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Chemically sumoylated histone H4 stimulates intranucleosomal demethylation by the LSD1-CoREST complex

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Abstract

Lysine-specific demethylase 1 (LSD1) downregulates eukaryotic gene activity by demethylating mono and dimethylated Lys4 in histone H3. Elucidating the biochemical crosstalk of LSD1 with histone post-translational modifications (PTMs) is essential for developing LSD1-targeted therapeutics in human cancers. We interrogated the small ubiquitin-like modifier (SUMO) driven regulation of LSD1 activity with semisynthetic nucleosomes containing site-specifically methylated and sumoylated histones. We discovered that nucleosomes containing sumoylated histone H4 (suH4), a modification associated with gene repression, stimulate LSD1 activity by a mechanism dependent upon the SUMO-interaction motif in CoREST. Furthermore, the stimulatory effect of suH4 was spatially limited and did not extend to the demethylation of adjacent non-sumoylated nucleosomes. Thus, we have identified histone modification by SUMO as the first PTM that stimulates intranucleosomal demethylation by the developmentally critical LSD1-CoREST complex.

Keywords

Chromatin; Nucleosome; Sumoylation; Demethylation; Semisynthesis

Histone lysine post-translational modifications (PTMs) such as acetylation, methylation and sumoylation play critical roles in regulating chromatin function in response to diverse cellular cues.¹ Specific PTMs act by recruiting a range of *reader*, *writer* and *eraser* proteins that modulate the transcriptional state of eukaryotic genes.² Lysine specific demethylase 1 (LSD1, or KDM1A) is a key eraser protein that mediates transcriptional repression by demethylating mono- and dimethylated Lys 4 in histone H3 (H3 K4me_{1/2}).³ LSD1 regulates multiple cellular pathways in eukaryotes including those involved in epithelial-mesenchymal transition,⁴ cell proliferation and survival.⁵ The misregulation of LSD1 is associated with prostate cancer, non-small cell lung cancer⁶ and neuroblastoma.⁷ Hence, understanding the regulation of LSD1 activity is critical from both developmental and therapeutic perspectives.

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LSD1 associates with the co-repressor for element 1 silencing transcription factor (CoREST) and histone deacetylase 1 (HDAC1) to form the LSD1-CoREST-HDAC1 complex.⁸ Although LSD1 can demethylate short H3 N-terminal *tail* peptides,⁹ its activity on nucleosomes requires CoREST, which provides additional interactions with nucleosomal DNA.^{8,10} Additionally, LSD1 activity is negatively regulated by PTMs in the H3 tail. Phosphorylation and acetylation at residues proximal to H3 K4 inhibit LSD1 activity to varying degrees.⁹ However, there are no reports of histone PTMs that positively stimulate LSD1 activity. This is because LSD1 lacks PTM-specific reader domains, such as PHD and Tudor domains that are known to mediate positive crosstalk of the Jumonji histone demethylases with histone PTMs.¹¹ Thus, our knowledge of LSD1 regulation remains incomplete, impeding efforts to control its activity in a locus-specific manner.

Recently, a non-canonical small ubiquitin-like modifier-3 (SUMO) interaction motif was discovered in CoREST.¹² This led us to ask if CoREST may function as a specific PTM reader for LSD1, bridging chromatin sumoylation with the demethylation of H3 K4me₂ by LSD1. We specifically focused on sumoylated histone H4 (suH4) to investigate biochemical crosstalk with the LSD1-CoREST complex because both suH4 and LSD1-CoREST are associated with transcriptional repression.^{13,14} Furthermore, the LSD1-CoREST mediated repression of sodium channel α -subunit genes depends upon SUMO-2/3 modification of a yet unknown chromatin-associated protein.¹² Nuclear desumoylation, by the overexpression of a SUMO protease, leads to reduced occupancy of CoREST and LSD1 at the α -subunit gene promoters and enhances their transcription. Given its correlation with gene repression, we hypothesized that suH4 may be a sumoylated component of chromatin that stimulates LSD1-CoREST mediated demethylation of H3 K4me₂.

