JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

Bioorthogonal Profiling of Protein Methylation Using Azido Derivative of *S*-Adenosyl-L-methionine

Kabirul Islam,[†] Ian Bothwell,[†] Yuling Chen,[‡] Caitlin Sengelaub,[†] Rui Wang,[†] Haiteng Deng,[‡,§] and Minkui Luo*[,†]

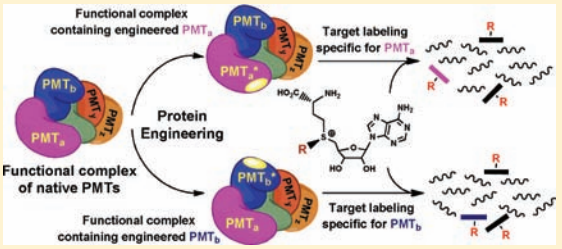
[†]Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10065, United States

[‡]School of Life Sciences, Tshinghua University, Beijing 100084, China

[§]Proteomic Resource Center, Rockefeller University, New York, New York 10065, United States

Supporting Information

ABSTRACT: Protein methyltransferases (PMTs) play critical roles in multiple biological processes. Because PMTs often function in vivo through forming multimeric protein complexes, dissecting their activities in the native contexts is challenging but relevant. To address such a need, we envisioned a Bioorthogonal Profiling of Protein Methylation (BPPM) technology, in which a SAM analogue cofactor can be utilized by multiple rationally engineered PMTs to label substrates of the corresponding native PMTs. Here, 4-azidobut-2-enyl derivative of *S*-adenosyl-L-methionine (Ab-SAM) was reported as a suitable BPPM cofactor. The resultant cofactor−enzyme pairs were implemented to label specifically the substrates of closely related PMTs (e.g., EuHMT1 and EuHMT2) in a complex cellular mixture. The BPPM approach, coupled with mass spectrometric analysis, enables the identification of the nonhistone targets of EuHMT1/2. Comparison of EuHMT1/2's methylomes indicates that the two human PMTs, although similar in terms of their primary sequences, can act on the distinct sets of nonhistone targets. Given the conserved active sites of PMTs, Ab-SAM and its use in BPPM are expected to be transferable to other PMTs for target identification.



INTRODUCTION

S-Adenosyl-L-methionine (SAM, **1**), one of the most commonly used enzyme cofactors,[1] serves as a ubiquitous methyl donor by SAM-dependent methyltransferases.[2] Protein methyltransferases (PMTs), in particular, have been the focus of much recent research due to their roles in epigenetic phenomena and disease-related processes.[3] PMTs catalyze the transfer of SAM's sulfonium methyl group to the amino acid side chains (mainly lysine or arginine) of specific proteins in a sequence-dependent fashion.[4] These methylation events can be subsequently recognized by "reader" proteins to render meaningful downstream signals.[5] The biological functions of an individual PMT are therefore tightly associated with its distinct methylome, a collection of substrates modified by the PMT.[6]

Several attempts have been made previously to profile methylome of specific PMTs using radioactive SAM and recombinant enzymes.[7] Novel PMT targets were also identified in vitro using arrayed peptide or protein libraries as substrate candidates.[8,9] Because PMT-mediated methylations often depend on specific in vivo conditions (e.g., methylation of Reptin by G9a only under hypoxic conditions and p65 by SET7/9 under TNFα stimulation),[10,11] substrate identification using PMT-knockout proteome is expected to be biologically more relevant under certain circumstances.[12] However, these prior approaches may possess limitations, such as low sensitivity for less abundant targets, low integrity using truncated substrates or enzymes, and disruption of functional complex in PMT-knockout systems.[7] For example, PMTs often associate with other proteins in vivo to form multimeric complexes; knocking down one PMT can disrupt the entire complex.[13] It is therefore important to overcome these limitations in the course of developing approaches to profile substrates of a given PMT.

Inspired by the emerging application of the terminal-alkynye-containing clickable SAM analogues to label targets of specific PMTs,[14−17] we envisioned a more general approach, which we termed Bioorthogonal Profiling of Protein Methylation (BPPM, Figure 1), for labeling and dissecting targets of multiple PMTs. The central tenet of the BPPM technology lies in developing a SAM analogue cofactor that is active for multiple rationally engineered PMTs but inert toward native PMTs in complex cellular milieu. Because only engineered PMTs utilize the SAM analogue to modify their targets in an efficient manner, the resultant labeled targets can be unambiguously characterized, when coupled to mass spectrometry analysis, and assigned to the designated (engineered) PMTs (Figure 1). Here, we report that the BPPM strategy is able to address several prior limitations and selectively profile the methylomes of PMTs

Received: December 19, 2011
Published: March 10, 2012

   dx.doi.org/10.1021/ja2118333 | *J. Am. Chem. Soc.* 2012, 134, 5909−5915

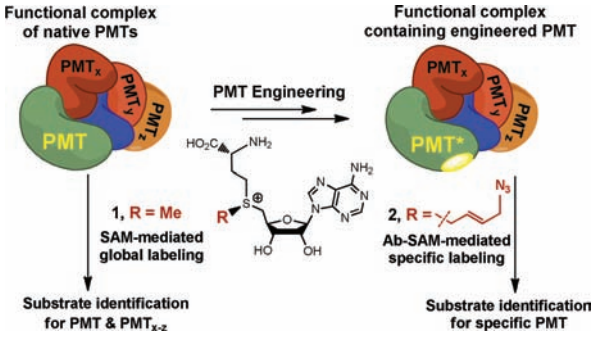


Figure 1. Schematic description of Bioorthogonal Profiling of Protein Methylation (BPPM). Designated PMT can be engineered to process selectively a clickable SAM analogue cofactor (e.g., Ab-SAM **2**) for the PMT-specific substrate labeling.

(e.g., EuHMT1 and EuHMT2) in cellular contexts. BPPM platform was also successfully applied to dissect and identify distinct nonhistone targets of the closely related PMTs.

RESULTS AND DISCUSSION

4-Azidobut-2-enyl *S*-Adenosyl-L-methionine (Ab-SAM) as a Cofactor for BPPM. Terminal alkyne-containing SAM analogues have been explored as cofactors for native as well as engineered PMTs.[14−16] Because terminal alkyne can be subject to copper-catalyzed azide−alkyne cycloaddition (CuAAC), these cofactors may have potential, yet to be tested, to identify novel substrates of PMTs. In the course of developing suitable SAM analogues for BPPM, we encountered a novel 4-azidobut-2-enyl SAM (Ab-SAM **2**, Figure 1), which was readily prepared from *S*-adenosyl-L-homocysteine. Such azide-containing SAM analogues have not been examined previously for PMTs. Ab-SAM contains a sulfonium-β-sp^2 moiety in the place of SAM's methyl group. This functionality has been shown to be essential for S_N2-type enzymatic transalkylation.[18] Ab-SAM also contains a distinct sulfonium-δ azido group, which can be combined with copper-free click chemistry for substrate labeling.[19] We further envisioned that the distinct geometry, size, and polarity of the azide group (a linear 3-atom dipolar) may restrain this cofactor from being recognized by native PMTs.

