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Expanding Cofactor Repertoire of Protein Lysine Methyltransferase for Substrate Labeling

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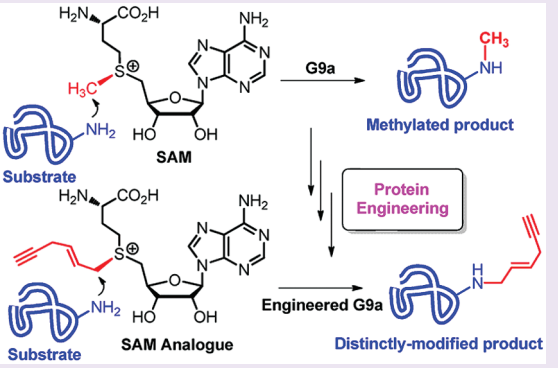
Supporting Information

ABSTRACT: Protein lysine methyltransferases (PKMTs) play crucial roles in normal physiology and disease processes. Profiling PKMT targets is an important but challenging task. With cancer-relevant G9a as a target, we have demonstrated success in developing *S*-adenosyl-L-methionine (SAM) analogues, particularly (*E*)-hex-2-en-5-ynyl SAM (Hey-SAM), as cofactors for engineered G9a. Hey-SAM analogue in combination with G9a Y1154A mutant modifies the same set of substrates as their native counterparts with remarkable efficiency. (*E*)-Hex-2-en-5-ynylated substrates undergo smooth click reaction with an azide-based probe. This approach is thus suitable for substrate characterization of G9a and expected to further serve as a starting point to evolve other PKMTs to utilize a similar set of cofactors.



Protein lysine methyltransferases (PKMTs) are a family of enzymes that deliver the sulfonium methyl group from *S*-adenosyl-L-methionine (SAM **1**) to specific lysine residues on histones (Figure 1a).[1−3] This sequence-specific posttranslational modification regulates numerous cellular processes through gene transcription or silencing.[4,5] The consequences of these methylations can be further dictated by the degree of modifications (*e.g.*, mono-, di-, or trimethylation).[4,5] PKMTs are also known to methylate many biologically relevant non-histone substrates.[6,7] For instance, the tumor suppressor p53 has been shown to be a substrate for multiple PKMTs, such as SET7/9, SET8, SMYD2, G9a, and GLP.[8−11] These methylation events regulate p53′s activity as well as its stability.[8−11] Aberrant PKMT activities are frequently associated with diseases, such as developmental abnormalities, neurodegenerative disorders, and cancer.[12,13] Given the importance of these enzymes in normal physiology and disease states, it is of great interest to develop small-molecule tools for functional analysis of PKMTs.[3]

It has been reported recently that some methyltransferases can promiscuously utilize SAM analogues, including those containing a "clickable" terminal alkynyl group, as cofactors.[16−21] These synthetic cofactors, coupled with Cu-catalyzed azide−alkyne cycloaddition (CuAAC or click chemistry),[22,23] proved to be suitable to label PKMT substrates. Some recent accomplishments in this direction include the use of cofactors **2** and **5** (Figure 1a) to label the substrates of human PKMTs SETDB1 and MLL4, respectively.[18,19] However, emerging evidence also indicates that certain SAM analogues may only act as cofactors of specific PKMTs. For example, although prop-2-ynyl SAM **2** is an active cofactor of SETDB1, it is inert toward SET7/9, SMYD2, PRDM8, -10, and -16.[18] This situation significantly hinders a broad application of SAM analogues for substrate labeling.

A strategy to address such limitation is to apply a protein-engineering approach to expand cofactor selectivity (a "bump-hole" approach), as shown for kinases, arginine methyltransferase (PRMT), and other enzymes.[24−26] Adenine-N6-derivatized ATP and SAM analogues have been used previously as cofactors of engineered kinases and yeast PRMT Rmt1, respectively.[24,26] In comparison with PRMTs, the majority of PKMTs share the structurally and functionally distinct SET domain for SAM recognition and enzyme catalysis.[14] However, no effort has been made thus far to explore whether SAM derivatives can be utilized by engineered PKMTs. A successful implementation of the bump-hole strategy is expected to examine the activity of a PKMT of interest even in the context of other closely related methyltransferases. Upon critically analyzing a series of PKMT structures, we envisioned the feasibility of tailoring SAM-binding pockets to accommodate and process terminal-alkyne-containing SAM analogues (Figure 1a). As a result, the substrates of a single PKMT can be labeled with distinct chemical moieties.

