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Traceless Synthesis of Asymmetrically Modified Bivalent Nucleosomes

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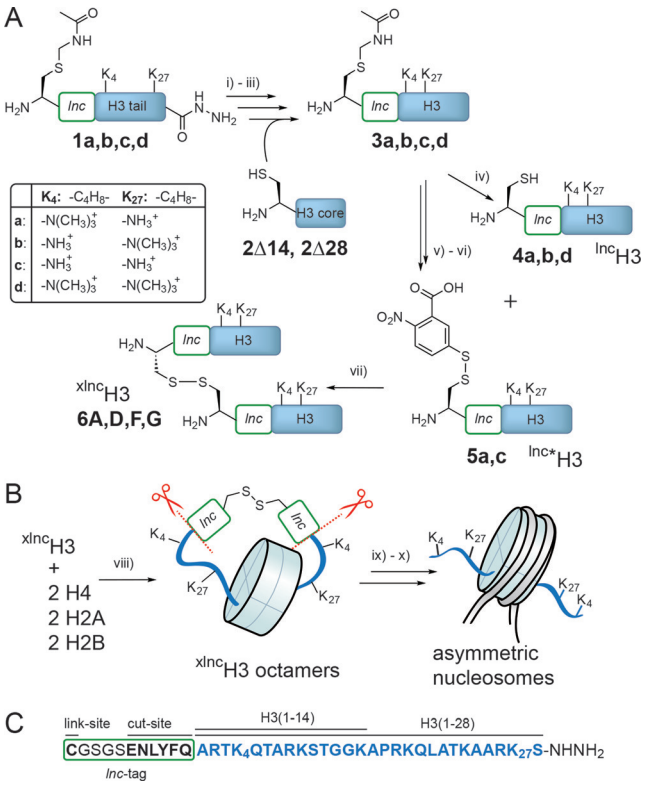
Abstract: *Nucleosomes carry extensive post-translational modifications (PTMs), which results in complex modification patterns that are involved in epigenetic signaling. Although two copies of each histone coexist in a nucleosome, they may not carry the same PTMs and are often differently modified (asymmetric). In bivalent domains, a chromatin signature prevalent in embryonic stem cells (ESCs), namely H3 methylated at lysine 4 (H3K4me3), coexists with H3K27me3 in asymmetric nucleosomes. We report a general, modular, and traceless method for producing asymmetrically modified nucleosomes. We further show that in bivalent nucleosomes, H3K4me3 inhibits the activity of the H3K27-specific lysine methyltransferase (KMT) polycomb repressive complex 2 (PRC2) solely on the same histone tail, whereas H3K27me3 stimulates PRC2 activity across tails, thereby partially overriding the H3K4me3-mediated repressive effect. To maintain bivalent domains in ESCs, PRC2 activity must thus be locally restricted or reversed.*

Nucleosomes form the basic unit of chromatin and are composed of two copies each of histones H2A, H2B, H3 and H4, around which a 147 base pair (bp) fragment of DNA is wrapped. All histones are subject to PTMs, which confer chromatin regulation and epigenetic signaling.[1] Nucleosomes have two pseudo-symmetrical halves, each composed of one copy of each histone.[2] Individual histone copies in a nucleosome may be differentially modified, thereby establishing a PTM-based asymmetry. Indeed, asymmetric nucleosomes have been detected by mass spectrometry,[3] as well as genome-wide mapping studies in several cell types.[4]

In embryonic stem cells (ESCs), an asymmetric chromatin signature could be tied to biological function. The two PTMs H3K4me3 and H3K27me3 are associated with active and repressed genes, respectively, and are deposited by specific lysine methyltransferases (KMTs). The mixed-lineage leukemia (MLL) family of KMTs is specific for H3K4, whereas polycomb repressive complex 2 (PRC2) methylates H3K27.[5] Owing to their antagonistic function, the two PTMs are found in distinct genomic regions.[6] In ESCs, as well as in some cancer cells, the two PTMs can coexist on a subset of promoters, forming "bivalent" chromatin domains.[3,7] Intriguingly, bivalent nucleosomes were found to be asymmetric for H3K4me3 and H3K27me3 (trans-bivalent).[3] It is not clear how such asymmetric patterns are established and maintained, however, a deeper investigation of the involved KMTs may provide important insight.

