Histone Modifications

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Differentially Isotope-Labeled Nucleosomes To Study Asymmetric Histone Modification Crosstalk by Time-Resolved NMR Spectroscopy

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Abstract: *Post-translational modifications (PTMs) of histones regulate chromatin structure and function. Because nucleosomes contain two copies each of the four core histones, the establishment of different PTMs on individual "sister" histones in the same nucleosomal context, that is, asymmetric histone PTMs, are difficult to analyze. Here, we generated differentially isotope-labeled nucleosomes to study asymmetric histone modification crosstalk by time-resolved NMR spectroscopy. Specifically, we present mechanistic insights into nucleosomal histone H3 modification reactions in cis and in trans, that is, within individual H3 copies or between them. We validated our approach by using the H3S10phK14ac crosstalk mechanism, which is mediated by the Gcn5 acetyltransferase. Moreover, phosphorylation assays on methylated substrates showed that, under certain conditions, Haspin kinase is able to produce nucleosomes decorated asymmetrically with two distinct types of PTMs.*

Post-translational modifications (PTMs) of histones work in concert through the formation of combinatorial patterns or histone codes to regulate a plethora of fundamental biological processes, including transcription, replication, recombination, and DNA repair.[1,2] Most of these PTMs occur in the unstructured N- or C-terminal tails of core histones and at closely spaced modification sites, thereby giving rise to functional networks of reciprocal PTM crosstalk.[3] Although a few PTMs are deposited on free histone substrates, the vast majority are placed on nucleosome-incorporated histones. The nucleosome is a symmetric structure consisting of approximately 147 bp of DNA wrapped around a histone octamer made up of two copies each of the four core histones.[4] Historically, and because of the inherent symmetry of the nucleosomal architecture, histone PTMs were thought to exclusively occur in a symmetrical fashion, with both copies of each of the core histones modified in exactly the same manner. This notion was recently challenged by two studies demonstrating a mechanistic basis for the establishment of asymmetric histone H3 phosphorylation in vitro by certain kinases,[5] and the existence of asymmetrically methylated nucleosomes in embryonic stem cells, fibroblasts, and cancer cells.[6] As such, asymmetrically modified nucleosomes represent a novel concept in chromatin biology and their existence raises important biological questions with regard to their establishment and the subsequent crosstalk in cis (within individual histone tails) and in trans (between the two copies of sister histones) that this asymmetry might impose on the propagation of histone marks.

Most analytical methods to investigate histone PTMs on nucleosomal substrates fall short in terms of verifying the copy-specific origin of the detected modifications. By proteolytically processing modified nucleosomes for mass spectrometry analysis, for example, individual histone tails are cleaved off from core particles and detected in mixtures of modified peptides, which effectively "erases" all information about whether they originated from the same or from different nucleosomes. To preserve this information, histone PTMs ought to be studied in a native nucleosomal context and with analytical tools that provide a means to distinguish between individual copies of assembled core histones, an undoubted experimental challenge altogether.

Herein, we present the generation of both differentially isotope-labeled and asymmetrically modified nucleosomes (hereafter referred to as "asNucs") to study modification crosstalk in cis and in trans. We reconstituted nucleosomes containing the individual copies of histone H3 incorporated in a ^{15}N- or ^{13}C-enriched form. Additionally, one of the two H3 copies was incorporated in a pre-modified mode. ^{15}N-, or ^{13}C-edited NMR experiments thus allow the selective detection of either nucleosomal copy of histone H3 in a completely unbiased and direct manner. Accordingly, by employing NMR routines developed over the past years in our laboratory[7,8] to detect different types of PTMs, we illustrate a unique ability to monitor individual histone modifications and modification crosstalk in cis and in trans through time-resolved NMR readouts. To prepare asNucs, we took advantage of a tandem affinity purification method for asymmetric histone subcomplexes that was previously introduced by Voigt et al.[6] Similarly, we prepared two versions of full-length histone H3, carrying either a His or a Strep affinity tag at their N termini and being asymmetrically modified by