Previous studies on SAM derivatives have shown that the sulfonium-β sp/sp^2 carbon, as present in **2** and **5**, favors efficient

Received: February 15, 2011
Accepted: April 15, 2011
Published: April 15, 2011

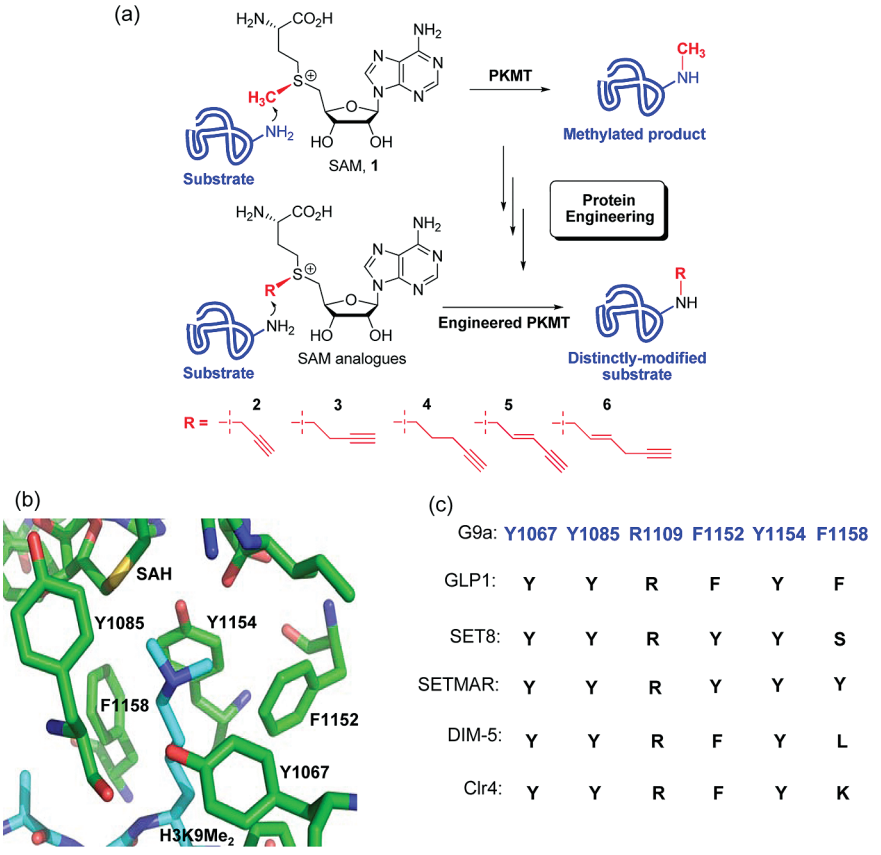


Figure 1. (a) SAM analogue cofactors in combination with protein-engineering approach for PKMT substrate labeling. SAM-binding sites of PKMTs will be tailored to recognize SAM derivatives. (b) Key catalytic residues for G9a-mediated methylation (PDB 2O8J). (c) Comparison of these residues between G9a and other SET-domain-containing PKMTs.[14,15]

enzymatic transalkylation.[16] Based on these prior observations, three new SAM derivatives **3**, **4**, and **6** along with previously reported SAM analogue cofactors **2** and **5** were synthesized from the common starting material *S*-adenosyl-L-homocysteine (Supporting Information).[27] Analogues **3** and **4**, containing a sp^3 carbon at the sulfonium-β position, were prepared with intent to evaluate the importance of the sulfonium-β sp^2 carbon in **5** for cofactor reactivity. In comparison with **5**, (*E*)-hex-2-en-5-ynyl SAM **6** (Hey-SAM) was prepared to examine the effect of the flexible methylene linker between its vinyl and terminal alkyne moieties.