Nucleosomes isolated from cellular chromatin contain a heterogeneous mixture of PTMs that preclude studies of specific biochemical crosstalk.¹⁵ To overcome this significant obstacle, we employed a semisynthetic approach to generate milligram quantities of homogenously methylated and sumoylated histones for the assembly of *designer* mononucleosomes (MNs). Two native chemical ligation (NCL)¹⁶ strategies were employed to generate full-length histone H3 K4me₂ and suH4 (Figure 1A–B). In brief, a 6-mer H3 N-terminal peptide containing dimethylated K4, ART(Kme₂)QT, was synthesized as the C-terminal hydrazide by solid phase peptide synthesis (SPPS) using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and the coupling agent *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexa-fluorophosphate (HBTU). Surprisingly, after coupling to the solid-phase, we found that the high basicity of the deprotonated K4me₂ side-chain led to premature deprotection of the base-labile Fmoc protecting group during subsequent amino acid coupling steps. To preclude this effect, ethyl(hydroxyimino)cynoacetate (Oxyma) and *N,N'*-diisopropyl-carbodiimide (DIC) were employed to maintain mildly acidic conditions in all subsequent coupling steps. The final peptide was deprotected and cleaved from the solid phase, and purified in 21% yield (Supplementary Figure S1). Following low-temperature diazotization and conversion to the C-terminal azide,¹⁷ the 6-mer was ligated with recombinant H3(7-135)A7C/C110A polypeptide in the presence of the thiol additive 4-mercaptophenylacetic acid. This yielded the full-length H3 A7C/C110A K4me₂ mutant (Supplementary Figure S2), which underwent radical-mediated desulfurization¹⁸ to generate

H3 K4me₂ with the functionally indistinguishable C110A mutation¹⁹ in 60% yield (Figure 1A and C).

Next, we focused on obtaining sumoylated histone H4. Multiple proteomic studies have identified K12 of H4 as a specific site of sumoylation *in vivo*.^{20–22} Sumoylation of the H4 N-terminal tail is associated with transcriptional repression in yeast and humans.^{13, 14} However, the lack of a known histone-specific SUMO ligase precludes enzymatic histone sumoylation as a viable strategy to interrogate this modification. Hence, we developed a chemical strategy to access suH4 (Figure 1B). The H4(1–14) peptidyl hydrazide was synthesized using Fmoc chemistry, and bromoacetic acid coupled selectively to the deprotected free ϵ -amine of K12 in the resin-bound peptide. Nucleophilic displacement of bromine by the NCL auxiliary *O*-(2-(tritylthio)ethyl)hydroxylamine²³ followed by acidolytic cleavage from the solid phase and global deprotection yielded peptide **1**, with Gly92 of SUMO-3 and the ligation auxiliary attached to Lys12 (Supplementary Figure S3). NCL of **1** with heterologously expressed SUMO-3(2–91)C47S- α -thioester, **2**, yielded the sumoylated peptide **3** (Supplementary Figure S4). The auxiliary N–O bond in **3** was selectively cleaved by Zn-mediated reduction at pH 3.0 to yield the sumoylated peptide **4**.²³ After conversion to the C-terminal α -thioester, **4** was ligated with the truncated H4(15–102) A15C protein, **5** (Supplementary Figure S5). Ligation over 24 h afforded 2.1 mg of the product **6** in 28% purified yield (Supplementary Figure S6). Finally, the full-length sumoylated histone H4 A15C mutant was desulfurized and purified by HPLC to yield the desired suH4, **7**, in 71% yield over two steps (Figure 1D and Supplementary Figure S7).

Wild-type unmodified H4 or semisynthetic suH4 were subsequently incorporated in octamers and MNs along with H3 K4me₂ (Figure 1E and Supplementary Figure S8). Methylated nucleosomes were then subjected to demethylation assays with the full-length LSD1-CoREST complex (Supplementary Figure S9). Non-specific DNA-binding by CoREST²⁴ results in non-productive enzyme-MN complex formation, and ultimately limits the rate of MN demethylation. We surmised that suH4 could, in principle, enhance demethylation activity by facilitating the formation of a productive Michaelis complex via its interaction with the SIM in CoREST (Figure 2A). Consistent with this model of enhanced productive binding by LSD1-CoREST, we observed that suH4-containing MNs were demethylated ~2-fold faster than non-sumoylated MNs in our biochemical assays (Figure 2B–C and Supplementary Figure S10). To ascertain if the enhancement of LSD1 activity was mediated by a specific CoREST-SUMO-3 interaction, we assembled an LSD1 complex with the CoREST 3A mutant where I270, I272 and V274 in the SUMO-interaction motif (SIM) were mutated to Ala in order to diminish SUMO-2/3 binding.¹² Consistent with a specific protein-protein interaction, demethylation assays with the LSD1-CoREST 3A complex showed no significant rate increases when suH4 was present in MNs (Figure 2B). Thus, we conclude that suH4 stimulates nucleosome demethylation by the LSD1-CoREST complex and that the mechanism of stimulation requires a functional SIM in CoREST.