To uncover the biochemical functions of asymmetric chromatin, chemically defined nucleosomes are required. Previous methods to generate PTM asymmetry were based on statistical histone octamer assembly from histone mixtures. Subsequently, asymmetric species were enriched through successive affinity pulldown steps with orthogonal peptide tags.[3,8] Owing to the preparative difficulty, only nucleosomes containing single-modified histones were assembled. In addition, remaining affinity tags may potentially interfere with biological assays. By using such tagged and affinity-enriched nucleosomes, PRC2 was found to be inhibited by



Scheme 1. Traceless chemical synthesis of asymmetrically modified nucleosomes. A) General synthetic route for the preparation of asymmetrically modified ^{xlnc}H3 dimers. i) Conversion of lnc-peptide hydrazide into the TFET thioester; ii) ligation of the lnc-peptide thioester to truncated H3 protein, iii) in situ desulfurization; iv) removal of Acm at the N-terminal cysteine or v, vi) Acm removal and in situ modification of cysteine with DTNB; vii) formation of the heterodisulfide. B) viii) Refolding of histone octamers using ^{xlnc}H3 dimers; ix) reconstitution of nucleosomes and release of xlnc; x) treatment of nucleosomes with TEV protease to remove non-native lnc peptide sequences. C) Design of lnc-peptides for the chemical synthesis of crosslinkable H3 proteins.

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H3K4 methylation.[3] By contrast, pre-existing H3K27me3 was found to stimulate the catalytic activity of the PRC2 complex.[3,9] To extend chromatin analysis to the bivalent state, novel methods are required that allow direct chemical control over the supramolecular assembly of nucleosomes while leaving no unnatural peptides or other traces in the final histones. Herein, we report a traceless and modular method to synthesize chemically pure asymmetrically modified nucleosomes containing arbitrary combinations of PTMs. We then applied this approach to generate a nucleosome library to probe the ability of PRC2 to modify and maintain bivalent chromatin.

We envisioned a transient crosslinking strategy for two H3 molecules carrying distinct PTMs, which would force their assembly into the same histone octamer and subsequently into an asymmetric nucleosome. Furthermore, all residues required for crosslink formation would need to be removable to ensure a traceless procedure (Scheme 1 A,B). These considerations led us to the design of the "link and cut" tag (lnc-tag), which carries a cysteine residue as well as a tobacco etch virus (TEV) protease site (Scheme 1 C). Both the cysteine-protected lnc-tag and the desired PTMs are incorporated into H3 through expressed protein ligation (EPL), followed by desulfurization to generate the native H3 sequence.[10] Deprotection of the cysteine (to yield ^{lnc}H3) and activation of the lnc-tag in a subset of H3 molecules (^{lnc}*H3) allows combinatorial assembly of heterodisulfides, which are used to generate histone octamers. Asymmetric nucleosomes devoid of any synthetic scars are then finally produced through proteolytic removal of the lnc-tag.

We thus set forth to synthesize lnc-tag-modified PTM-carrying N-terminal H3 peptides as C-terminal hydrazides (**1a–d**),[11] which are converted to thioesters in situ through oxidation with sodium nitrite followed by thiol addition.[12] For protecting the critical cysteine in the lnc-tag during desulfurization, we employed an *S*-acetamidomethyl (Acm) protecting group. We thus synthesized Cys(Acm)-lnc-H3(1–14)K4me3 (**1a**) on a hydrazine resin (Figure 1 A). We then generated Cys(Acm)-lnc-H3K4me3 (**3a**) in a one-pot ligation and desulfurization reaction[13] by first converting the peptide hydrazide into a trifluoroethanethiol (TFET) thioester (**1a′**) under denaturing conditions. Ligation was initiated by the addition of truncated H3(Δ1-14), which carries an N-terminal cysteine as well as the commonly used C110A mutation (**2Δ14**), and proceeded to completion within 3 h at room temperature. Subsequent desulfurization and HPLC purification produced **3a** in good yield (51 %, see the Supporting Information).