G9a is a protein lysine methyltransferase that is predominantly involved in methylating histone H3 at lysine 9 (H3K9).[28] It also acts on other non-histone substrates, such as DNA-methyltransferase 1 and the chromatin-remodeling factor reptin.[28−30] Human G9a has been implicated in oncogenesis through silencing tumor suppressors.[31] With this cancer-relevant PKMT as a target, we describe herein the successful development of synthetic SAM analogue cofactors, in particular Hey-SAM **6**, with a rational protein-engineering approach to label PKMT substrates (Figure 1a).

Extensive structural and biochemical characterization of SET-domain-containing methyltransferases revealed several conserved aromatic residues (*e.g.*, Y1067/Y1085/F1152/Y1154 in G9a) that line up to form a catalytic channel for methylation (Figure 1b,c).[14] We reasoned that mutating these residues or their adjacent regions (*e.g.*, R1109 and F1158 in G9a) to smaller amino acids may extend the cofactor binding pocket for SAM derivatives. As a proof-of-concept, we performed alanine screening on Y1067, Y1085, R1109, F1152, Y1154, and F1158 residues of G9a. To identify functional cofactor−enzyme pairs to a maximal degree, the entire suite of SAM analogues **2**−**6** along with SAM **1** were screened against native G9a and its mutants (Figure 2a, Supporting Information). After incubating these enzymes and histone H3 peptide (a peptide substrate of G9a)[29] in the presence of cofactors **1**−**6**, the reaction products were analyzed by MALDI mass spectrometry.

Among the examined 6 × 7 combinations, **5** and **6** were shown to be active toward the Y1154A mutant (Figure 2a). The desired (*E*)-pent-2-en-4-ynyl and (*E*)-hex-2-en-5-ynyl modifications on histone peptide were discernible in MALDI mass spectra (Figure 2b,c). This novel activity of the Y1154A variant must be acquired through protein engineering because native G9a is inactive toward these synthetic cofactors (no modification as shown in Supplementary Figure S1). In contrast, SAM was efficiently used by G9a and its mutants Y1067A, Y1085A, R1109A, F1152A, and F1158A and to a minor extent by Y1154A (methylated products as shown in Supplementary Figures S2−8). Alanine mutations of Y1067 and F1152 mutants showed the processivity to trimethylated product (Figure 2a and Supplementary Figures S3 and S6), consistent with their roles as mono/di/trimethylation switches.[14,32] F1152A mutant was also active toward Hey-SAM **6**, albeit to a less degree than Y1154A (Figure 2a and Supplementary Figure S9). Collectively, the functional G9a mutants that process SAM analogues **5** and **6** were identified *via* the rational protein-engineering approach combined with the MS-based screening strategy.