Identification of PMT Variants That Can Recognize Ab-SAM. As the first proof-of-principle example of BPPM, we focused on dissecting methylation activities of EuHMT1 (GLP1/KMT1D) and EuHMT2 (G9a/KMT1C). The two human PMTs harbor potential oncogenic activities by methylating histone H3 lysine 9 (H3K9) as well as nonhistone targets.[20] The full-length EuHMT1/2 contain >1200 amino acids with their C-termini (only 15−20% of the full-length constructs) sufficient for methylation in vitro.[20] In contrast, the rest of the 80% of EuHMT1/2 play regulatory roles through modulating their localization and binding partners.[20] In cellular contexts, EuHMT1/2, together with other PMTs such as SUV39H1 and SETDB1, function via a multimeric complex.[13] These findings therefore argue the importance of using full-length enzymes as well as the native context to recapitulate EuHMT1/2's activities.

SAM-binding site of EuHMT1 was first modified to accommodate Ab-SAM **2** for BPPM. Conserved SAM-binding motifs of SET-domain-containing PMTs led us to perform alanine replacement of EuHMT1's Y1124, Y1142, F1144, F1209, Y1211, and F1215 residues, which either directly interact with or are adjacent to SAM's sulfonium methyl group (crystallographic analysis, Figure S1).[21] After incubating Ab-SAM **2** or SAM **1** with the EuHMT1 mutants and H3K9 peptide substrate, the products were analyzed with MALDI MS (Figures 2a, S2). The functional enzyme−cofactor pairs were

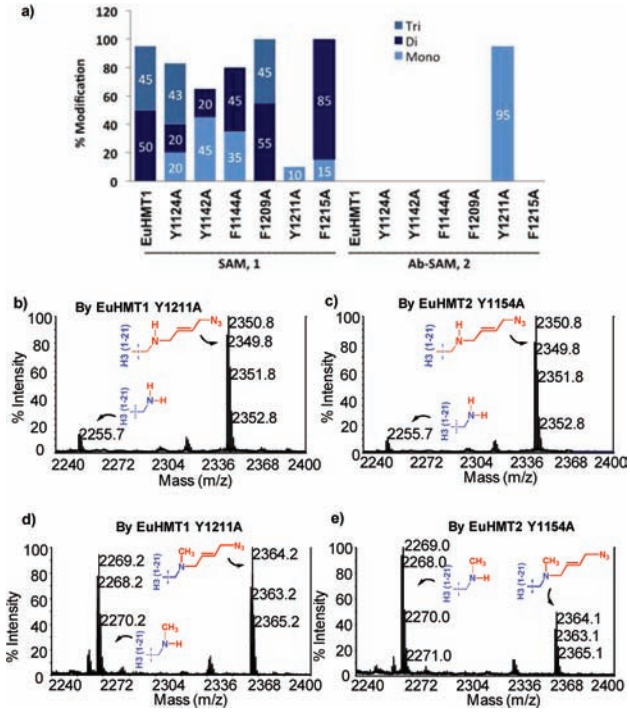


Figure 2. Activities of EuHMT1/2 variants on H3 peptide substrate. (a) Modification % of H3 peptide by EuHMT1/2 variants with SAM (left) or Ab-SAM (right) as cofactors. Bar diagram represents MALDI-MS-based qualification of methylation (left) and 4-azido-2-butenylation (right) of H3 peptide labeled as conversion %. Colors are coded for mono-/di-/trialkylation products, respectively. (b and c) MALDI-MS spectra of 4-azido-2-butenylation of H3K9 peptide by Y1211A and Y1154A, respectively. (d and e) MALDI-MS spectra of 4-azido-2-butenylation of H3K9Me peptide by Y1211A and Y1154A, respectively. These enzymatic reactions were carried out at Ab-SAM concentration and incubation time that are 10-fold higher than those for (b) and (c).

identified upon detecting the expected alkylated products (Figure 2a). The reactivity toward SAM **1** and the inertness to Ab-SAM **2** were observed for native EuHMT1 and most of the EuHMT1 mutants (Figures 2a, S2). In terms of product specificity (ratios of mono-/di-/trimethylation), only F1209A mutant is comparable to native EuHMT1. In contrast, the alanine mutants of Y1124, Y1142, F1144, and F1215 alter EuHMT1's product specificity from di-/trimethylation to mono-/dimethylation. Remarkably, EuHMT1-Y1211A mutant is almost inert toward SAM but active to Ab-SAM **2** (Figures 2b, S2). This orthogonal preference thus presents EuHMT1-Y1211A mutant and Ab-SAM **2** as a suitable enzyme−cofactor pair for BPPM.

Given the conserved SAM-binding motifs of PMTs,[21] the corresponding mutants of EuHMT2 were cross-examined against SAM **1** and Ab-SAM **2**. The MALDI-MS screening results confirmed that the EuHTM2 mutants recapitulate the cofactor-recognizing pattern of EuHMT1 counterparts with the preference of EuHMT2−Y1154A mutant to Ab-SAM **2** and others to SAM **1** (Figures 2c, S3). Strikingly, these results

dx.doi.org/10.1021/ja2118333 | J. Am. Chem. Soc. 2012, 134, 5909−5915

reflect the cofactor-recognizing pattern of the EuHTM2 variants on terminal-alkyne-containing SAM analogues,[22] despite the structural difference between these cofactors. While the molecular mechanism for the recognition is intriguing (will be reported elsewhere), our current findings demonstrate that active sites of PMTs can be engineered to accommodate bulky synthetic cofactor in a bioorthogonal manner.

