer Biol.* **2018**, *2*, 337–351.
 - (18) Amatori, S.; Tavolaro, S.; Gambardella, S.; Fanelli, M. The Dark Side of Histones: Genomic Organization and Role of Oncohistones in Cancer. *Clin. Epigenetics* **2021**, *13*, 1–21.
 - (19) Bagert, J. D.; Mitchener, M. M.; Patriotis, A. L.; Dul, B. E.; Wojcik, F.; Nacev, B. A.; Feng, L.; Allis, C. D.; Muir, T. W. Oncohistone Mutations Enhance Chromatin Remodeling and

- Alter Cell Fates. *Nat. Chem. Biol.* **2021**, *17*, 403–411.
- (20) Mohammad, F.; Helin, K. Oncohistones: Drivers of Pediatric Cancers. *Genes Dev.* **2017**, *31*, 2313–2324.
- (21) Nacev, B. A.; Feng, L.; Bagert, J. D.; Lemiesz, A. E.; Gao, J.; Soshnev, A. A.; Kundra, R.; Schultz, N.; Muir, T. W.; Allis, C. D. The Expanding Landscape of “oncohistone” Mutations in Human Cancers. *Nature* **2019**, *567*, 473–478.
- (22) Zhao, S.; Bellone, S.; Lopez, S.; Thakral, D.; Schwab, C.; English, D. P.; Black, J.; Cocco, E.; Choi, J.; Zammataro, L.; et al. Mutational Landscape of Uterine and Ovarian Carcinosarcomas Implicates Histone Genes in Epithelial–Mesenchymal Transition. *Proc. Natl. Acad. Sci.* **2016**, *113*, 12238–12243.
- (23) Koufaris, C.; Kirmizis, A. N-Terminal Acetyltransferases Are Cancer-Essential Genes Prevalently Upregulated in Tumours. *Cancers* **2020**, *12*, 2631.
- (24) Chew, G. L.; Bleakley, M.; Bradley, R. K.; Malik, H. S.; Henikoff, S.; Molaro, A.; Sarthy, J. Short H2A Histone Variants Are Expressed in Cancer. *Nat. Commun.* **2021**, *12*, 1–9.
- (25) Schiza, V.; Molina-Serrano, D.; Kyriakou, D.; Hadjiantoniou, A.; Kirmizis, A. N-Alpha-Terminal Acetylation of Histone H4 Regulates Arginine Methylation and Ribosomal DNA Silencing. *PLoS Genet.* **2013**, *9*, e1003805.
- (26) Molina-serrano, D.; Schiza, V.; Demosthenous, C.; Stavrou, E.; Oppelt, J.; Kyriakou, D.; Liu, W.; Zisser, G.; Bergler, H.; Dang, W.; et al. Loss of Nat 4 and Its Associated Histone H 4 N-Terminal Acetylation Mediates Calorie Restriction-Induced Longevity. *EMBO Rep.* **2016**, *17*, 1829–1843.

- (27) Ju, J.; Chen, A.; Deng, Y.; Liu, M.; Wang, Y.; Wang, Y.; Nie, M.; Wang, C.; Ding, H.; Yao, B.; et al. NatD Promotes Lung Cancer Progression by Preventing Histone H4 Serine Phosphorylation to Activate Slug Expression. *Nat. Commun.* **2017**, *8*, 928.
- (28) Fulton, M. D.; Zhang, J.; He, M.; Ho, M.-C.; Zheng, Y. G. Intricate Effects of α -Amino and Lysine Modifications on Arginine Methylation of the N-Terminal Tail of Histone H4. *Biochemistry* **2017**, *56*, 3539–3548.
- (29) Demetriadou, C.; Pavlou, D.; Mpekris, F.; Achilleos, C.; Stylianopoulos, T.; Zaravinos, A.; Papageorgis, P.; Kirmizis, A. NAA40 Contributes to Colorectal Cancer Growth by Controlling PRMT5 Expression. *Cell Death Dis.* **2019**, *10*, 236.
- (30) Fulton, M. D.; Dang, T.; Brown, T.; Zheng, Y. G. Effects of Substrate Modifications on the Arginine Dimethylation Activities of PRMT1 and PRMT5. *Epigenetics* **2020**, 1–18. <https://doi.org/10.1080/15592294.2020.1864170>.
- (31) Ho, Y.-H.; Chen, L.; Huang, R. Development of A Continuous Fluorescence-Based Assay for N-Terminal Acetyltransferase D. *Int. J. Mol. Sci.* **2021**, *22*, 594.
- (32) Richardson, S. L.; Hanjra, P.; Zhang, G.; Mackie, B. D.; Peterson, D. L.; Huang, R. A Direct, Ratiometric, and Quantitative MALDI–MS Assay for Protein Methyltransferases and Acetyltransferases. *Anal. Biochem.* **2015**, *478*, 59–64.
- (33) Richardson, S. L.; Mao, Y.; Zhang, G.; Hanjra, P.; Peterson, D. L.; Huang, R. Kinetic Mechanism of Protein N-Terminal Methyltransferase 1. *J. Biol. Chem.* **2015**, *290*, 11601–11610.
- (34) Dong, G.; Yasgar, A.; L. Peterson, D.; Zakharov, A.; Talley, D.; Chih-Chien Cheng, K.;

Jadhav, A.; Simeonov, A.; Huang, R. Optimization of High-Throughput Methyltransferase Assays for the Discovery of Small Molecule Inhibitors. *ACS Comb. Sci.* **2020**, 22, 422–432.