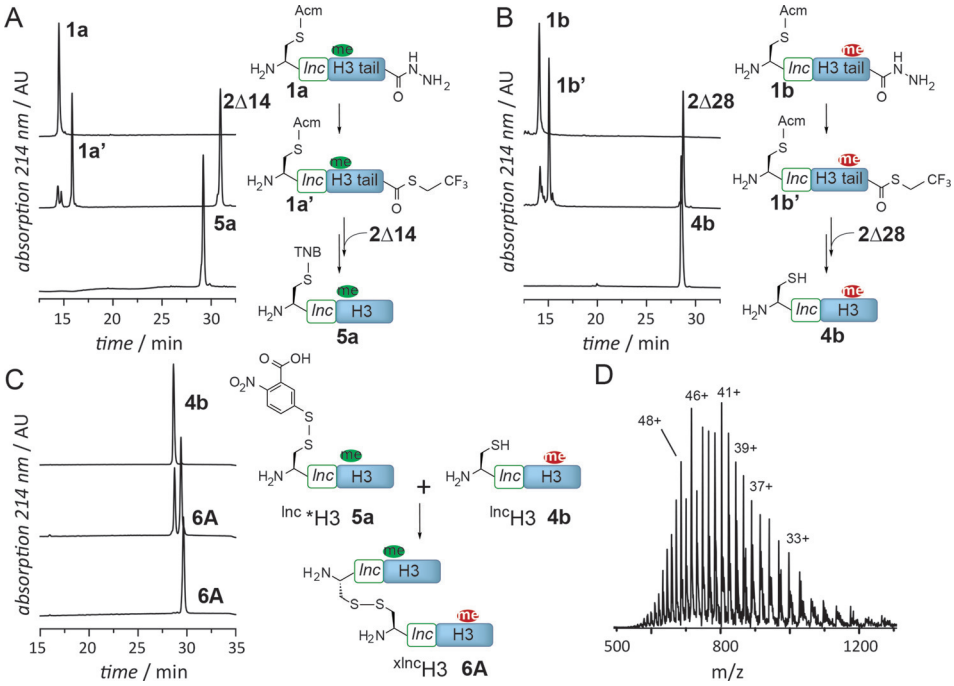

Figure 1. Synthesis of ^{xlnc}H3 **6A**. A) Preparation of ^{lnc}*H3K4me3 (**5a**) by one-pot ligation/desulfurization, followed by activation of the N-terminal cysteine using DTNB. B) Preparation of ^{lnc}H3K27me3 (**4b**) by one-pot ligation/desulfurization followed by removal of Acm. C) Conversion of **5a** and **4b** into ^{xlnc}H3K4me3/H3K27me3 (**6A**). D) MS analysis of **6A** (calc. MW: 32874 Da, observed MW: 32877 ± 4 Da).

Silver acetate (AgOAc)-mediated deprotection of the N-terminal cysteine residue followed by same-pot modification with 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) yielded activated ^{lnc}*H3K4me3 (**5a**), the first member of our lnc-tag histone library (yield 73 %). By following a similar procedure but omitting the final activation of cysteine with DTNB, we synthesized ^{lnc}H3K27me3 (**4b**) from Cys(Acm)-lnc-H3(1–28)K27me3 (**1b**) and H3(Δ1–28) (**2Δ28**; Figure 1 B). With ^{lnc}*H3K4me3 (**5a**) and ^{lnc}H3K27me3 (**4b**) in hand, we proceeded to form the heterodisulfide-linked H3 dimer. **4b** was added to **5a** (in 20 % excess) under denaturing and slightly acidic conditions, which resulted in the rapid formation of ^{xlnc}H3K4me3/H3K27me3 (**6A**; Figure 1 C, 30–40 % yield of purified product), which was stored as lyophilized product until further use. Taken together, we have developed an efficient convergent synthesis approach by maximizing one-pot reactions and reducing HPLC purification steps.

We then assembled trans-bivalent histone octamers from **6A** by following standard procedures,[14] including final purification by gel-filtration chromatography (Figure 2 A). As judged by the symmetrical gel-filtration elution profile and SDS-PAGE, the produced octamers were homogenous, with each containing one ^{xlnc}H3 molecule. At this point, the disulfide crosslink was still intact but could be readily opened with a reducing agent (Figure 2 B). Refolded octamers were kept oxidized and stored in 50 % glycerol at −20 °C until further use.

