Acetyltransferase p300/CBP Associated Factor (PCAF) Regulates Crosstalk-Dependent Acetylation of Histone H3 by Distal Site Recognition

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ABSTRACT: Epigenetic regulation is directed, in part, by the correlated placement of histone post-translational modifications, but the mechanisms controlling correlated modifications are incompletely understood. Correlations arise from crosstalk among modifications and are frequently attributed to protein–protein interactions that recruit enzymes to existing histone modifications. Here we report the use of a peptide array to discover acetyltransferase-mediated crosstalks. We show that p300/CBP associated factor (PCAF)/GCN5 activity depends on the presence of a distal arginine residue of its histone H3 substrate. Modifications to H3 Arg8 decrease PCAF acetylation of H3 Lys14, and kinetic data indicate that arginine citrullination has the strongest effect in decreasing acetylation. Mutagenesis experiments demonstrate that PCAF specifically interprets H3 Arg8 modifications through interaction with residue Tyr640 on the surface of its catalytic domain, and this interaction regulates Lys14 acetylation by substrate discrimination. PCAF discriminates modified peptides as well as semisynthetic proteins and reconstituted nucleosomes bearing Arg8 modifications. Together, this work describes a method for systematically mapping crosstalks and illustrates its application to the discovery and elucidation of novel PCAF crosstalks.

Histone proteins are subject to a broad array of post-translational modifications that regulate gene expression, replication, and repair.1,2 These modifications include acetylation, methylation, and phosphorylation, and their presence or absence at multiple sites generates staggering complexity in histone structure and function. Post-translational modification (PTM) of histone H3, for example, can theoretically generate over ten million distinct forms, and systems-level studies have catalogued a fraction of these. They reveal that distinct PTMs are generally correlated within observed histone states3−5; however, the mechanisms by which cells install and maintain these correlated modifications are much less clear. For example, many of the enzymes that modify histones lack specificity for individual sites and in many cases are localized to their substrates through interactions of adaptor domains. In this way, the action of such enzymes on their substrates generally depends on the presence or absence of other histone PTMs. A complete understanding of these indirect relationships between enzyme expression and histone modification is important for understanding regulation in the cell and in the development of drugs for so-called “epigenetic targets”. In this paper, we employ a biochip-based strategy for identifying post-translational modifications that regulate the actions of histone modifying enzymes, and we show specifically that acetylation of H3 Lys14 by the lysine acetyltransferase p300/CBP associated factor (PCAF) is inhibited by methylation or deimination of the distal residue H3 Arg8.

This report is the first to identify a mechanism of crosstalk between methylation/deimination and acetylation on the histone. Significant earlier work has identified several examples of crosstalk where the presence or absence of a modification enhances or represses another modification on the same histone (in cis) or a different histone (in trans).6,7 For example, Allis and co-workers discovered that H2B ubiquitination is a master switch of gene silencing through its effects on H3 methylation.8 Oliviero and co-workers reported that phosphorylation of H3 Ser10 induces the acetylation of H4 Lys16 and directs gene activation, revealing the downstream regulatory impact of a single crosstalk activity.9 The discovery of such crosstalks usually relies on a pair of antibodies to isolate histones having a first modification and to probe for the presence of a second modification, but the development of antibodies having specificity for each of the relevant modifications is challenging. In the present work, we use a combination of mass spectrometry and a peptide array, the
SAMDI method, to efficiently identify an example of crosstalk at the biochemical level where the ability of an enzyme to modify a histone sequence depends on modifications to distal residues.

We compared the acetylation of a peptide library representing mutants of the H3 Lys14 site by the acetyltransferases (KATs) PCAF and GCN5. Using this positional scanning approach, we found that the enzymes interact with Arg8 and that modification of this residue, either by methylation or deimination, significantly reduces acetylation of Lys14. Kinetic analyses confirmed the extent to which various Arg8 modifications fine-tune PCAF activity at Lys14, and we used high-resolution mass spectrometry to quantify this effect on semisynthetic H3 protein substrates and reconstituted nucleosomes. We also prepared a series of PCAF mutants and found that residue Tyr640 is necessary for recognition of Arg8 in the substrate. Together, this work provides mechanistic support for a novel in cis crosstalk between Arg8 methylation/deimination and Lys14 acetylation, and it also introduces a systematic and label-free strategy for identifying crosstalks that regulate the modification of histone substrates.