dx.doi.org/10.1021/cb2000567 |*ACS Chem. Biol.* 2011, 6, 679–684

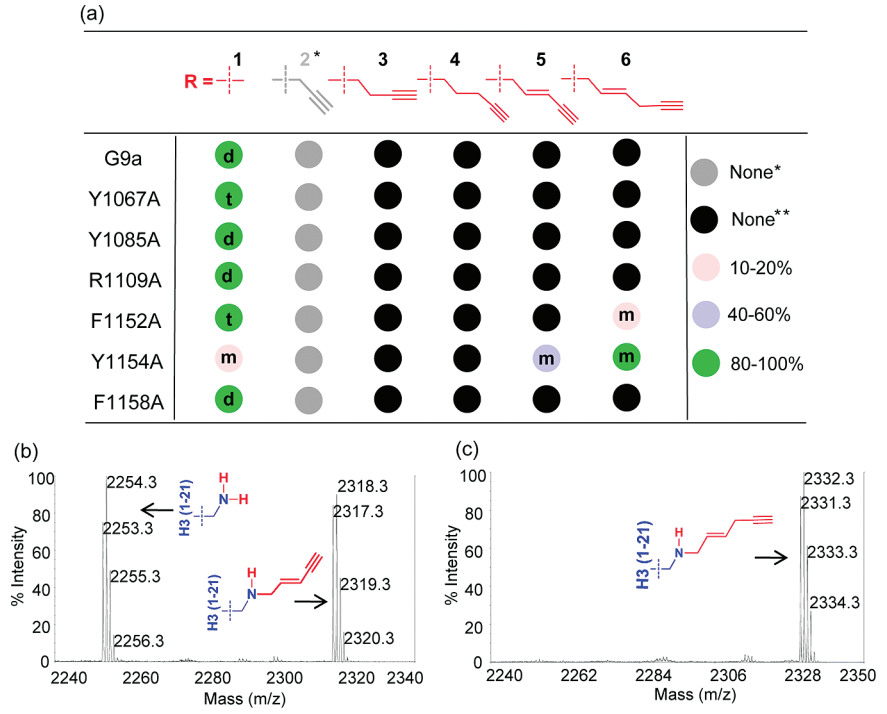


Figure 2. (a) Screening SAM analogues against engineered G9a. After incubating H3K9 peptide, SAM analogues and G9a mutants, reaction products were examined with MALDI mass spectrometry. m/d/t for mono/di/trimodifications. *SAM analogue **2** rapidly decomposes under our assay conditions (Supporting Information). **These negative hits were confirmed with high concentrations of proteins and SAM analogues, as well as extended reaction time (Supporting Information). (b) MALDI mass spectrum of H3K9 peptide partially modified by Y1154A and SAM analogue **5**. (c) MALDI mass spectrum of H3K9 peptide fully modified by Y1154A and SAM analogue **6**.

In a dose-dependent experiment, 50 μM of Hey-SAM was sufficient to completely modify 10 μM of peptide substrate (Supplementary Figure S10). In contrast to **5**, Hey-SAM **6** exhibited higher substrate-labeling efficiency as shown by its dual reactivity for F1152A and Y1154A mutants as well as a more rapid turnover (100% versus 50% conversion, Figure 2b,c). For a stringent comparison, a competition assay between these two cofactors was performed. In the presence of equal amounts of **5** and **6**, the (*E*)-hex-2-en-5-ynylated peptide derived from **6** was found to be the dominant product (Figure 3a). Even the presence of a 10-fold higher concentration of **5** did not significantly affect the product distribution (Supplementary Figure S11). For the Y1154A mutant, Hey-SAM **6** was also shown to efficiently compete with native SAM as a cofactor (Figure 3b). These results therefore present **6** as an excellent SAM surrogate for substrate labeling. Given that Hey-SAM **6** differs from **5** only by a flexible methylene linker, this moiety may impart favorable steric or electronic attributes that render **6** a more effective cofactor than **5**.

Remarkably, among the panel of structurally related SAM analogues, **2**−**4** are completely inert toward the G9a mutants examined so far (no modification as shown in Supplementary Figures S12−14). The lack of activity of prop-2-ynyl SAM **2** could be due to its rapid decomposition as observed under our experimental conditions (Supporting Information and Supplementary Figure S15).[19] In contrast, analogues **3** and **4** are relatively stable (data not shown). These observations led us to probe the origin of the lack of activity. Inhibition assays were carried out with G9a Y1154A mutant and its matched cofactor **5** in the presence of inactive SAM analogues **3** and **4**. The activity of Y1154A toward **5** was not significantly altered even in the presence of 10-fold higher concentrations of **3** and **4** (Supplementary Figure S16). This result argues that the lack of activity of **3** and **4** to the Y1154A mutant is due in part to their significantly lower affinity compared with that of **5**.