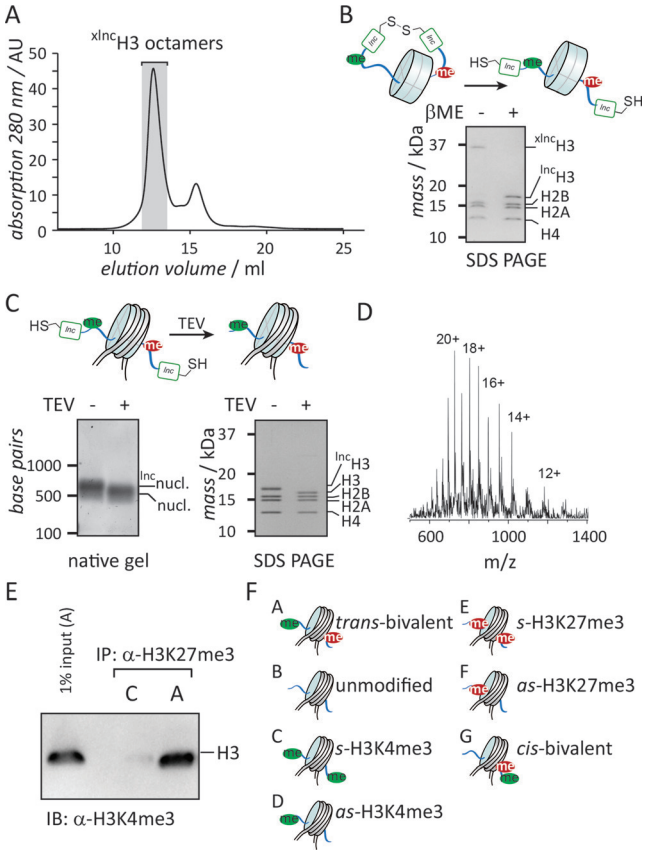

Figure 2. Generation of asymmetric nucleosomes. A) Refolding of asymmetric octamers by using **6A**, and purification by gel-filtration chromatography. Fractions within the shaded region are combined, and the peak at 16 mL corresponds to H2A–H2B dimers (added in slight excess). B) Reduction of the disulfide crosslink, as analyzed by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. C) TEV protease treatment to yield native asymmetric trans-bivalent nucleosomes (**A**) as analyzed by native agarose gel electrophoresis and Gel Red staining, as well as SDS-PAGE and CBB staining. D) MS analysis of H3K4me3/H3K27me3 in the final trans-bivalent nucleosomes (calc. MW: 15252 Da, obs. MW: 15249 ± 5 Da). E) IP of the indicated nucleosomes, showing the bivalent state of **A**. F) The nucleosome library for enzymatic assays.

Finally, we were in a position to reconstitute trans-bivalent nucleosomes. We mixed **6A**-containing octamers with a 153 bp fragment of the "601" nucleosome-positioning DNA sequence[15] and dialyzed gradually from high-salt (1.4 M KCl) to low-salt (10 mM KCl)conditions. ^{xlnc}H3 may remain oxidized until the nucleosomes are stably formed. After reduction with 1 mM dithiothreitol (DTT) and addition of TEV protease, trans-bivalent nucleosomes (**A**) were recovered (Figure 2C). We subsequently analyzed the nucleosomes by mass spectrometry and found the expected mass of H3K4me3/H3K27me3 (15252 Da; Figure 2D). In addition, we confirmed the trans-bivalent modification state by immunoprecipitation (IP) with an antibody against H3K27me3, followed by H3K4me3 detection by immunoblotting (Figure 2E).

Having established a robust synthetic method, we generated a library of symmetric and asymmetric nucleosomes suitable for investigating PRC2 activity on bivalent chromatin domains (Figure 2F). In particular we generated nucleosomes carrying no modification (**B**), H3K4me3 on both (symmetric, s-H3K4me3, **C**) or solely on one H3 molecule (asymmetric, as-H3K4me3, **D**). Similarly, we assembled nucleosomes that are symmetric (s-H3K27me3, **E**) or asymmetric (as-H3K27me3, **F**) in H3K27me3. Finally, we generated cis-bivalent nucleosomes carrying both K4me3 and K27me3 on the same H3 tail (**G**).

We then performed methyltransferase assays with recombinant PRC2, employing ^{3}H-*S*-adenosyl methionine (^{3}H-SAM) for detection of methylation. PRC2 activity was found to be completely ablated by s-H3K4me3 (**C**) and reduced by 50% on as-H3K4me3 (**D**) compared to unmodified nucleosomes (**B**; Figure 3A,C). Similarly, PRC2 did not methylate s-H3K27me3 (**E**), thus demonstrating its selectivity for H3K27. In contrast, as-H3K27me3 (**F**) demonstrated stimulation of PRC2 activity.