Another possible mechanism for the stimulation of nucleosome demethylation may be the allosteric activation of LSD1 upon CoREST binding to SUMO. To investigate allosteric activation by SUMO, and the importance of its nucleosomal context, we tested the effect of free unconjugated SUMO on LSD1-CoREST activity on methylated nucleosomes

(Supplementary Figure S11) and measured the kinetics of demethylation of the H3(1-21)K4me₂ tail peptide (Supplementary Figures S12 and S13). In each case, no significant stimulation of demethylation was observed upon the addition of free SUMO, indicating that the precise nucleosome context of SUMO is critical for its ability to stimulate LSD1 function.

Although the magnitude of stimulation by suH4 is moderate, it is identical to the 1.5 to 2-fold changes in H3K4me₂ levels observed at the promoters of LSD1-regulated genes after RNAi-mediated knockdown of LSD1 in embryonic stem cells.²⁵ Importantly, such 2-fold changes in H3 K4me₂ levels correspond to 2-fold changes in transcription of the developmentally critical *oct4* and *sox2* genes and dramatic changes in cell proliferation.²⁵

One key aspect of epigenetic regulation is the spreading of histone modifications, such as H3 K9me_{1/2}, to adjacent nucleosomes by the recruitment of histone code *writers*.²⁶ This is important toward ensuring a high local concentration of specific PTMs in chromatin. Therefore, we asked if the stimulatory effect of histone sumoylation would also extend to the demethylation of adjacent non-sumoylated nucleosomes by LSD1-CoREST. Toward this, mononucleosomes with overlapping DNA ends were ligated with T4 DNA ligase²⁷ to generate asymmetric dinucleosomes (aDNs) containing H3 K4me₂ on one nucleosome, and either suH4 or *wt* H4 on the adjacent nucleosome (Figure 3A–B). Interestingly, we observed significantly greater demethylation of aDNs containing suH4-H3 K4me₂ than aDNs containing *wt* H4-H3 K4me₂ (Figure 3C–D and Supplementary Figure S14). However, we also noted statistically indistinguishable rates of demethylation when aDNs containing suH4-H3 K4me₂ were compared with non-sumoylated mononucleosomes containing H3 K4me₂ (Figure 3C–D). This indicates that sumoylation does not significantly stimulate LSD1-CoREST activity on adjacent MNs. Instead, we attribute the enhanced demethylation of aDNs with suH4-H3 K4me₂ to a less compacted dinucleosome structure, which was demonstrated in biophysical investigations with disulfide-linked sumoylated chromatin arrays.²⁸ In the case of aDNs without SUMO, the partial occlusion of K4me₂ in the H3 tails due to greater dinucleosome compaction and aggregation likely underlies diminished demethylation by LSD1-CoREST. Thus, we have established that the suH4-CoREST interaction, while enhancing the demethylation of sumoylated nucleosomes, does not similarly extend to adjacent nucleosomes.

In summary, our semisynthesis of suH4 and H3 K4me₂ enabled the first demonstration of positive biochemical crosstalk between a histone PTM and the developmentally critical histone mark *eraser* LSD1. Our results highlight the need for protein semisynthetic techniques in testing hypotheses otherwise untestable by current molecular biology techniques. Indeed, studies of histone sumoylation and its biochemical crosstalk with other histone modifications have long been limited by the heterogeneity of histone modifications in cells and the lack of a suH4-specific antibody. We established that the mechanism of LSD1 stimulation by suH4 requires a functional SUMO-interacting motif in CoREST, and likely proceeds by recruiting the LSD1-CoREST complex proximal to the H3 tail. Consistent with this mechanism, the stimulatory effect of suH4 did not extend to the demethylation of adjacent MNs and unconjugated free SUMO did not stimulate the LSD1-CoREST complex. The fact that suH4 directly prevents chromatin compaction,²⁸ but also

stimulates the removal of gene-activating histone methylation suggests a model wherein sumoylation may serve as a transient modification to change the methylation state of chromatin (Figure 4). Ultimately, however, suH4 may be removed to facilitate the formation of transcriptionally silenced heterochromatin. This is consistent with the low abundance of sumoylated H4 in cells.¹⁴