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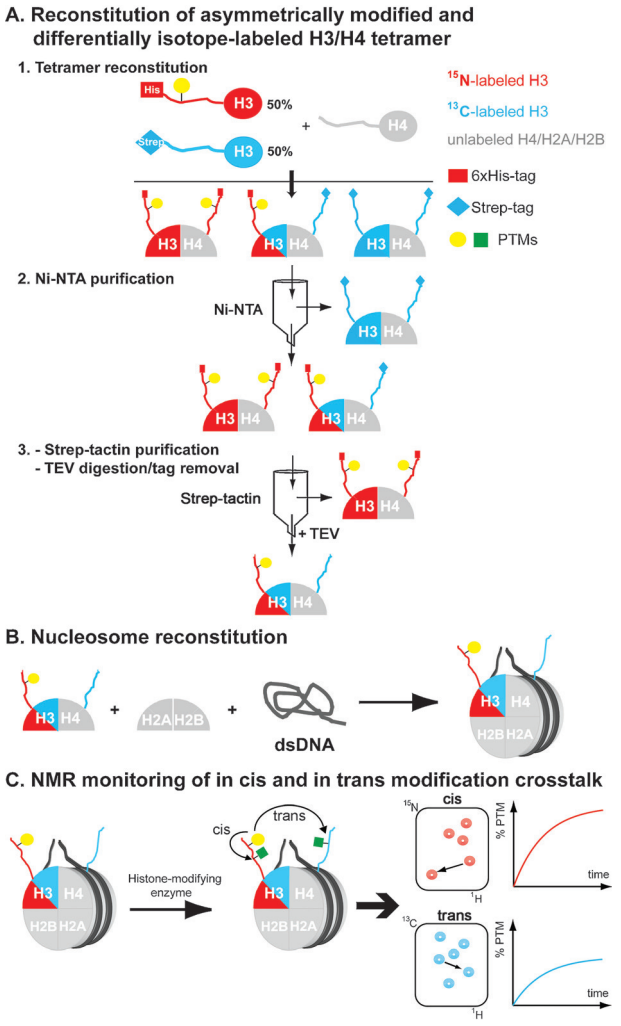
reacting one of the two pools with a specific enzyme. Additionally, we recombinantly expressed and purified the two H3 pools from either ^{15}N or ^{13}C isotope-enriched growth media. After following standard methods,[9] we reconstituted H3/H4 tetramers and subsequently asNucs with unlabeled histones H2A, H2B, H4, and DNA (Scheme 1 a, b). Next, we reacted these asNucs with H3-modifying enzymes, followed by time-resolved NMR monitoring of the ^{15}N or ^{13}C "channel" to report on cis or trans modification crosstalk, respectively (Scheme 1 c).

To exemplify the power of this approach in delineating subtle differences on copy-specific histone modification rates, we studied acetylation of H3K14 (to give H3K14ac) by the acetyltransferase Gcn5 in the absence or presence of H3 S10 phosphorylation, which has previously been shown to enhance Gcn5 activity both in vitro[8] and in vivo.[10] To this



Scheme 1. Reconstitution of asymmetrically modified and isotope-labeled nucleosomes for NMR-monitoring of asymmetric modification patterns. A) Tandem affinity purification with Ni–NTA and Strep–tactin chromatography of asymmetrically modified and differentially isotope-labeled H3/H4 tetramers (asH3/H4). B) Nucleosome reconstitution by combining asH3/H4 with unlabeled H2A/H2B dimers and a DNA nucleosome-positioning sequence. C) Mixing of asNucs with histone-modifying enzymes enables NMR-monitoring of modification crosstalk in cis and in trans.

end, we reconstituted asNucs phosphorylated on S10 of histone H3. Additionally, we incorporated different isotopes (the phosphorylated H3S10ph species was ^{15}N-labeled and the unmodified species ^{13}C-labeled). To prepare fully phosphorylated H3 on S10, we reacted the ^{15}N-labeled H3 with Aurora B kinase and assessed complete phosphorylation by following the characteristic shift of the S10 amide NMR signal (Figure S1 and the Methods section in the Supporting Information). We used this H3 pool to initially reconstitute asymmetric H3/H4 tetramers. To verify that the tandem purification scheme resulted in highly pure asymmetric complexes, we took advantage of the only tryptophan that exists in the system, which comes from the Strep-tag, since histones lack this amino acid. We followed the characteristic NMR resonance of the tryptophan side chain NHε throughout the purification process, assessing the presence of the Strep-tag and hence the ^{13}C-labeled H3 copy. Indeed, pure asymmetric H3/H4 tetramers were isolated, as concluded after comparing the proton (^{1}H) NMR spectra of H3/H4 tetramer pools from different purification phases (Figure S2). The latter were mixed with H2A/H2B dimers and a 165 bp-long DNA fragment containing a strong nucleosome-positioning sequence, and by using the gradient-dialysis method,[9] we reconstituted asNucs phosphorylated on H3S10 (Figure S3). Subsequently, we reacted this preparation with Gcn5 and monitored H3K14ac on both H3 tails by recording interleaved $^{1}H/^{15}N$ and $^{1}H/^{13}C$ correlation spectra (Figure 1 a,b and the Methods section in the Supporting Information). We followed H3K14ac on Ser10-phosporylated, ^{15}N isotope-enriched H3 by monitoring the characteristic chemical shift changes of the K14 amide signal, whereas $CH_2\varepsilon$ resonance displacements reported on K14ac of the unmodified, ^{13}C-enriched copy of nucleosomal H3. The latter was deduced from a test acetylation reaction with a free H3 tail peptide (Figure S4). By comparing the K14ac levels over time on individual copies of H3, we confirmed that H3S10ph exerted a clear stimulatory effect on the Gcn5 reaction at K14 in cis (Figure 1 c).