The superior substrate-labeling ability of Hey-SAM **6** and Y1154A prompted us to further characterize this cofactor-mutant pair. We first investigated whether the pair can act on full-length histone H3, a biologically relevant substrate of G9a.[28] Human histone H3 was modified efficiently by Y1154A in the presence of Hey-SAM **6**, though to a slightly less degree than the H3K9 peptide (50% modification on H3 versus 100% on the peptide, Figures 2c and 3c). A mixture of human core histones was then examined as a substrate. Among the four histone subunits (H2A, H2B, H3, and H4), the modification was observed exclusively on the H3 subunit (Supplementary Figure S17), indicating the same substrate specificity of G9a and its Y1154A mutant. The characteristic tandem MS data of the tryptic peptide also revealed that H3K9 was the only site of modification (Supplementary Figure S18). Our data therefore demonstrate that Hey-SAM **6** and its matched Y1154A mutant unequivocally act on the H3K9 site in the context of over 50 distinct lysines on histone octamers.[1] Such capability of the engineered G9a-cofactor pair is remarkable and strongly suggests that the Y1154A mutant coupled with Hey-SAM **6** maintains the substrate specificity of native G9a and SAM.

To apply the SAM analogues and engineered PKMTs for substrate labeling, it would be ideal if cofactor−enzyme pairs could compete with native PKMTs and SAM. Upon incubating a 1:1 ratio of G9a and the Y1154A mutant with the physiological concentration of SAM [33] and Hey-SAM **6** (50 μM), approximately equal amounts of H3 peptide was methylated and (*E*)-hex-2-en-5-ynylated, respectively (Figure 3d). Since native G9a and its mutant predominantly recognize SAM and Hey-SAM,

dx.doi.org/10.1021/cb2000567 |*ACS Chem. Biol.* 2011, 6, 679–684

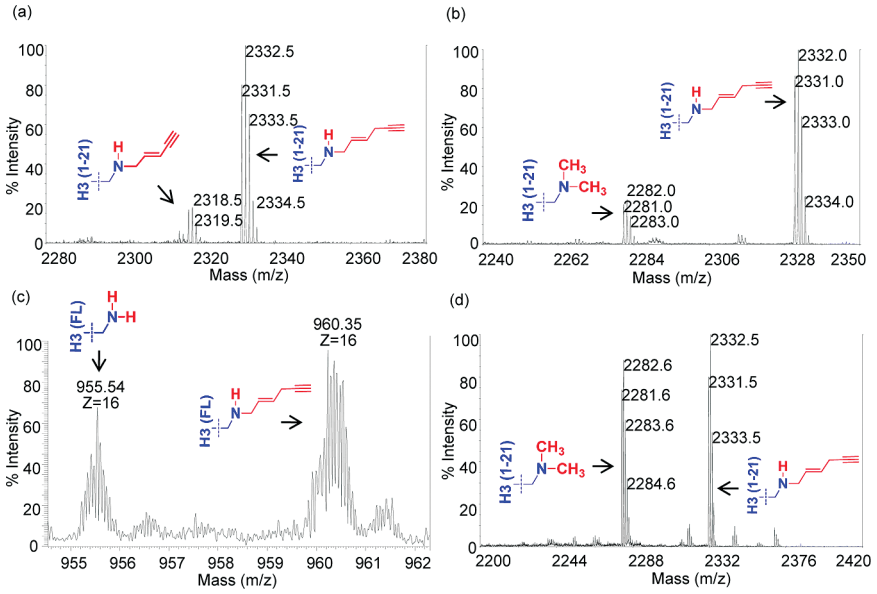


Figure 3. Characterization of Hey-SAM **6** and Y1154A mutant. (a) Product distribution of modified peptides in the presence of 1:1 ratio of (*E*)-pent-2-en-4-ynyl SAM **5** and Hey-SAM **6**. (b) Product distribution of modified peptides in the presence of 1:10 ratio of Hey-SAM **6** and native SAM **1**. (c) ESI-MS spectrum of histone H3 modified by Hey-SAM **6** and Y1154A mutant. (d) Competition assay between native G9a-SAM and engineered Y1154A-Hey-SAM pairs. Equal molar of native G9a and its Y1154A mutant in the presence of 50 μM SAM **1** and Hey-SAM **6** furnished approximately 1:1 ratio of dimethylated and (*E*)-hex-2-en-5-ynylated peptides.