**RESULTS AND DISCUSSION**

**PCAF-Mediated Acetylation of H3 Fragment.** PCAF is an acetyltransferase that uses the cofactor acetyl coenzyme A (AcCoA) to acetylate lysine 14 of histone 3 (H3 Lys14). We prepared a peptide representing residues 6–16 of H3 (TARKS\(^r^\)STGGK\(^r^\)APC), which includes the Lys14 acetylation site. The Lys9 residue in the peptide was synthetically acetylated in order to eliminate the possibility for enzymatic immobilization to a self-assembled monolayer. We prepared a monolayer presenting maleimide groups at a density of 10% against a background of tri(ethylene glycol) groups. The former are used to immobilize cysteine-terminated peptides, and the latter prevent the nonspecific adsorption of protein to the monolayer. The monolayer is well suited for analysis by matrix-assisted laser desorption-ionization mass spectrometry to reveal the masses of the peptide–alkanethiolate conjugates and therefore can identify products of enzyme-mediated reactions and the yields in which these products are formed. Further, the SAMDI mass spectrometry method is compatible with the analysis of peptide arrays and was used in the present work to profile acetyltransferase activities across 73 distinct substrates.

To assay PCAF activity, we applied a solution containing the enzyme and cofactor (1 μM PCAF, 100 μM AcCoA, 50 mM Tris pH 8.0, 0.1 mM EDTA) to a monolayer presenting the H3 peptide substrate. The reaction was incubated for 1 h. The monolayer was then rinsed, treated with matrix, and analyzed by mass spectrometry (Figure 1). The SAMDI spectrum of the original monolayer has a peak at \(m/z = 2110\) that corresponds to the peptide–alkanethiolate conjugate. Following the reaction, a spectrum revealed a new peak at \(m/z = 2152\) that corresponds to the acetylated form of the peptide. Integration of peak areas showed that acetylation proceeded in 70% yield.

**Distal Sequence Scanning.** We designed a library of peptide substrates that could be used to identify whether PCAF interacts with H3 residues distal to the Lys14 acetylation site. The library included the following 73 peptides that represent the wild-type sequence and each possible mutant at positions 7–10 of histone H3: TXR\(^r^\)STGGK\(^r^\)APC, TAX\(^r^\)STGGK\(^r^\)APC, TAR\(^r^\)STGGK\(^r^\)APC, and TAR-7\(^r^\)X\(^r^\)STGGK\(^r^\)APC, where X is substituted with any of the natural amino acids except for cysteine. Again, lysine 9 was synthetically acetylated to restrict enzymatic acetylation to lysine 14. The peptides were immobilized on an array of monolayers arranged in standard 384-well format as recently described. The reaction conditions were adjusted to give an approximate 85% conversion on the wild-type sequence. Reaction mixtures were automatically dispensed to the array and incubated for 1 h. Next, the monolayers were rinsed and analyzed by SAMDI mass spectrometry to determine the extent of reaction of each peptide, which is shown in a bar graph plot (Figure 2). These data reveal a striking dependence on the Arg8 residue for acetylation at Lys14 by PCAF. Substitution of this amino acid with either histidine or lysine gives weak activity, while peptides having other residues at this position are not acetylated at all. By contrast, PCAF is remarkably tolerant to mutations at positions 7, 9, and 10, with only K9ac → D and S10 → G/P showing at least a 3-fold reduction in acetylation of Lys14. Hence, PCAF recognizes the Arg8 residue of its substrate.

To assess whether distal site dependence might be a common feature of KATs, we tested the acetyltransferase GCN5 and p300. GCN5 is structurally similar to PCAF, while...
p300 is structurally distinct. These KATs also prefer different histone and non-histone substrates.\textsuperscript{14,15} p300 was weakly active at Lys14 and did not reveal a strict dependence on distal sequence variation. Instead, it preferred substrates that included a lysine mutation, consistent with reports that p300 prefers substrates with a distal lysine residue.\textsuperscript{15,16} GCN5 is structurally homologous to PCAF and shared the same requirement for Arg8. It was similarly tolerant to substrate mutations at positions 7, 9, and 10.