Based on our discovery of suH4 as the first histone modification to stimulate LSD1 activity, future studies in our laboratory will test its role in stimulating HDAC1 activity in the expanded LSD1-CoREST-HDAC1 complex, and its effect on gene-specific changes in chromatin methylation and acetylation.

Methods

Semisynthesis Of Full-length H3 K4me₂

See Supporting Information for full experimental details. The 6-mer H3 N-terminal peptide (H₂N-ARTKme₂QT-C(O)NHNH₂) containing dimethylated Lys4 and a C-terminal acyl hydrazide was synthesized on 2-chlorotrityl hydrazide resin using a Liberty Blue microwave-assisted peptide synthesizer (CEM, Matthews, NC). The peptide was cleaved from the resin, dissolved in 0.2 ml of 0.2 M NaP_i buffer containing 6 M Gn-HCl, pH 3.0 and incubated at -20 °C. Next, 2 mg of truncated H3(7-135)A7C/C110A were mixed with 2 mg of 4-mercaptophenylacetic acid (MPAA) and dissolved in 0.2 ml of 0.2 M NaP_i solution containing 6 M Gn-HCl at pH 3.0 by vortexing. The pH of this mixture was adjusted to 6.5 with 6 M NaOH and the solution was cooled to -20 °C. To oxidize the peptide hydrazide to the corresponding azide, 20 µL of 0.5 M NaNO₂ were added into the pre-cooled solution and gently agitated for 15 min at -20 °C. The 6-mer was then ligated to the H3(7-135)A7C/C110A polypeptide by mixing the two solutions at room temperature for 30 min after adjusting the pH to 6.8 – 7.0. This yielded the full-length H3 K4me₂ A7C/C110A mutant, which was desulfurized in 0.2 ml of 1.0 M TCEP, 40 µL of ^tBuSH and 20 µL of 0.1 M VA-044 solution at 37 °C for 2 h to yield H3 K4me₂ containing the C110A mutation in 17% overall yield. The product was purified to homogeneity by RP-HPLC and characterized by ESI-MS.

LSD1 Assays With Mononucleosomes And Asymmetric Dinucleosomes

Demethylation of mononucleosomes and asymmetric dinucleosomes was performed by incubation of H3 K4me₂ containing substrates (50 nM) with varying concentrations of LSD1 (2 to 25 µM) in 50 mM HEPES, pH 8.0, 5% (v/v) glycerol, 1 mM DTT, 50 mM KCl buffer at 25 °C. Aliquots were withdrawn at t=0, 30, 60 and 120 min, quenched by mixing with 6x SDS-containing Laemmli dye, and boiled for 2 min to stop all enzymatic activity. Samples were resolved by 15% SDS PAGE gels at 200 V for 40 min and transferred to Immunoblot PVDF membranes (Bio-Rad) for 1 hr at 100 V. Membranes were blocked at 25 °C using 4% fat-free milk in PBS buffer for 60 min and then incubated overnight at 4 °C with an H3 K4me₂-specific antibody (Upstate 07-030, Lot: 2017310) and H4-specific antibody (Active Motif 39269, Lot: 11908001) at 1:10,000 and 1:1,000 dilutions, respectively, in a PBST buffer containing 4% fat-free milk. Membranes were subsequently washed and incubated with goat anti-rabbit secondary antibody (LI-COR 926-68021, Lot:

C10628-01) at 1:20,000 dilutions in PBST buffer containing 4% fat-free milk for 30 min at room temperature. Following this, membranes were washed and visualized using Odyssey Application Software (LI-COR Biosciences).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Semisynthesis of H3 K4me₂ and suH4 and reconstitution in mononucleosomes