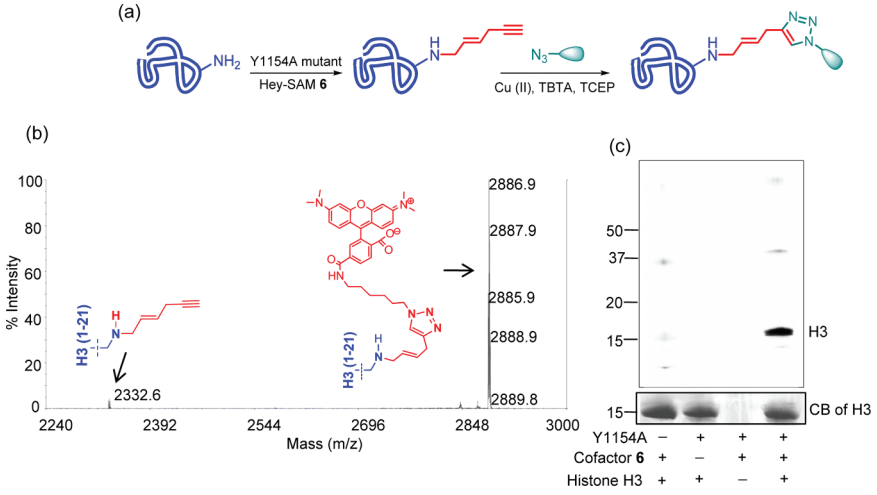


Figure 4. Clickable feature of Hey-SAM-modified substrates. (a) Schematic presentation of the reaction sequence. H3K9 peptide and full-length histone H3 were modified by G9a Y1154A mutant with Hey-SAM **6**. The resultant product was subjected to Cu(I)-catalyzed click ligation with an azido-tetramethylrhodamine probe (TBTA, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine; TCEP, tris(2-carboxyethyl)phosphine hydrochloride). (b) MALDI-MS spectrum of the enzymatically modified, azido-probe-derivatized H3K9 peptide product. (c) In-gel fluorescence of the enzymatically modified, azido-probe-derivatized H3 product (top panel). Coomassie Blue staining (CB, bottom panel) as a loading control.

respectively, the 1:1 product ratio indicates that Y1154A and Hey-SAM **6** can efficiently label the substrate even in the presence of comparable amount of native G9a and SAM.

Enzymatic incorporation of the (*E*)-hex-2-en-5-ynyl group makes modified substrates amenable to copper(I)-catalyzed azide−alkyne cycloaddition (Figure 4a).[22,23] We observed that (*E*)-hex-2-en-5-ynylated peptide, which was generated by Y1154A mutant and Hey-SAM **6**, underwent a smooth "click" reaction with azide-containing tetramethylrhodamine (Figure 4b). Full-length histone H3 was also labeled in a similar manner (in-gel fluorescence, Figure 4c). A concentration-dependent experiment revealed that as low as 1 μg of (*E*)-hex-2-en-5-ynylated, rhodamine-labeled histone H3 can be visualized readily through in-gel fluorescence (Supplementary Figure S19). Taken together, our data suggest that Hey-SAM **6** can be processed efficiently by an engineered PKMT and the resultant (*E*)-hex-2-en-5-ynylation can be probed readily with azido-based reporters.

In summary, our success in identifying clickable SAM analogue cofactors for engineered G9a has demonstrated the feasibility to apply a protein-engineering approach to tailor the cofactor selectivity of PKMTs. The remarkable efficiency of the SAM analogues, in particular (*E*)-hex-2-en-5-ynyl SAM, in the context

of mutated G9a, also allows the active enzyme-cofactor pair to compete with native G9a and SAM for substrate labeling. Several prior work showed that the clickable SAM analogues **2** and **5** are promiscuous toward native SETDB1 and MLL4, respectively.[18,19] In contrast to the prior approaches, the current effort to develop clickable SAM analogues for engineered PKMTs, though less straightforward, provides the flexibility to improve the catalytic efficiency aimed at labeling PKMT targets in the context of complex cellular mixture. More importantly, given the limited promiscuity of native PKMTs for SAM analogue cofactors[18,19] and the highly conserved SET domains, the current work can serve as a general starting point to evolve other SET-domain-containing PKMTs to use a similar set of cofactors. The clickable feature of (*E*)-hex-2-en-5-ynyl SAM can be further coupled with azide-based reporters for substrate characterization from complex cellular proteomes.[34] Profiling substrates of PKMTs is an important step toward understanding their biological roles and can be accelerated by developing novel chemical tools.[3] The newly characterized SAM analogue cofactors in combination with a protein-engineering approach are expected to play an important role in dissecting PKMT-mediated protein posttranslational methylation.