Further Validation of Activities of EuHTM1/2 Variants on Ab-SAM. Native EuHMT1 and 2 are known to transfer at least two methyl groups to their substrates.[23] In vitro, the extended incubation also led to trimethylation of the peptide substrate (Figures 2, S2). In contrast, EuHMT1's Y1211A and EuHMT2's Y1154A mutants can transfer only one 4-azidobut-2-enyl group even upon extending reaction time and increasing cofactor concentration by 10-fold (Figure S4). This observation is likely due to the bulky size of the 4-azidobut-2-enyl group, which may not allow the EuHMT1/2 variants to accommodate the transferring of the second group of a similar size. To gain further insight into the product specificity of the engineered PMTs, we examined Ab-SAM cofactor with H3K9Me peptide as a substrate of the Y1211A/Y1154A mutants. A small amount (<5%) of 4-azido-but-2-enylation of the premethylated substrate was observed under the standard assay conditions (Supporting Information and Figure S5). Such conversion then reached around ~30−50% when a 10-fold increase of concentration of Ab-SAM and incubation time was applied (Figure 2d,e). Albeit at slower rates, the dialkylation pattern partially mimics the dimethylation character of native EuHMT1/2 and SAM and therefore argues the feasibility to modify EuHMT1/2's targets with the 4-azidobut-2-enyl group even in the prior presence of monomethylation.

After confirming the activities of engineered EuHMT1/2 and Ab-SAM **2** on the peptide substrates, we examined whether the two enzyme−cofactor pairs, like native EuHMT1/2 and SAM, can act on a known protein target, full-length histone H3.[24] After treating H3 with Ab-SAM **2** and the engineered EuHMT1/2, the resultant tandem MS of tryptic peptide revealed the expected 4-azido-but-2-enylation on H3K9 (Figure S6), a site known to be methylated by EuHMT1/2 in vivo.[24] These results thus suggest that the engineered enzyme−cofactor pairs largely maintain the substrate recognition integrity of their native counterparts.

Functionalization of Modified Substrates of EuHMT1/2 by Copper-Free "Click" Chemistry. Enzymatic incorporation of an azide group either on peptide or on protein substrate makes them amenable to strain-promoted azide−alkyne cycloaddition (SPAAC).[19] This chemical ligation has been proved invaluable in biomolecule imaging and identification.[25] We tested whether 4-azido-but-2-enylated peptide can undergo SPAAC with the fluorescent dye tetramethylrhodamine-dibenzocyclooctyne (TAMRA-DIBO). H3K9 peptide treated with Ab-SAM and Y1211A/Y1154A mutants underwent smooth cycloaddition reaction with TAMRA-DIBO as observed in MALDI spectra (Figure 3a,b). Enzymatically installed 4-azidobut-2-enyl group is therefore well poised for further functionalization at the peptide level.

The azido-modified full-length histone H3 can also be readily detected via SPAAC ligation followed by in-gel fluorescence. The recombinant human H3 was modified with Ab-SAM **2** by EuHMT1-Y1211A/EuHMT2−Y1154A mutants. The modified H3 was then "clicked" with TAMRA-DIBO and resolved by SDS PAGE. Strong fluorescence signal was observed only when

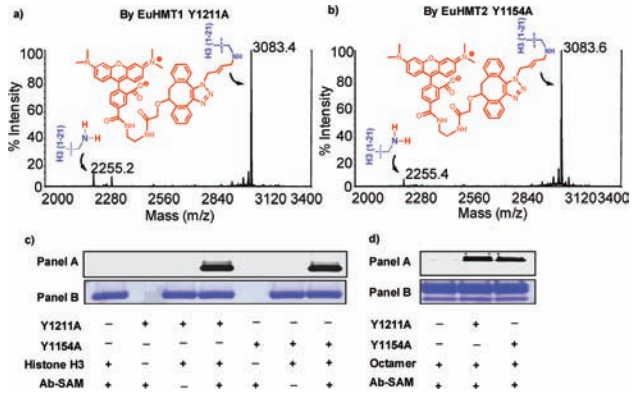


Figure 3. Strain-promoted azide−alkyne cycloaddition on modified substrates. (a,b) H3K9 peptide was modified by Y1211A/Y1154A mutants and Ab-SAM. The resultant modified peptides were treated with TAMRA-DIBO and analyzed by MALDI spectra. (c,d) In-gel fluorescence of labeled histone H3 and histone octamer, respectively. Substrates were modified by engineered EuHMT1/2 and Ab-SAM, followed by treatment with TAMRA-DIBO, SDS-PAGE separation, and in-gel fluorescence (panel A, in-gel fluorescence; panel B, Coomassie staining as loading controls).

H3 was subject to the enzyme-catalyzed modification, but was absent in no-enzyme/substrate/cofactor controls (Figure 3c). The established protocol also laid the ground for in-gel fluorescence as readout of BPPM (results below). With a similar protocol, human histone octamer (H2A/H2B/H3/H4) was also confirmed as a substrate. Here, the engineered EuHMT1/2 and Ab-SAM **2** solely labeled H3 but not other histone subunits (Figure 3d). These observations further argue that the EuHMT1/2 variants maintain the substrate specificity of native EuHMT1/2 (selective for H3 versus other histone subunits).

Application of BPPM: Proteome-Wide Labeling of Substrates of EuHMT1/2. The success in generating the bioorthogonal mutant−cofactor pairs allowed us to further implement and validate the BPPM approach in cellular contexts with known substrates and visualize the proteome-wide targets of EuHMT1/2 (Figure 4a). Here, human embryonic kidney 293 cells (HEK293T) were transfected with full-length native EuHMT1/2, mutants (Y1211A, Y1154A), and empty vector plasmids (Figures 4a, S7). The use of full-length PMT constructs is essential for substrate labeling because *E. coli*-expressed, catalytically active EuHMT1/2 truncates only gave barely detectable background labeling as will be described below.

To validate the BPPM approach in a cellular context, we first focused on hisone H3 as a common target of EuHMT1 and 2. Transfected HEK293T cells were lysed and treated with Ab-SAM **2** followed by click ligation with a biotin-conjugated DIBO probe (biotin-DIBO). The modified substrates were then pulled down with streptavidin beads and blotted with antihistone H3 antibody (Figure 4b). The EuHMT1/2 substrate H3 was pulled down only from the lysates containing the engineered EuHMT1/2 and Ab-SAM **2** but not from negative controls (empty vectors or without cofactor treatment). This result therefore demonstrated the feasibility and robustness of BPPM technology to modify and enrich EuHMT1/2 targets in complex cellular milieu.

