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LETTER TO THE EDITOR

A systematic evaluation of the compatibility of histones containing methyl-lysine analogues with biochemical reactions

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Dear Editor,

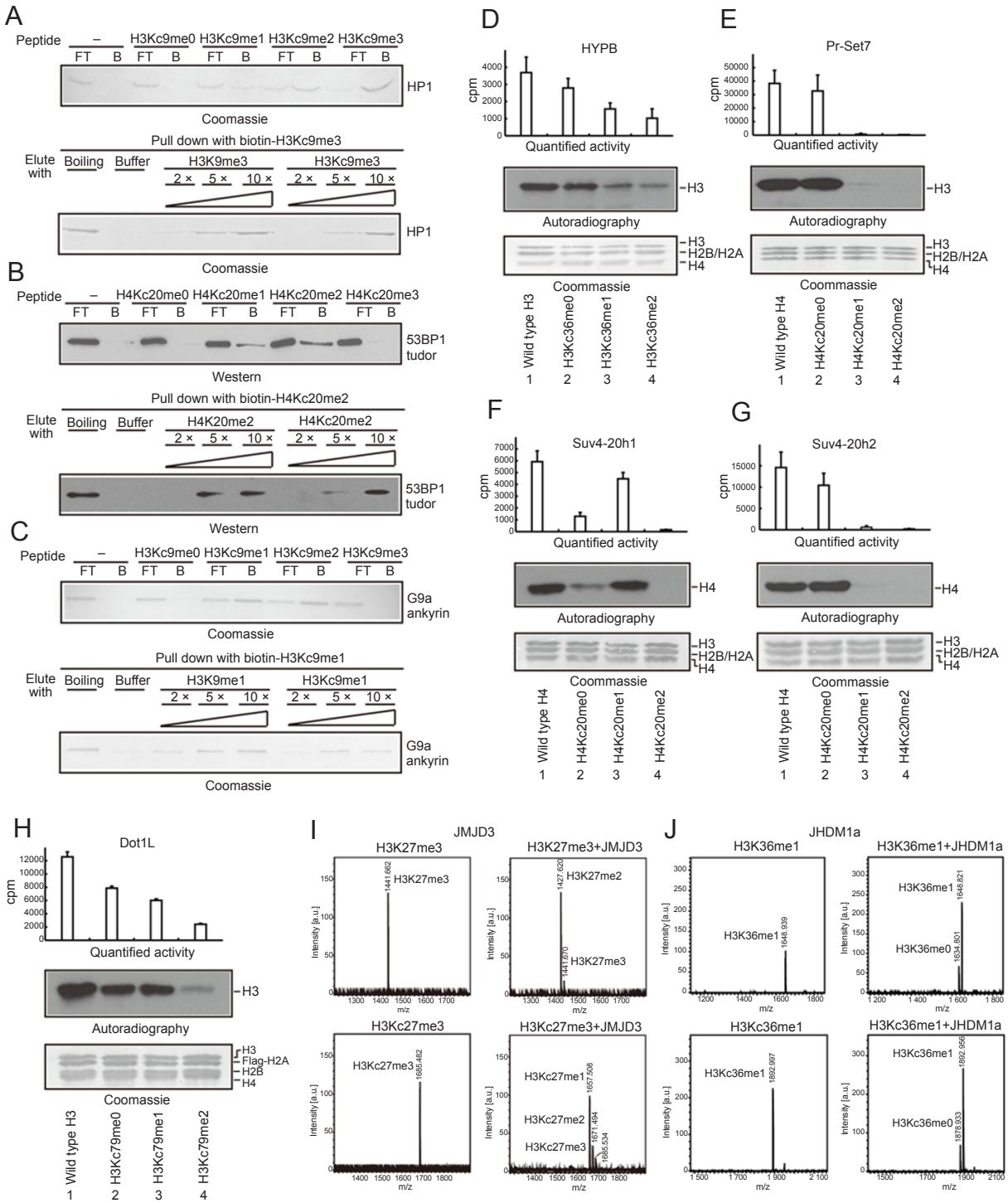
Histone lysine methylation has received a great deal of attention from the chromatin field over the past 10 years. To date, histone lysine methylations have been demonstrated to play pivotal roles in nearly all biological processes involving chromatin, including replication, transcription, DNA repair *etc.* [1]. One major challenge in the field has been that of obtaining homogenously methylated histones, i.e., histones methylated at a specific lysine residue with a particular methyl status. Such histone materials could be of important use in many functional applications. In 2007, Shokat's lab invented an easy, robust alkylating reaction (Shokat's reaction) that chemically installs specific methyl-lysine analogues (MLAs) onto specific histone residues [2]. It is a very promising technology that may revolutionise our understanding of methylated histone lysine-mediated biological functions. However, Shokat's reaction is yet to be widely adopted by biologists. Some researchers may worry about the compatibility of the "pseudo lysine" generated by Shokat's reaction with subsequent biochemical assays. Shokat's reaction product carries an *N*-methylated aminoethylcysteine (Kc or "pseudo lysine"), which replaces the γ-methylene with a sulphide, resulting in a slight lengthening of the side chain (0.28 Å) and a small increase in acidity (−1.1 pKa unit) [2]. Here, we report a systematic evaluation of the products of Shokat's reaction in various biochemical assays, using different classes of methylated histone-binding proteins, histone lysine methyltransferases (HKMTs) and histone demethylases.