**PCAF Kinetics on Arg8-Modified Substrates.** Known PTMs to H3 Arg8 include methylation\textsuperscript{17} and deimination,\textsuperscript{18} and we therefore determined whether those modifications to the peptide substrates had an impact on acetylation by PCAF. We synthesized peptides having each of the four possible modified forms, monomethyl (R\textsubscript{me}), symmetric dimethyl (R\textsubscript{me2s}), asymmetric dimethyl (R\textsubscript{me2a}), and the deiminated form having the amino acid citrulline (cit) forms. We characterized their activity toward PCAF (Figure 3). Determining Michaelis–Menten kinetics in cases where the enzyme is soluble and the substrate is immobilized can be challenging.\textsuperscript{19} For this reason, we employed a solution-phase spectroscopic assay developed by Denu and co-workers.\textsuperscript{20} We determined kinetic profiles for the reaction across varying substrate concentrations (10–1300 μM), while maintaining PCAF and AcCoA concentration at 1 and 100 μM, respectively. The initial velocities displayed a stepwise increase in \( K_M \) as the native Arg8 was mutated to R\textsubscript{me}, R\textsubscript{me2s}, R\textsubscript{me2a}, and finally cit. Generally, \( k_{cat} \) remained constant between the mutants, apart from the citrulline mutant, which was near the limit of resolution. The dimethyl and citrullinated peptides had a 10-fold reduction in catalytic efficiency (\( k_{cat}/K_M \)) owing primarily to an increase in \( K_M \) (Table 1).

**Histone and Nucleosome Substrates.** We next verified that our finding of an Arg8-dependent PCAF activity was not an artifact stemming from the use of a peptide substrate but also operated for protein and nucleosome substrates. We used

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**Histone and Nucleosome Substrates.** We next verified that our finding of an Arg8-dependent PCAF activity was not an artifact stemming from the use of a peptide substrate but also operated for protein and nucleosome substrates. We used
native chemical ligation to prepare full-length H3 proteins that had modified forms of Arg8.21 We synthesized appropriate peptide thioesters representing residues 1–24 of H3 containing the desired Arg8 modification and performed ligation reactions with a recombinant truncated H3 protein bearing an N-terminal cysteine in place of the native alanine residue at position 25. For these experiments, Lys9 was not synthetically modified forms (Rme2a, Rme2s) or deiminated (cit), the peptide precursor also contained the desired Arg8 modifications and was observed across the Arg8-modified forms (Figure 4, Table 2). The trend is consistent with that observed using the peptide substrates but less pronounced in that the most disruptive modifications, Rme2a and cit, yielded a 3-fold reduction in acetylation versus an approximate 10-fold reduction observed for peptides. We tested a commercially available recombinant H3 protein standard and found approximately 80% Lys14 acetylation, suggesting that semisynthetic substrates are not perfect biological mimics of endogenous H3.

Because H3 is found almost exclusively within chromatin, we reconstituted nucleosomal particles that were modified at H3 Arg8. We used a commercially available H3 that was
dimethylated at Arg8 (H3R8me2a) and assembled a histone octamer according to the method of Luger.
Nucleosome core particles (NCPs) were prepared in the presence of a standard DNA sequence amplicon by a gradual reduction in ionic strength. Confirmation of nucleosome formation was visualized by a gel-shift assay on native polyacrylamide gels stained for DNA. NCPs were assayed and analyzed by mass spectrometry according to the preceding protocol. Relative PCAF acetylation of Lys14 was determined to be 62% for the unmodified NCP and 19% for the H3R8me2a NCP.

**PCAF Mutagenesis.** We reasoned that this crosstalk is mediated by an interaction of the Arg8 residue with PCAF by way of a site on the surface of the enzyme. To examine this possibility, we generated several PCAF mutants that substituted residues near the enzyme active site with an alanine. We used structural data for PCAF and the homologous GCN5 to target mutations on the surface of the protein near the peptide binding cleft.

We site-specifically mutated amino acids situated in the catalytically important α2 helix region (residues S30–S40) and the α5–β6 loop (residues S35–S64), which are known to rearrange during substrate binding (Figure 5). Following verification by SDS-PAGE and DNA sequencing, each mutant was tested for its ability to preserve or impair crosstalk.

![Diagram](https://via.placeholder.com/150)

**Figure 5.** Mutation of Tyr640 eliminates crosstalk capability. (A) PCAF illustration highlighting the amino acids mutated to alanine. PCAF H3 Lys14 acetylation activity by SAMDI on Arg8-modified peptides for wild-type PCAF (B) and Y640A mutant (C). Bars indicate relative acetylation ± SD (n = 3).