As the next step, this methodology was advanced to probe global methylation activities of EuHMT1/2. The cell lysates of the transfected HEK293T were treated with Ab-SAM followed

dx.doi.org/10.1021/ja2118333 | J. Am. Chem. Soc. 2012, 134, 5909−5915

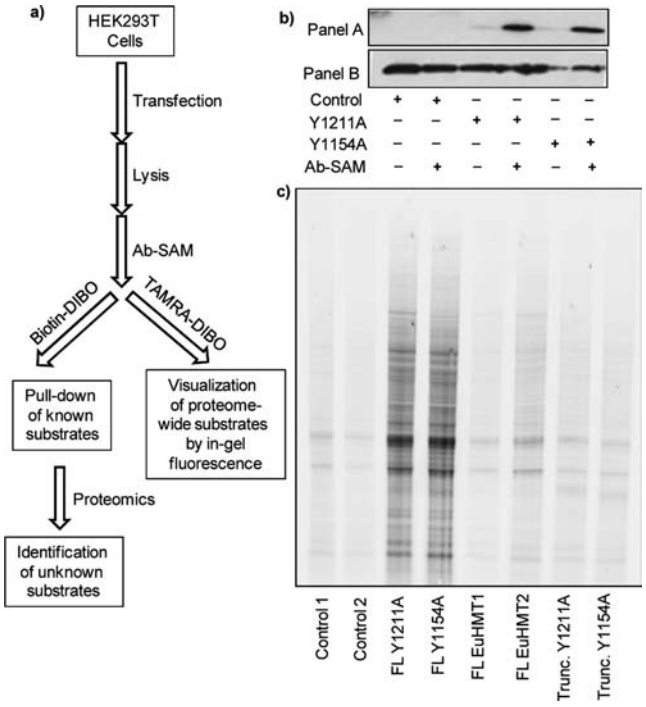


Figure 4. BPPM of EuHMT1/2. (a) Schematic description of protocols for BPPM. (b) Pulldown of 4-azido-2-butenylated histone H3. Lysates of control (empty vector)- and EuHMT1/2-transfected HEK293T cells were treated with or without Ab-SAM and then with biotin-DIBO. 4-Azido-2-butenylated histone H3 was pulled down by Streptavidin bead and blotted with anti-H3 antibody (panel A). Panel B served as loading controls (samples prior to the pull-down treatment). (c) BPPM of EuHMT1/2 with in-gel fluorescence as readout. Lysates of empty vector- and EuHMT1/2 (native and mutants)-transfected HEK293T cells were treated with Ab-SAM and then TAMRA-DIBO. Labeled proteins were resolved by SDS-PAGE and visualized with in-gel fluorescence. Exogenously added, bacterially expressed SET domain of EuHMT1/2 shows the activities (Trunc. Y1211A/Y1154A) comparable with background labeling. Control 1 and control 2 represent cell lysates transfected with empty vector and treated with Ab-SAM **2** and SAM **1**, respectively. See Figure S8 for the whole cell lysate loading controls.

by TAMRA-DIBO click conjugation and in-gel fluorescence imaging (Figure 4c). Numerous proteins (putative substrates of EuHMT1/2) were labeled in the cell lysates containing in vivo expressed EuHMT1/2 variants. The efficient labeling (lanes 3 and 4) relied on the presence of the full-length EuHMT1/2 variants to act on Ab-SAM **2** because the controls with empty-vector (lane 1), native full-length EuHMT1/2 (lane 5 and 6), or exogenously added EuHMT1/2 catalytic domains (lanes 7 and 8, Figure 4c) only showed marginal background labeling. Although the truncated EuHMT1/2 (only catalytic domains) were reported to recognize substrates in vitro,[21,23] the distinct in-gel fluorescence labeling patterns can only be observed in the presence of the full-length EuHMT1/2. This result argues the importance of using the full-length PMTs to profile targets in the cellular context. The barely detectable background labeling in the absence of engineered PMTs (Figure 4) further suggests that endogenously expressed native PMTs in HEK293T cells did not utilize Ab-SAM **2** to a significant degree. The observed background labeling likely arises from the click chemistry because the labeling patterns show no significant difference when either SAM **1** or Ab-SAM **2** was used in the assay (lane 2 versus lane 1). Ab-SAM **2** therefore stands as an ideal bioorthogonal SAM analogue cofactor for BPPM.

Application of BPPM to Profile and Characterize Methylomes of EuHMT1/2. The ability to identify known target H3 and visualize a series of labeled proteins in in-gel fluorescence prompted us to undertake mass spectrometry-based proteomic analysis to identify these putative substrates (EuHMT1/2's methylomes). After treating the lysates of HEK293T with Ab-SAM **2** in the presence or absence of EuHMT1/2's Y1211A/Y1154A mutants (sample versus control as described in Figure 4c), the cellular mixtures were subject to the "click" reaction with biotin-DIBO as a probe and then streptavidin bead to enrich the putative targets. The proteins of streptavidin pulldown were resolved in SDS-PAGE, subject to in-gel trypsin digestion, and then characterized by LCMS−MS. After subtracting the control (empty vector) from the samples (transfection with EuHMT1/2's Y1211A/Y1154A mutants) and with the criteria of more than 10 spectral counts, we identified 64 proteins as potential substrates for EuHMT1 and 82 for EuHMT2 (Figure 5, Tables S1,2 in the Supporting

a)

	Mutant-transfected	Control-transfected
Histone H1.4	YES	NO
Histone H3	YES	NO
EuHMT1/2 (automethylation)	YES	NO
Widely Interspaced Zinc Finger Motifs Protein (WIZ)	YES	NO
Chromodomain Y-Like Protein (CDYL)	YES	NO

b)

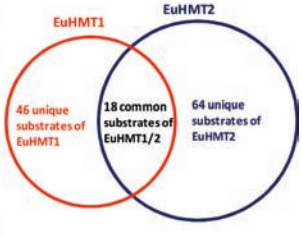


Figure 5. BPPM-derived proteomic analysis of putative substrates of EuHMT1/2. (a) The representative list of the known targets of EuHMT1/2 identified in the current study by the BPPM approach. These substrates are only present in the cell lysates transfected with engineered EuHMT1/2 but not in the control containing empty vector. (b) Comparative analysis. Venn diagram shows the 64 and 82 proteins as potential targets of EuHMT1 and EuHMT2, respectively, with 18 commonly shared targets (for details, see Supporting Information Tables S1,2).

Information for a comprehensive list). Known targets of EuHMT1/2 such as histone H3 and automethylation were readily detected in the samples containing the engineering full-length EuHMT1/2 but not in the control containing the empty vector (Figure 5a).[9,26] In contrast to prior EuHMT1/2 substrates that were identified in vitro and in vivo,[9,10,12,27,28] our BPPM results suggest that EuHMT1/2 may act on a more diverse set of targets. Interestingly, the BPPM-derived methylomes cover some but not all of the known substrates of EuHMT1/2. It therefore remains to investigate whether the difference is simply due to the low abundance or absence of the targets in HEK293T context or because BPPM-derived methylomes better reflect EuHMT1/2's methylation activities in a cellular context or in vivo.