METHODS

For complete materials and methods, including synthesis, protein expression and purification, assay protocol, and figures, see the Supporting Information.

General. *S*-Adenosyl-L-methionine (SAM), *S*-adenosyl-L-homocysteine (SAH), and other reagents for chemical synthesis were obtained from Aldrich Chemical or Acros Organics and used without further purification. Reactions to prepare SAM analogues were generally carried out in capped 1 dram vials (4 mL) stirred with Teflon-coated magnetic stir bars. Analytical and preparative HPLCs were carried out on Waters 600 Controller HPLC/2998 diode array detector using XBridge reverse phase columns. G9a plasmid PET28a-LIC was a kind gift from Prof. Jinrong Min (University of Toronto). Histone H3 peptide (amino acids 1−21) was obtained from Proteomics Resource Center of the Rockefeller University. Recombinant full-length human histones were obtained from New England Biolabs.

Cofactor Synthesis. SAM analogues were synthesized on the basis of reported protocols with some modifications, particularly for the new compounds **3**, **4**, and **6**. Briefly, 100 equiv of activated electrophiles was reacted with SAH (1 equiv) in 1:1 acetic acid/formic acid mixture (1.5 mL) at RT. Upon completion of the reaction, product was extracted into water and purified by HPLC. For details of the synthetic procedure and characterization data, see Supporting Information.

Recombinant G9a and Its Mutants. The plasmid was transformed into *E. coli* Rosetta-2(DE3) strain (Novagen) using pET28a-LIC vector. Protein expression was induced in the presence of 25 μM $ZnSO_4$ at 17 °C overnight with 0.5 mM IPTG. Protein was purified using Ni-NTA agarose resin (Qiagen) followed by gel filtration chromatography (Superdex-75, GE Healthcare). The concentrated protein was stored at −80 °C before use. G9a mutants were generated by the QuickChange site-directed mutagenesis method (Stratagene) by following manufacturer's instruction.

Methyltransferase Assay. Enzymatic reactions were performed in 50 mM tris buffer (pH 8.0). Typically, 1 μM of native/mutant enzyme was incubated with 10 μM of histone peptide or full-length histone H3 and 50 μM cofactors. Samples were then subject to MALDI-MS or LC-MS analyses. See the Supporting Information for details.

Cu(I)-Catalyzed Click Chemistry. Enzymatically modified (*E*)-hex-2-en-5-ylated peptide or full-length histone was subject to click ligation by incubating with 100 μM tetramethylrhodamine 5-carboxamido-(6-azidohexanyl) (Invitrogen), 1 mM $CuSO_4$, 2 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and 100 μM tris-[(1-benzyl-1*H*-1,2,3-triazol-4yl)methyl]amine (TBTA). Upon completion, peptide samples were purified by Sep-Pak Vac C18 Cartridge (Waters) and analyzed with MALDI-MS. Histone samples were subject to in-gel fluorescence.

ASSOCIATED CONTENT

Supporting Information. his material is available free of charge *via* the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

We thank Dr. Jinrong Min for plasmids; Dr. George Sukenick, Ms. Hui Liu for small-molecule NMR and mass analyses.; This work is supported by the NIH through NIGMS (1R01GM096056-01) and the NIH Director's New Innovator Award Program (1-DP2-OD007335-01). M.L. is a 2009 V Foundation Scholar. This work is dedicated to the memory of Prof. David Gin for his critical comments on the manuscript and his model role as a respected scientist, colleague, and mentor.

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