Recognition of methylated histones by effector proteins (or "readers") is critical for the biological function of histone methylation. Thus, it is important to test whether the artificially "methylated" histones generated by MLA technology can still be recognised by their concomitant methylated histone-binding proteins. In the initial report of MLA technology, HP1α displayed binding activity towards H3Kc9me2 (H3 with its amino acid No. 9 converted to *N*-dimethyl-aminoethylcysteine or "pseudo" dimethyl-lysine) [2]. We repeated this experiment with all four methyl statuses (me0/1/2/3). Indeed, HP1α bound "methylated" H3Kc9, but not its unmethylated form (Figure 1A, upper panel). Moreover, it preferentially bound the trimethyl and dimethyl forms (Figure 1A, upper panel), which is a characteristic feature of HP1's interaction with native methylated H3K9. In a competition experiment, both H3K9me3 and H3Kc9me3 peptides were capable of eluting HP1α protein bound by biotin-labelled H3Kc9me3 peptide at similar concentrations (Figure 1A, lower panel).

We then tested other domains that recognise methylated histones. 53BP1, the mammalian homologue of the fission yeast protein Crb2, specifically binds methylated H4K20 through its tudor domain [3]. Interestingly, 53BP1 cannot bind H4K20me3 due to structural hindrance [3]. In our peptide pull-down assay, recombinant 53BP1 tudor domain recognised MLA-generated H4Kc20me1 and H4Kc20me2, but not the trimethyl or unmethylated forms (Figure 1B, upper panel). In a competition experiment, a slightly higher concentration of H4Kc20me2 peptide was required to elute bound 53BP1 (Figure 1B, lower panel), suggesting that 53BP1 has slightly lower binding affinity towards H4Kc20me2 peptide compared with the authentic H4K20me2 peptide. Recently, G9a and GLP were demonstrated to bind methylated H3K9 through a new methylated histone recognition domain, ankyrin repeats [4]. We thus analysed the G9a ankyrin repeats in the peptide pull-down assay. H3Kc9me1 and H3Kc9me2 were capable of being recognised by the recombinant G9a ankyrin repeats, but not by the trimethyl or unmethylated forms (Figure 1C, upper panel). This methyl status specificity is consistent with the previous report on native methylated peptides [4]. In addition,

G9a ankyrin repeats displayed comparable binding affinity towards H3K9me1 and H3Kc9me1 peptides in a competition experiment (Figure 1C, lower panel).

The above experiments demonstrated the compatibility of MLA products with methylated histone-binding proteins in binding assays that do not involve any enzymatic reaction. Therefore, we extended our evaluation of MLA products to enzymatic reactions that directly use methylated histones as substrates. In the initial report of MLA technology, the founding member of the HKMT family, Suv39h1, displayed comparable activity towards MLA-generated H3 tail peptide versus



regular H3 tail peptide [2]. Here, we analysed four different SET domain-containing HKMTs and one non-SET domain HKMT, Dot1L. Moreover, reconstituted nucleosomes were used as substrates instead of the histone tail peptides because many HKMTs are nucleosome-specific enzymes that do not react on histone peptides or core histones [1].

We first evaluated HYPB, an H3K36-specific HKMT. Recombinant HYPB SET domain displayed similar activity towards nucleosomes containing H3Kc36me0 (Figure 1D, lane 2) and nucleosomes containing wild-type H3 (Figure 1D, lane 1). This activity gradually declined when H3Kc36me1- and H3Kc36me2-containing nucleosomes were tested (Figure 1D, lanes 3-4), which is consistent with the fact that HYPB is a trimethylase [5, 6].