It was worth noticing that, despite the highly conserved catalytic domain and the overall topology of EuHMT1 and EuHMT2,[20] the two PMTs display distinct substrate preferences (Figure 5b). Only about 12% of the targets identified here are commonly shared by EuHMT1 and 2. The distinct methylation profiles of the closely related EuHMT1 and 2 most likely reflect the different roles of their regulatory domains. Together, the proteomic data suggest that our BPPM

dx.doi.org/10.1021/ja2118333 | J. Am. Chem. Soc. 2012, 134, 5909−5915

approach can be reliably applied to identify substrates of PMTs in complex cellular contexts.

CONCLUSION

In the present work, we demonstrated the application of BPPM technology to dissect methylation activities of closely related PMTs. This bioorthogonality was achieved by evolving designated PMTs to recognize a novel SAM analogue **2**, which is distinct enough from SAM and thus cannot be recognized by native PMTs (a "bump-hole" approach as applied for kinases and other enzymes[17,29−32]). Our approach is highlighted by its ability to probe the activity of individual PMTs with the minimal interference from irrelevant PMTs in native cellular contexts. In contrast to Ab-SAM **2**, some less sterically hindered SAM analogues were reported to be active for native PMTs.[14,15,17] These promiscuous cofactor-enzyme pairs are not suitable for BPPM because of the uncertainty that other native PMTs may act on these SAM analogues. In addition, the catalytic efficiency and generality using SAM analogues and native PMTs for substrate labeling are low.[14,15] These limitations have been addressed in the current work by developing a bulky, bioorthogonal SAM analogue and coevolving matched PMT variants. In contrast to 4-porpargyloxybut-2-enyl SAM (Pob-SAM),[16,17] another bulky SAM analogue recently developed by our laboratory for PRMT1, Ab-SAM is further featured by its azido group for Cu-free click chemistry and avoids toxic copper for target labeling in a cellular context. In addition, Ab-SAM **2** was demonstrated to be active toward two engineered PMTs, likely more PMT variants given the conserved SET domain, and therefore may serve as a general BPPM reagent.

EuHMT1/2 mutants and Ab-SAM were implemented in search for novel protein targets in cellular context. Here, we noticed the importance of using in vivo expressed full-length PMTs for substrate identification. Robust substrate labeling was observed only when the full-length engineered PMTs (Figure 4) were applied in contrast to the lack of labeling with the truncated EuHMT1/2, which carries only catalytic domains. Prior to this work, a limited number of proteins were reported as EuHMT1/2 substrates in vitro.[9,10,12,27,28] However, extensive protein labeling as revealed in our in-gel fluorescence experiment and mass spectrometric analysis indicates that substrate profiles of EuHMT1/2 are more diverse (Figure 4). Another feature is that EuHMT1/2 likely act on the distinct classes of nonhistone substrates. The ongoing project is to validate these newly identified PMT targets in vitro and in vivo with native enzymes and SAM via well-established biochemical and genetic methods.[9,10,12,27,28] The diverse targets of EuHMT1/2 and their respective preference, as revealed by the current BPPM, may link the two PMTs to unknown pathways in the course of defining their biological functions. The success in probing the activities of multiple PMTs with a commonly shared SAM analogue further presents the feasibility to generalize the BPPM technology for other PMTs. Revealing substrate profile at the level of individual PMTs via the BPPM approach will accelerate our understanding of epigenetic functions of the important class of enzymes.

EXPERIMENTAL SECTION

Synthesis, Purification, and Characterization of Ab-SAM 2. *S*-Adenosyl-L-homocystine (12 mg, 0.031 mM) was placed in a capped 4 mL glass vial and dissolved into a freshly prepared mixture of formic and acetic acids (1:1, 1 mL) and placed in an ice bath. To this acidic solution were added (*E*)-1-azido-4-bromobut-2-ene (269 mg, 1.55 mM) and $AgClO_4$ (5.4 mg, 0.031 mM). After the mixture was stirred for 5 min, the ice bath was removed, and the reaction was allowed to warm to ambient temperature (22 °C). Reaction progress was monitored by analytical reversed-phase HPLC (XBridge C18 5 μm 4.6 × 150 mm) at 260 nm eluting with acetonitrile (linear gradient to 10% in 15 min and then to 70% in 5 min) in aqueous trifluoroacetic acid (0.01%) at a flow rate of 1 mL/min. After 5 h, the addition of (*E*)-1-azido-4-bromobut-2-ene (269 mg, 1.55 mM) and $AgClO_4$ (5.4 mg, 0.031 mM) was repeated to drive the reaction to completion. The resultant reaction mixture was quenched by adding 20 mL of distilled water containing 0.01% TFA (v/v). The aqueous phase was washed three times with diethyl ether (3 × 15 mL) and then passed through a Nalgene 0.2 μm syringe filter. Ab-SAM **2** was purified with preparative reversed-phase HPLC (XBridge Prep C18 5 μm OBD 19 × 150 mm) eluting at a flow rate of 10 mL/min with acetonitrile (linear gradients to 10% in 30 min and then to 70% in 5 min) in aqueous trifluoroacetic acid (0.01%). Because the stereochemistry at sulfonium center could not be assigned unambiguously, a diastereomeric mixture of Ab-SAM was collected. The mixture was concentrated by SpeedVac for 2 h, followed by lyophilization overnight. The dried product was redissolved in water containing 0.01% TFA (v/v) and stored at −80 °C before use. The concentrations of Ab-SAM analogue were determined by UV absorption with ε_{260} = 15 400 L mol^{-1} cm^{-1}. The compound was isolated in ∼40% yield. T_R = 10 min. ^{1}H NMR (600 MHz, D_2O): δ 8.41 (s, 0.5H), 8.39 (s, 1H), 8.38 (s, 0.5H), 6.1 (t, 1H, *J* = 3.4 Hz), 6.09−6.06 (m, 0.5H), 5.96−5.91 (m, 0.5H), 5.79−5.73 (m, 0.5H), 5.67−5.62 (m, 0.5H), 4.76 (q, 1H, *J* = 5.22 Hz), 4.6 (t, 0.5H, *J* = 6.54 Hz), 4.58 (t, 0.5H, *J* = 5.7 Hz), 4.5−4.47 (m, 1H), 4.15 (d, 1H, *J* = 7.56 Hz), 4.11 (d, 1H, *J* = 7.56 Hz), 3.88−3.8 (m, 5H), 3.56−3.38 (m, 2H), 2.3−2.28 (m, 2H). ^{13}C NMR (150 MHz, D_2O): δ 171.59, 171.49, 163.06, 162.83, 150.15, 148, 144.78, 144.72, 143.5, 143.46, 138.23, 138.06, 119.37, 119.3, 117.92, 117.4, 117.28, 115.35, 90.1, 78.67, 78.41, 73.05 (2C), 72.88, 72.66, 52.11, 52.08, 51.2, 51.15, 42.03, 41.2, 40.81, 35.84, 35.55, 25.3, 25.16. ESI-MS (*m*/*z*): 480.1[M]$^+$, 379.09 [5′-(4-azidobut-2-en-1-ynyl)thio-5′-deoxyadenosine+H]$^+$, 250.2 [5′-deoxyadenosine]$^+$. HRMS: calculated for $C_{18}H_{26}N_9O_5S$, 480.1778; obtained, 480.1759.