We assayed each mutant by SAMDI-MS against a panel of peptides having R, Rme, Rme2a, Rme2s, or cit at position 8. Most of the mutants displayed a trend in activity on these substrates that was similar to wild-type, suggesting that their respective residues do not participate in the putative interaction with Arg8. One mutant, Y640A, had a reduced, but near-constant, activity on each of the substrates (Figure 5). Hence, this mutant uncoupled acetylation of Lys14 from recognition of Arg8, suggesting that the tyrosine residue mediates the interaction with Arg8, perhaps using a cation–π interaction.

Tyr244 in yGCN5 is analogous to PCAF Tyr640 and is also located within the peptide binding cleft distal to the active site. Interestingly, this single point mutant has been found to decrease acetylation activity in vivo. Despite the slight structural differences between GCN5 and PCAF, we believe that our results extend the importance of this residue as a substrate reader required for crosstalk functionality.

The dependence of PCAF-mediated acetylation of H3 Lys14 on the Arg8 residue establishes a novel crosstalk between these sites. Modifications at Arg8 impaired PCAF-mediated acetylation of Lys14 with dimethyl arginine and citrulline proving most significant. Kinetic analyses of Arg8-modified peptides verified a stepwise reduction in acetylation from the native substrate to the monomethylated, dimethylated, and deiminated forms. We also observed this crosstalk on full-length H3 protein substrates prepared semisynthetically and on reconstructed nucleosomes, although the observed effect was less pronounced. In these instances, the availability of additional sequence contacts may have compensated for the disrupted interaction with PCAF Tyr640 caused by Arg8 modifications, which our kinetic studies revealed to affect peptide affinity.

Studies using peptide substrates provide a convenient platform for investigating biochemical properties of enzymes but often do so at the expense of true biological context. Our experiments on the histone H3 positional mutant peptide library allowed the facile determination of Arg8 dependence for PCAF/GCN5 when arranged in array format for automated handling and analysis. The more challenging characterization on semisynthetic protein substrates and reconstructed nucleosomes revealed that our peptide-level work reliably approximated the solution-phase in vitro outcomes. Still, the mechanism of PCAF regulation by distal site recognition remains to be verified in vivo in order to truly appreciate the biological importance of this crosstalk and the many other crosstalks that support correlated histone states.

New examples of crosstalk continue to redefine the epigenetic landscape, but in most cases, these examples do not include a biochemical rationale. In those cases that do, the mechanistic insight generally focuses on adaptor-mediated recognition rather than substrate discrimination by catalytic domains. Our mutagenesis experiments revealed that Tyr640 of the PCAF catalytic domain participates in recognizing the methylation/deimination state of Arg8 via a distal site interaction. Biochemical studies of the PCAF parologue GCN5 by Marmorstein and Poux hypothesized that residues N-terminal to H3 Lys14 are particularly important to substrate affinity, and our data support this notion of distal site dependence. Mutagenesis experiments by Allis and co-workers found several GCN5 surface residues critical to catalytic function in vivo, including Tyr244, the analogue of PCAF Tyr640. Hence, the catalytic importance of these residues may then derive from their role as crosstalk mediators, but this association needs to be studied further.

This and related forms of crosstalk may underlie the correlations of PTMs that are often observed on histones. Kelleher and co-workers, for example, noted such correlations in HeLa nuclei arising from the placement of methyl and acetyl marks on histone H4. Correlations of modifications at Arg8 and Lys14 have not been reported, but this work illustrates a mechanism by which PCAF could maintain a correlated state via these two residues. Given the overwhelming combinatorial complexity of theoretical histone modifications, we believe it reasonable that histone modifiers participate in crosstalk to engage a more tractable number of states.

Known H3 Arg8 post-translational modifications include symmetric dimethylation by protein arginine methyltransferase 5 (PRMT5) and deimination to citrulline by protein arginine deiminase 4 (PAD4), but their impact on Lys14 is not yet...
clear. Genome-wide epigenetic profiles in mice have linked Lys14 acetylation to a subset of inactive promoters “poised” for transcripational activation.32 The repressive link between Arg8 modifications and PCAF-mediated Lys14 acetylation may then predict a repressive function overall, but such speculation awaits in vivo demonstration.