A) Semisynthesis of H3 K4me₂. (i) a) 6 M Gn-HCl, 200 mM Na₂HPO₄, 10 mM NaNO₂, pH 3, –20 °C, 15 min, b) 6 M Gn-HCl, 200 mM Na₂HPO₄, 150 mM MPAA, pH 6.8–7.0, 25 °C, 30 min, (ii) 6 M Gn-HCl, 100 mM Na₂HPO₄, 500 mM TCEP, 100 mM MESNa, 280 mM t-BuSH, 10 mM VA-044, 37 °C, 2 h. B) Semisynthesis of suH4. (i) SUMO-3(2-91)C47S-MESNa, 6 M Gn-HCl, 100 mM Na₂HPO₄, 10 mM TCEP, pH 7.3, 25 °C, 24 h, (ii) 6 M Gn-HCl, pH 3, Zn²⁺, 37 °C, 24 h, (iii) a) 6 M Gn-HCl, 200 mM Na₂HPO₄, 10 mM NaNO₂, pH 3, –20 °C, 15 min, b) 5, 6 M Gn-HCl, 200 mM Na₂HPO₄, 130 mM MPAA, pH 6.8–7.0, 25 °C, 24 h, (iv) 6 M Gn-HCl, 100 mM Na₂HPO₄, 500 mM TCEP, 100 mM MESNa, 280 mM t-BuSH, 10 mM VA-044, 37 °C, 24 h. C) ESI-MS of purified H3 K4me₂, calculated m/z [M + H]⁺ 15,256.7 Da, observed 15,256.7 ± 0.7 Da. D) ESI-MS of purified suH4 (7), calculated m/z [M + H]⁺ 21,596.7 Da, observed 21,602.9 ± 5.5 Da. E) Ethidium bromide-stained 5% TBE gel of mononucleosomes (MN) reconstituted with H3K 4me₂ and either suH4 or wild-type (*wt*) H4.

Figure 2. The effect of sumoylated H4 on LSD1-CoREST activity

A) Model for the stimulation of LSD1 activity by CoREST SUMO-interaction motif (SIM) binding to suH4. The crystal structure of LSD1(171-836)-CoREST(286-482) complex is shown and relative position of the N-terminal SIM in CoREST is indicated, PDB code 2V1D. AOD= amine oxidase domain, SANT2= DNA-binding domain. B) Western blots showing the time-course of MN demethylation with the LSD1-CoREST or CoREST 3A complex. Asterisk indicates a non-specific band. C) MNs containing either H3 K4me₂ alone (white) or suH4 and H3 K4me₂ (black) were assayed with full-length LSD1-CoREST complex or the CoREST 3A mutant (grey). The signal from an H3 K4me₂-specific antibody in (B) was normalized to total histone H4 detected with an H4-specific antibody in each gel lane. n = 3, error bars show standard deviation of the mean. Student's two-tailed t-test, *P < 0.05.

Figure 3. The effect of sumoylated H4 on demethylation of adjacent nucleosomes by LSD1-CoREST

A) Scheme for generating an asymmetrically modified dinucleosome (aDN). B) Ethidium bromide stained 5% TBE gel showing assembly of suH4-H3 K4me₂ containing aDNs. C) Western blots showing time-course of LSD1-CoREST-mediated demethylation of aDNs containing either *wt*H4 and H3 K4me₂, or suH4 and H3 K4me₂, and comparison with MNs containing H3 K4me₂. D) Quantification of H3 K4me₂ demethylation by LSD1-CoREST. The signal from an H3 K4me₂-specific antibody was normalized to total histone H4 detected with an H4-specific antibody in each gel lane. Asterisk indicates a non-specific band. n = 3, error bars show standard deviation of the mean. Student's two-tailed t-test, *P < 0.05.

Figure 4. A model for suH4 function in chromatin repression by LSD1-CoREST

Histone H4 sumoylation activates gene repressive complexes with SIMs, such as the LSD1-CoREST-HDAC1 complex. The presence of suH4 stimulates demethylase activity of LSD1, and potentially deacetylation by HDAC1, leading to the removal of gene-activating histone PTMs. Finally, the removal of SUMO, which inhibits chromatin compaction, permits the formation of compact gene structures associated with silenced heterochromatin.