Expression and Purification of EuHMT1/2 and Their Variants in *E. coli*. Plasmids containing N-terminal His_6-tagged methyltransferase SET domain of human EuHMT1 (aa 951−1235) and EuHMT2 (aa 913−1193) were obtained from Dr. Jingrong Min at the University of Toronto.[21] EuHMT1/2 mutants were generated by the QuickChange site-directed mutagenesis method (Stratagene) by following manufacturer's instruction. The resulting mutant plasmids were confirmed by DNA sequencing. Native proteins and EuHMT2 mutants were expressed and purified as described earlier.[22] EuHMT1 mutant plasmids were transformed into *E. coli* Arctic Express (DE3) strain (Novagen) using pET28a-LIC kanamycin-resistant vector. A single colony was picked up and grown at 37 °C in 5 mL of Difco LB broth in the presence of 50 μg/mL kanamycin overnight. The culture was diluted 100-fold and allowed to grow at 30 °C to A_{600} = 0.7 and induced with 0.6 mM IPTG in the presence of 25 μM $ZnSO_4$ at 10 °C for 24 h. Proteins were purified as follows: harvested cells were resuspended in 50 mM Tris-HCl (pH = 8.0) containing 50 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 25 mM imidazole, and Roche protease inhibitor cocktail. The cells were lysed by French Press Cell Disruptor (Thermo) and centrifuged at 13 000*g* for 1 h at 4 °C. The soluble extracts were subject to Ni-NTA agarose resin (Qiagen) according to manufacturer's instructions. After passing six volumes of washing buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol and 25 mM imidazole), proteins were eluted with a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and 400 mM imidazole, and further purified by gel filtration chromatography (Superdex-75, GE Healthcare) using the buffer containing 25 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 15% glycerol. Purified proteins were concentrated using Amicon Ultra-10K centrifugal filter device. The protein concentration was determined with Bradford assay kit

dx.doi.org/10.1021/ja2118333 | J. Am. Chem. Soc. 2012, 134, 5909−5915

(BioRad) with BSA as a standard. The concentrated protein was stored at −80 °C before use.

Cloning and Mutagenesis of Full-Length Human EuHMT1/2. For eukaryotic expression, pCDNA3-FLAG vector encoding full-length human EuHMT1 and pFLAG-CMV2 vector encoding human EuHMT2 were obtained from Dr. Jing Huang at the National Cancer Institute (NCI). To clone human EuHMT2 into pCDNA3 vector, a KPNI restriction site was first inserted in pFLAG-CMV2 vector upstream flag sequence by the QuickChange site-directed mutagenesis method (Stratagene) by following manufacturer's instruction. Cloning was performed using KPNI and ECORI.HF restriction enzymes and confirmed by DNA sequencing. Y1211A and Y1154A mutants were generated on these mammalian vectors as described above.

Transient Transfection and Cell Lysis. Human embryonic kidney (HEK) 293T cells were grown in Dulbecco modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2 in T25 flask. At 40% confluence stage, transient transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Twelve hours after transfection, cells were treated with 15 μM of adenosine-2′,3′-dialdehyde (Adox, Sigma A7154) for 48 h to generate hypomethylated proteome. The Adox-treated cells were harvested and lysed with cold RIPA buffer (Sigma) supplemented with EDTA-free Roche protease inhibitor cocktail (1×) and 5 mM TCEP. Cell lysates were centrifuged at 21 000*g* for 30 min at 4 °C to remove cell debris. The supernatant was then passed through the detergent removal spin column (Pierce, cat. no.: 87778) and eluted with Tris buffer (50 mM Tris-HCl, pH = 8.0, 10% glycerol, 2 mM TCEP, 1 Roche protease inhibitor) following manufacturer's protocol. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories). Protein was diluted to 2 mg/mL using Tris buffer. This stock solution was used for Western blotting and methylation assay.

In Vitro Methyltransferase Assay with *E. coli* Expressed Proteins. For the initial screening, a 20 μL mixture contains 1.0 μM PMTs (EuHMT1/2 and their variants), 10 μM histone H3 peptide, and 100 μM of cofactor (**1**−**2**) in 50 mM Tris-HCl buffer (pH = 8.0). The assay was carried out for 45 min at 25 °C. The samples were then subject to MALDI mass analysis. For the reactions containing histone substrate, 1 μM of EuHMT1 Y1211A or EuHMT2−Y1154A mutant was incubated with 20 μM recombinant human histone H3 and 100 μM cofactor **2** for 2 h at 25 °C in 40 μL of buffer (50 mM Tris-HCl, pH = 8.0, 1 mM TCEP). In the assay to determine the substrate specificity, an equimolar mixture of human recombinant histones H2A, H2B, H3, and H4 (10 μM each) was incubated with 1 μM of either mutant and 100 μM SAM analogue **2** for 2 h at 25 °C in 40 μL of buffer (50 mM Tris-HCl, pH = 8.0, 1 mM TCEP). The samples were then subject to strain-promoted cycloaddition/mass spectroscopic analysis as described below.

Strain-Promoted Azide−Alkyne Cycloaddition (SPAAC). The modified H3K9 peptide was directly treated with 100 μM Click-iT TAMRA-DIBO alkyne (cat. no. C10410, Invitrogen) and shaken gently for 1 h at room temperature and subject to MALDI mass analysis. Histone H3 or octamer was modified by either of the mutants in the presence of cofactor **2** (100 μM) as described above. Enzyme-, cofactor-, and histone-negative controls were carried out similarly. Samples were then subject to SPAAC by adding 100 μM TAMRA DIBO alkyne for 1 h at room temperature in darkness. After the ligation, samples were diluted with 600 μL of methanol, 200 μL of chloroform, and 400 μL of water and centrifuged for 10 min at 15 000 rpm. Top aqueous methanol phase was discarded. The residual sample was further diluted with 450 μL of methanol, vortexed briefly, centrifuged for 10 min at 15 000 rpm, and supernatant was removed. Methanol washing was repeated once more. After supernatant was removed , the resultant sample was dried in air for 25 min in darkness. The dried sample was then dissolved in 20 μL of 1× loading buffer and heated for 10 min at 70 °C, followed by SDS-PAGE separation (Criterion Precast gel, 18% Tris-HCl, Bio-Rad). The gel was washed in washing buffer (10% acetic acid, 40% methanol, and 60% water) for a few hours. The fluorescence band was visualized with Amersham Biosciences Typhoon 9400 (excitation of 532 nm, 580 nm filter, and 30 nm band-pass). Coomassie Blue staining was used for loading controls.