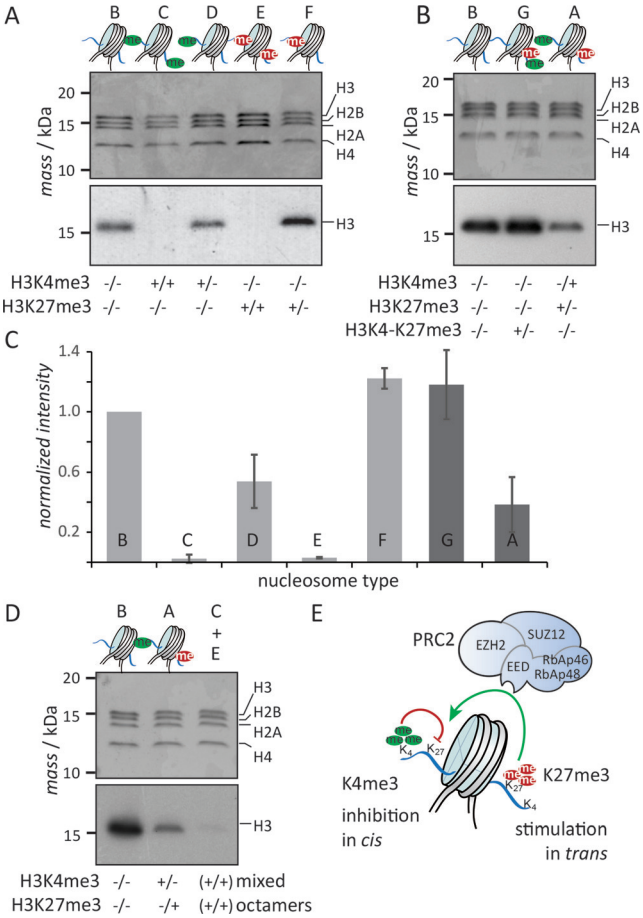

Figure 3. Regulation of PRC2 in asymmetric bivalent nucleosomes. A) The indicated symmetric or asymmetric nucleosomes were used as substrates for PRC2 methyltransferase assays with ^{3}H-SAM. Histones were separated by SDS-PAGE, stained with CBB (upper panel), and probed for ^{3}H-methyl incorporation by fluorography (lower panel). B) As in (A) but using trans- or cis-bivalent nucleosomes. C) Densitometry quantification of ^{3}H incorporation over multiple experiments ($n = 3$–4). D) As in (A) but using either trans-bivalent nucleosomes or nucleosomes prepared from a 1:1 mix of symmetric H3K4me3- or H3K27me3-containing octamers. E) Model for PRC2 regulation (for details see text).

Mechanistic insight could be gleaned from bivalent nucleosomes (Figure 3B,C). Firstly, PRC2 exhibited increased activity on cis-bivalent nucleosomes (**G**), in spite of the presence of the inhibitory H3K4me3 mark. The inhibitory effect of H3K4me3 thus does not extend to its partner H3 molecule in a nucleosome. By contrast, trans-bivalent nucleosomes (**A**) showed low but detectable activity for PRC2. Hence, the stimulatory function of H3K27me3 across the nucleosome can partially override the local inhibition of H3K4me3. Importantly, nucleosomes assembled from a mixture of s-H3K4me3 and s-H3K27me3 octamers showed no measurable PRC2 activity (Figure 3D). This finding shows that 1) during nucleosome assembly no histone exchange occurs between octamers; 2) nucleosomes remain stable during the enzymatic assays and, 3) stimulation of PRC2 by H3K27me3 does not occur in trans between individual nucleosomes. Taken together, these results demonstrate that within a nucleosome, H3K4me3 blocks PRC2 activity only in cis, that is, on the same H3 molecule. In contrast, H3K27me3 has a long-range effect, presumably owing to PRC2 recruitment and allosteric enzyme activation (Figure 3E).

In summary, we have developed a modular and traceless method to reconstitute asymmetrically modified nucleosomes from transiently linked H3 precursor molecules. These asymmetrically modified nucleosomes have wide applicability in epigenetic research, for example, in mechanistic or high-throughput in vitro studies of histone PTM crosstalk[16] or in quantitative chromatin immunoprecipitation procedures.[17] We used a library of asymmetric nucleosomes to study the mechanisms of PRC2 regulation by the histone PTM combination H3K4me3 and H3K27me3. Together, these results indicate that pre-existing H3K4me3 on one histone tail inhibits PRC2 locally, but this inhibitory effect can be partially overridden by nearby H3K27me3. We thus speculate that bivalent domains have to be constantly maintained in ESCs by restricting PRC2 activity or local reversal of H3K27 methylation.

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Keywords: bivalent domains · chromatin · epigenetics · expressed protein ligation · PRC2

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