To further demonstrate the flexibility of the employed labeling approach, we performed the same experiment, but this time having prephosphorylated the ^{13}C-enriched H3, using the Cβ group of S10 to assess efficient phosphorylation (Figure S5 and Note in the Supporting Information). Accordingly, we reconstituted the asNucs and re-determined the level of K14ac formation upon Gcn5 treatment. As before, kinetic analyses with these nucleosomes showed faster acetylation of the S10 prephosphorylated H3 copy (Figure S6), thus confirming the stimulatory role of S10ph on Gcn5 activity in cis compared to in trans. This effect is nucleosome-specific since Gcn5 processes both unmodified and H3S10ph tail peptides similarly[5] and is directly linked to the influence that charge-modulating PTMs have on electrostatic H3 tail/DNA interactions. Particularly, H3S10ph disrupts the aforementioned transient contacts and hence promotes K14ac formation by rendering H3 tail more accessible to Gcn5.[8]

Having established the functionality of our approach, we set out to test its applicability to another important histone modification, namely lysine methylation. We focused on

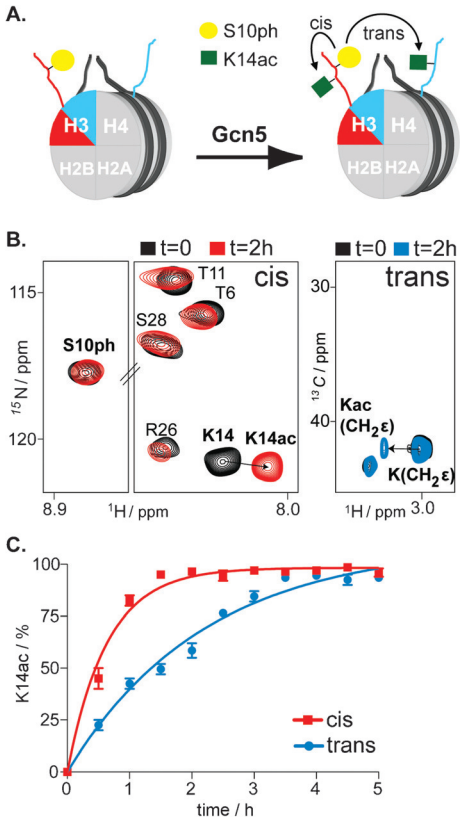


Figure 1. Modification crosstalk imposed in cis and in trans by H3S10ph on the H3K14 acetylation activity of Gcn5. A) Schematic illustration of asymmetrically phosphorylated on H3S10 nucleosomes reacted with the Gcn5 acetyltransferase enables H3K14ac mapping in cis and in trans. B) $^{1}H/^{15}N$ SOFAST-HMQC and $^{1}H/^{13}C$-HSQC spectra (selected regions) of asNucs before and after reaction with Gcn5. C) Time-resolved NMR readouts of H3K14 acetylation by Gcn5 on H3S10ph asNucs.