Western Blotting. Equal amounts of cell lysate (50 μg of protein) were separated in a 4−12% Bis-Tris SDS-PAGE (Criterion XT Precast Gel, Bio-Rad) and then transferred to a Supported Nitrocellulose Membrane (Bio-Rad). Immunoblotting was performed with 1:1000-diluted primary antibodies for 10 h in cold room. Membrane was washed with PBS-T buffer (3 × 10 min). Primary antibodies were then recognized by HRP-conjugated secondary antibodies (1:5000 dilutions) by gentle shaking for 1 h at room temperature. Upon similar washing, bands were detected using Luminata Crescendo Western HRP Substrate (Millipore) following manufacturer's protocol.

In-Gel Labeling of Proteome-Wide Substrates of EuHMT1/2. 40 μg of HEK293T cell lysates of mock-, Y1211A-, and Y1154A-transfeted was incubated with 250 μM of Ab-SAM for 2 h at room temperature in the final volume of 20 μL. For samples containing bacterially expressed truncated Y1211A and Y1154A mutants (2 μM final concentration), mock-treated HEK293T cell lysates were used. Upon enzymatic reaction, samples were passed through the detergent removal spin column and eluted with Tris buffer (50 mM Tris-HCl, pH = 8.0, 10% glycerol, 2 mM TCEP, 1 × Roche protease inhibitor). This helps in removing excess cofactor present in the enzymatic reaction. "Click" chemistry followed by in-gel fluorescence was performed as described above.

Pull-Down of Known Substrate Histone H3. Two milligrams of HEK293T cell lysates of mock-, Y1211A-, and Y1154A-transfeted was incubated with/without 200 μM of Ab-SAM for 2 h at room temperature. Lysates were then treated with 200 μM of Click-iT biotin DIBO alkyne (cat. no. C10412, Invitrogen) and gently shaken for 1 h at room temperature. Five milliliters of methanol was added to each sample and kept at −80 °C overnight. Precipitated proteins were centrifuged for 30 min at 4000 rpm at 4 °C and washed twice with 5 mL of cold methanol. Protein residues were dried for 20 min and redissolved with 400 μL of dilution buffer (50 mM triethylamine at pH 7.4, 150 mM NaCl, Roche protease inhibitor cocktail, 0.5% SDS) with brief sonication. Protein concentration was determined by Bradford assay. 50 μg of proteins from each sample was separated for Western blotting. Twenty-five microliters of streptavidin bead in dilution buffer was added to the remaining protein and rotated end-over-end at room temperature for 1 h. Samples were diluted with 10 mL of PBS buffer supplemented with 0.2% SDS and centrifuged for 2 min at 2000 rpm. Beads were successively washed with 10 mL of PBS and 250 mM ammonium bicarbonate buffer and transferred to 1.5 mL micro centrifuge tubes. 40 μL of 1× sample buffer (Bio-RAD) was added and heated at 100 °C for 10 min. Samples were subject to Western blotting with antihistone H3 antibody as described above.

Pull-Down and Mass Spectroscopic Analysis of Proteome-Wide Substrates of EuHMT1/2. The pull-down protocol is essentially the same as described for H3 above. In the present case, 10 mg of HEK293T cell lysates of mock-, Y1211A-, and Y1154A-transfeted was used. Prior to heating the streptavidin bound proteins with loading buffer, beads were treated with freshly made reduction buffer (500 μL of 8 M urea, 25 μL of 200 mM TCEP, and 25 μL of 400 mM iodoacetamide) for 40 min in dark and washed thoroughly with ammonium bicarbonate buffer. The eluted proteins were separated on SDS-PAGE. Each lane on the 1D SDS PAGE was cut into seven pieces, which were subjected to in-gel digestion. The gel bands ware reduced with 25 mM of DTT, and then alkylated with 55 mM iodoacetamide. The in-gel digestion was implemented with the trypsin (Promega, Fitchburg, WI) in 50 mM ammonium bicarbonate at 37 °C overnight. The peptides were extracted with 0.1% formic acid in 50% acetonitrile at 37 °C for 30 min. The peptide extraction was concentrated in a speedvac, decreasing the volume and increasing the peptide concentration.

For LC−MS/MS analysis, the digestion product was separated by a 65 min gradient elution at a flow rate 0.250 μL/min with the EASY-nLCII integrated nano-HPLC system (Proxeon, Denmark), which is directly interfaced with the Thermo LTQ-Orbitrap mass spectrometer. The analytical column was a homemade fused silica capillary column

(75 μm ID, 150 mm length; Upchurch, Oak Harbor, WA) packed with C-18 resin (300 A, 5 μm, Varian, Lexington, MA). Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid. The LTQ-Orbitrap mass spectrometer was operated in the data-dependent acquisition mode using the Xcalibur 2.0.7 software, and there is a single full-scan mass spectrum in the Orbitrap (400−1800 m/z, 30 000 resolution) followed by 20 data-dependent MS/MS scans in the ion trap at 35% normalized collision energy.

The Thermo Proteome Discoverer 1.2.0 software was used to search the MS/MS data against in ipi.HUMAN.v3.82 database. The searching parameters included peptide mass tolerance of 10 ppm, ms/ms tolerance of 0.8 Da, and two missed cleavages allowed. The fixed modification of carbamidomethylation on Cys and variable modifications of oxidation on Met, deamidated on Asn and Gln, and $C_{34}H_{40}N_7O_4S$ (Ab-SAM+DIBO-biotin) on Lys were also used in the database searching. The decoy database search was added with the criteria of FDR at 0.01. The criteria used for filtering peptide were the following: 2, 2.75, and 3 for singly charged, doubly charged, and triply or higher charged ions, respectively.

MALDI-MS and Tandem MS Analysis for Peptide and Histone Samples. This was carried out as described earlier.[22]

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedure and characterization of the precursors for Ab-SAM, supplementary figures and NMR spectra for relevant compounds, and tables for pulled-down proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

luom@mskcc.org

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Drs. Jinrong Min and Jing Huang for PMT plasmids; Dr. George Sukenick, Hui Liu for small-molecule NMR and mass analyses; the financial supports from NIGMS (1R01GM096056) and the NIH Director's New Innovator Award Program (1DP2-OD007335), the V Foundation for Cancer Research (2009 V Foundation Scholar Award), March of Dimes Foundation (Basil O'connor Starter Scholar Award), Starr Cancer Consortium, and the Alfred W. Bressler Scholars Endowment Fund. We thank Dr. Howard C. Hang for his helpful discussion on the protocol of proteomic analysis.