Whether modification states codify defined regulatory signatures remains to be firmly established. Still, aberrant modifications are observed in diseased cells, and the enzymes that regulate histone modification have become important targets in drug development programs. For example, many cancers are associated with hypoacetylation mediated possibly by the overexpression of deacetylases.33,34 The drugs vorinostat targets in drug development programs. For example, many that regulate histone modi-

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**METHODS**

**General.** Unless specified otherwise, laboratory chemicals and reagents were purchased from Sigma-Aldrich and used without additional purification. Peptide synthesis reagents, including Fmoc amino acids and Rink-amide resin, were purchased from AnaSpec. Selectively methylated Fmoc-arginine amino acids and H-Ala-sulfamylbutylryl NovaSyn TG resin are products of Novabiochem (EMD Millipore). Plasmids containing human PCAF and histone H3.3 were purchased from ATCC, and remaining molecular biology materials were purchased from Invitrogen. Oligonucleotide primers were from Integrated DNA Technologies. Restriction endonucleases, recombinant human histones, and control DNA were purchased from New England Biolabs. Histone H3R8me2a protein was purchased from ActiveMotif. Spectrophotometric data were collected on a DU-640 spectrophotometer (Beckman Coulter) unless indicated other-

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**Protein Expression and Purification.** Recombinant plasmids (pTriEx3, Novagen EMD Millipore) harboring genes for human PCAF catalytic domain (aa 492–658), full-length human GCN5, or human p300 catalytic domain (aa 1284–1673) were confirmed via bidirectional DNA sequencing and expressed in *Escherichia coli* [BL21(DE3)] (Invitrogen) by isopropyl-β-D-galactopyranoside (IPTG) induction (0.5 μM final) for 4 h at 37 °C after reaching an OD of 0.4–0.6. After lysis by sonication in the presence of protease inhibitors (Roche), the soluble 6× His-tagged proteins were purified on a prepared IMAC column containing Co2+-HisPur affinity resin (Thermo) and eluted across a stepwise imidazole gradient (10–150 mM) in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5% glycerol, and 5 mM β-mercaptoethanol. Pure fractions were determined by SDS-PAGE on a 4–12% polyacrylamide gel (Lonza), concentrated with centrifugal filters (Millipore), dialyzed against storage buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 M EDTA), flash frozen, and stored at ~80 °C. Protein concentration was determined on a NanoDrop spectrophotometer (Thermo Scientific) using calculated extinction coefficients (PCAF ε280 = 2025 M−1 cm−1, GCN5 ε280 = 5390 M−1 cm−1, p300 ε280 = 42860 M−1 cm−1). Expression was confirmed by SDS-PAGE (Figure 1).

Truncated human histone H3.3 protein was expressed in BL21-(DE3) harboring pET21a(+) according to standard protocols.37,38 Briefly, the gene encoding truncated H3 (amino acids 26–135) was amplified from the plasmid containing full-length human histone H3.3 with a forward primer designating an Ndel site, a start codon, codons was amplified. A Factor Xa protease cleavage site (amino acids IEGR), and a codon for an N-terminal cysteine residue (′-ACTG CTC GAG AGC CTC TCC CTC CGG TAT CGG G-3′), the reverse primer encoded an Xhol site (′-ACTG CTC GAG AGC CTC TCC CTC CGG TAT CGG G-3′). Plasmids were maintained and propagated in *E. coli* (DH5α, Novagen), and DNA sequencing validated the identity of the expression plasmid. Following induction by 0.2 μM IPTG (final) for 2 h at 37 °C, the 6× His-tagged protein was purified by inclusion bodies with Co2+ affinity chromatography as described above. Fractions were dialyzed against three changes of 5 mM dithiothreitol (DTT), analyzed by SDS-PAGE on a 15% polyacrylamide gel (Lonza) and by MALDI-TOF mass spectrometry (linear midmass positive mode, m/z = 14335). Protein concentration was determined spectrophotometrically (ε280 = 4470 M−1 cm−1), and the protein was flash frozen and stored at ~80 °C.

**Peptide Synthesis.** Solid-phase peptide synthesis was performed on Rink-amide lanterns (Mimotopes) housed in 96-well filter plates. Deprotection of N-terminal fluorenlymethyloxycarbonyl (Fmoc) protecting groups was achieved with 20% piperidine in dimethylfor-

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assembled monolayers of mixed alkyl thiolates on gold were prepared (DIUF) water, analyzed by MALDI-TOF MS in

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Notes
The authors declare no competing financial interest.

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