We then systematically analysed the three H4K20-specific HKMTs, Pr-Set7 (also known as Set8), Suv4-20h1 and Suv4-20h2. In all three cases, MLA products were able to serve as substrates for the methylation reaction (Figure 1E-1G). Pr-Set7 displayed minimal activity towards H4Kc20me1 (Figure 1E, lane 3), consistent with the fact that Pr-Set7 is a mono-methylase [1]. To our surprise, despite the requirement of Suv4-20 family HKMTs for H4K20me3 *in vivo* [7], neither Suv4-20h1 nor Suv4-20h2 displayed detectable activity towards H4Kc20me2 (Figure 1F, lane 4; Figure 1G, lane 4). This is unlikely to be due to the MLA reaction, because both enzymes were capable of methylating H4Kc20me0/1 (Figure 1F, lanes 2-3; Figure 1G, lane 2). This implies that additional factor(s) might exist that regulate the methyl status specificity of Suv4-20 family HKMTs, especially that of Suv4-20h2, because it is the main enzyme responsible for maintaining H4K20me3 levels *in vivo* [7]. This is an intriguing hypothesis because, unlike the global distribution of H4K20me2 and the Suv4-20 family HKMTs [7], H4K20me3 is specifically enriched at pericentric heterochromatin [1], which suggests a requirement for a methyl status regulatory mechanism. Notably, the methyl status specificity of certain HKMT complexes has been shown to be regulated by regulatory subunits [8]. It is also worth noting that Suv4-20h1 displayed robust activity towards H4Kc20me1 (Figure 1F, lane 3), unlike Suv4-20h2 (Figure 1G, lane 3). This implies that Suv4-20h1 may favour the H4K20me1 generated by Pr-Set7 as its substrate, whereas Suv4-20h2 may prefer to directly methylate unmethylated H4. Next, we tested Dot1L (the human homologue of yeast Dot1), which is the only known non-SET domain HKMT that specifically methylates H3K79 [1]. Once again, nucleosomes with MLAs could serve as substrates for Dot1L (Figure 1H).

In the previous section, we have shown the ability of HKMTs to add new methyl groups onto MLA products (Figures 1D-1H). Next, we assessed whether histone demethylases can remove the methyl groups from MLA products. JMJD3 is an H3K27-specific KDM that reacts with trimethylated substrate [9]. Incubation with recombinant JMJD3 effectively demethylated peptides containing H3K27me3 (Figure 1I, upper panel) as well as MLA-generated H3Kc27me3 (Figure 1I, lower panel). In addition, the H3K36-specific demethylase JHDM1a [10] effectively demethylated H3K36me1 (Figure 1J, upper panel) and H3Kc36me1 peptides (Figure 1J, lower panel). Our results collectively indicate that MLA products can serve as substrates for histone demethylases.

We have thus systematically evaluated the biochemical reaction compatibility of MLA products in different systems, including methylated histone-binding assays, histone methyltransferase assays and demethylase assays. The products of Shokat's reactions served as substrates for all the above biochemical reactions and retained

Figure 1 MLA products are compatible with various biochemical reactions. **(A-C)** MLA products are compatible with methylated histone-binding proteins. Upper panels, peptide pull-down assays. Lower panels, competition assays. FT: 10% of total flow through. B: 100% of bound material eluted from the magnetic streptavidin beads. **(A)** Recombinant HP1 binds MLA products with correct methyl status specificity. **(B)** Recombinant 53BP1 tudor domain binds the MLA products with correct methyl status specificity. **(C)** Recombinant G9a ankyrin repeats bind the MLA products with correct methyl status specificity. **(D-H)** MLA-containing nucleosomes are compatible with various HKMTs. Bottom panels show the coomassie blue-stained membranes for nucleosome loading; middle panels are autoradiography results for the HKMT activity assay; upper panels are quantified data obtained by liquid scintillation counting, the standard deviations were obtained from three parallel experiments. **(D)** HYPB. **(E)** Pr-Set7. **(F)** Suv4-20h1. **(G)** Suv4-20h2. **(H)** Dot1L. **(I-J)** MLA products are compatible with histone demethylases. **(I)** MALDI-TOF mass spectrometry analysis of H3K27me3 peptide before (upper left panel) and after incubation with recombinant JMJD3 (upper right panel); MALDI-TOF mass spectrometry analysis of H3Kc27me3 peptide before (lower left panel) and after incubation with recombinant JMJD3 (lower right panel). **(J)** MALDI-TOF mass spectrometry analysis of H3K36me1 peptide before (upper left panel) and after incubation with recombinant JHDM1a (upper right panel); MALDI-TOF mass spectrometry analysis of H3Kc36me1 peptide before (lower left panel) and after incubation with recombinant JHDM1a (lower right panel).

excellent substrate specificity in most cases, demonstrating their great potential as a powerful tool for histone lysine methylation studies. We hope that our results will encourage more researchers to incorporate Shokat's reaction into their studies.

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Abbreviations: MLA (methyl-lysine analogue); HKMT (histone lysine methyltransferase); KDM (Lysine demethylase); Kc ("pseudo lysine" or aminoethylcysteine); H3Kc9me2 (H3 with its amino acid No. 9 converted to N-dimethyl-aminoethylcysteine or "pseudo" dimethyl-lysine)

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