■ REFERENCES

(1) Fontecave, M.; Atta, M.; Mulliez, E. *Trends Biochem. Sci.* **2004**, *29*, 243.

(2) Schubert, H. L.; Blumenthal, R. M.; Cheng, X. *Trends Biochem. Sci.* **2003**, *28*, 329.

(3) Bhaumik, S. R.; Smith, E.; Shilatifard, A. *Nat. Struct. Mol. Biol.* **2007**, *14*, 1008.

(4) Smith, B. C.; Denu, J. M. *Biochim. Biophys. Acta* **2009**, *1789*, 45.

(5) Taverna, S. D.; Li, H.; Ruthenburg, A. J.; Allis, C. D.; Patel, D. J. *Nat. Struct. Mol. Biol.* **2007**, *14*, 1025.

(6) Huang, J.; Berger, S. L. *Curr. Opin. Genet. Dev.* **2008**, *18*, 152.

(7) Rathert, P.; Dhayalan, A.; Ma, H. M.; Jeltsch, A. *Mol. Biosyst.* **2008**, *4*, 1186.

(8) Levy, D.; Liu, C. L.; Yang, Z.; Newman, A. M.; Alizadeh, A. A.; Utz, P. J.; Gozani, O. *Epigenet. Chromatin* **2011**, *4*, 19.

(9) Rathert, P.; Dhayalan, A.; Murakami, M.; Zhang, X.; Tamas, R.; Jurkowska, R.; Komatsu, Y.; Shinkai, Y.; Cheng, X. D.; Jeltsch, A. *Nat. Chem. Biol.* **2008**, *4*, 344.

(10) Lee, J. S.; Kim, Y.; Kim, I. S.; Kim, B.; Choi, H. J.; Lee, J. M.; Shin, H. J.; Kim, J. H.; Kim, J. Y.; Seo, S. B.; Lee, H.; Binda, O.; Gozani, O.; Semenza, G. L.; Kim, M.; Kim, K. I.; Hwang, D.; Baek, S. H. *Mol. Cell* **2010**, *39*, 71.

(11) Ea, C. K.; Baltimore, D. B. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 18972.

(12) Sampath, S. C.; Marazzi, I.; Yap, K. L.; Krutchinsky, A. N.; Mecklenbrauker, I.; Viale, A.; Rudensky, E.; Zhou, M. M.; Chait, B. T.; Tarakhovsky, A. *Mol. Cell* **2007**, *27*, 596.

(13) Fritsch, L.; Robin, P.; Mathieu, J. R.; Souidi, M.; Hinaux, H.; Rougeulle, C.; Harel-Bellan, A.; Ameyar-Zazoua, M.; Ait-Si-Ali, S. *Mol. Cell* **2010**, *37*, 46.

(14) Binda, O.; Boyce, M.; Rush, J. S.; Palaniappan, K. K.; Bertozzi, C. R.; Gozani, O. *ChemBioChem* **2011**, *12*, 330.

(15) Peters, W.; Willnow, S.; Duisken, M.; Kleine, H.; Macherey, T.; Duncan, K. E.; Litchfield, D. W.; Luscher, B.; Weinhold, E. *Angew. Chem., Int. Ed.* **2010**, *49*, 5170.

(16) Wang, R.; Zheng, W.; Yu, H.; Deng, H.; Luo, M. *J. Am. Chem. Soc.* **2011**, *133*, 7648.

(17) Luo, M. *ACS Chem. Biol.* **2012**, *7*, 443.

(18) Dalhoff, C.; Lukinavicius, G.; Klimasauskas, S.; Weinhold, E. *Nat. Chem. Biol.* **2006**, *2*, 31.

(19) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046.

(20) Shinkai, Y.; Tachibana, M. *Gen. Dev.* **2011**, *25*, 781.

(21) Wu, H.; Min, J. R.; Lunin, V. V.; Antoshenko, T.; Dombrovski, L.; Zeng, H.; Allali-Hassani, A.; Campagna-Slater, V.; Vedadi, M.; Arrowsmith, C. H.; Plotnikov, A. N.; Schapira, M. *PLoS One* **2010**, *5*, e8570.

(22) Islam, K.; Zheng, W.; Yu, H.; Deng, H.; Luo, M. *ACS Chem. Biol.* **2011**, *6*, 679.

(23) Esteve, P. O.; Patnaik, D.; Chin, H. G.; Benner, J.; Teitell, M. A.; Pradhan, S. *Nucleic Acids Res.* **2005**, *33*, 3211.

(24) Tachibana, M.; Ueda, J.; Fukuda, M.; Takeda, N.; Ohta, T.; Iwanari, H.; Sakihama, T.; Kodama, T.; Hamakubo, T.; Shinkai, Y. *Gen. Dev.* **2005**, *19*, 815.

(25) Laughlin, S. T.; Baskin, J. M.; Amacher, S. L.; Bertozzi, C. R. *Science* **2008**, *320*, 664.

(26) Weiss, T.; Hergeth, S.; Zeissler, U.; Izzo, A.; Tropberger, P.; Zee, B. M.; Dundr, M.; Garcia, B. A.; Daujat, S.; Schneider, R. *Epigenet. Chromatin* **2010**, *3*, 7.

(27) Pless, O.; Kowenz-Leutz, E.; Knoblich, M.; Lausen, J.; Beyermann, M.; Walsh, M. J.; Leutz, A. *J. Biol. Chem.* **2008**, *283*, 26357.

(28) Huang, J.; Dorsey, J.; Chuikov, S.; Perez-Burgos, L.; Zhang, X.; Jenuwein, T.; Reinberg, D.; Berger, S. L. *J. Biol. Chem.* **2010**, *285*, 9636.

(29) Bishop, A. C.; Ubersax, J. A.; Petsch, D. T.; Matheos, D. P.; Gray, N. S.; Blethrow, J.; Shimizu, E.; Tsien, J. Z.; Schultz, P. G.; Rose, M. D.; Wood, J. L.; Morgan, D. O.; Shokat, K. M. *Nature* **2000**, *407*, 395.

(30) Hoffman, H. E.; Blair, E. R.; Johndrow, J. E.; Bishop, A. C. *J. Am. Chem. Soc.* **2005**, *127*, 2824.

(31) Kapoor, T. M.; Mitchison, T. J. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9106.

(32) Lin, Q.; Jiang, F. Y.; Schultz, P. G.; Gray, N. S. *J. Am. Chem. Soc.* **2001**, *123*, 11608.