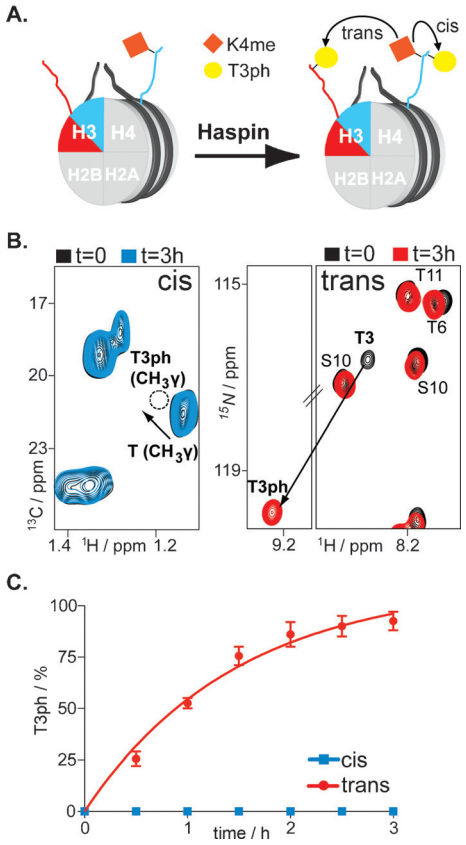


Figure 2. Modification crosstalk imposed in cis and in trans by H3K4me on the H3T3 phosphorylation activity of Haspin. A) Schematic illustration of asymmetrically methylated H3K4 nucleosomes reacted with Haspin kinase enables H3T3ph mapping in cis and in trans. B) $^{1}H/^{15}N$ SOFAST-HMQC and $^{1}H/^{13}C$ HSQC spectra (selected regions) of asNucs before and after reaction with Haspin kinase. C) Time-resolved NMR readouts of H3T3 phosphorylation by Haspin on H3K4me asNucs.

methyl/phospho crosstalk between the adjacent T3 and K4 on histone H3. H3T3ph is a characteristic PTM of mitotic chromatin.[11] The methylated H3K4me species has been correlated with promoters of active genes,[12] even though certain methylated states of H3K4 have also been mapped in centromeric chromatin,[13] with their levels persisting during mitosis.[14] We reacted ^{13}C-labeled histone H3 with the SET7/9 methyltransferase, which specifically methylates K4.[15] By following the corresponding $CH_2\varepsilon$ peak of lysines, we monitored efficient H3K4 monomethylation (Figure S7). Subsequently, by using an unmodified/^{15}N-labeled His-H3 pool, we reconstituted asNucs on H3K4me1 (Figure S8). We reacted the latter with the Haspin kinase, which specifically phosphorylates H3T3.[11] We monitored efficient phosphorylation of the unmodified copy of H3, whereas we detected no modification of the K4-methylated sister histone (Figure 2 Note in the Supporting Information). In the latter case, H3K4me1 completely abolished Haspin activity. This behavior was similarly displayed with isolated H3 tail peptides (Figure S9). Thus, the observed negative regulation resulted from an effect of H3K4me1 on Haspin's catalytic activity or substrate recognition and was not imposed by the nucleosomal environment, as was the case with Gcn5 and the H3S10ph/K14ac crosstalk. Even though we could not test the effect of higher H3K4 methylation states owing to inefficient activity of SET7/9 to produce relevant substrates, it is fair to expect a similar inhibitory effect in those cases as well. Interestingly, asymmetrically modified H3K4me3 nucleosomes have been found in embryonic stem cells, and their levels were partially retained upon differentiation.[6] Based on our data, if these nucleosomes encounter Haspin during mitosis, they will become asymmetrically modified with regard to H3T3ph as well. Do such nucleosomes containing two different types of PTMs (K methylation and T phosphorylation), albeit on different sister histones, exist in native chromatin? Immunoaffinity purification coupled with quantitative LC/MS analysis[6] of mitotic extracts could initially identify such species before setting out to address their biological role.

In summary, we introduce an approach to selectively detect and monitor asymmetric modification states of individual histone copies in fully assembled nucleosomes. By employing differential isotope-labeling and site-selective incorporation of histone PTMs, asymmetrically modified nucleosomes can be generated for time-resolved studies to

address PTM crosstalk in cis and in trans. We used a well-characterized crosstalk example to test the functionality of our approach. Additionally, we showed that, under certain conditions, Haspin kinase can generate asymmetrically phosphorylated and methylated nucleosomes. While we have outlined the applicability of our method for monitoring PTM reactions on individual sites of histone H3, this could be extended to other histones or combinations of different histones. We analyzed crosstalk involving the most common PTMs (S/T phosphorylation, K acetylation/methylation), but we have already delineated the NMR characteristics of equally or less abundant PTMs, such as arginine methylation, lysine crotonylation/propionylation, and tyrosine phosphorylation.[16] Of outmost importance for the utility of our approach is the ability to prepare specifically and quantitatively modified histones that can be used for asNucs reconstitution. In cases where histone modifications cannot be established enzymatically, alternative schemes for the specific incorporation of modified amino acids can be employed, including the use of nonnatural amino acids[17] or the use of cysteine-mutant-derived PTM mimetics (histones are devoid of cysteine residues).[18] In addition, heteromeric DNA ligation of two mononucleosomes can be exploited to construct dinucleosome templates that can be used to analyze internucleosomal effects.[19] Currently, limited data are available and only few PTMs have been found to occur asymmetrically in vivo, but many more are expected to come in the near future. At the same time, new chemical tools are emerging for the reconstitution of asymmetrically modified nucleosomes to be used for functional studies.[20] Into this highly growing field, our approach aims to uncover mechanistic facets on the establishment and propagation of asymmetrically modified nucleosomes.

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