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Using Azido Analogue of S-Adenosyl-L-methionine for Bioorthogonal Profiling of Protein Methylation (BPPM)

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Abstract

Protein methyltransferases (PMTs) utilize *S*-adenosyl-*L*-methionine as a cofactor and deliver its sulfonium methyl moiety to diverse substrates. These methylation events can lead to meaningful biological outcomes from transcriptional activation/silencing to cell cycle regulation. With the long-term goal of elucidating the substrates and defining the functions of PMTs, our laboratory recently developed technology based on protein engineering in tandem with SAM analogue cofactors and bioorthogonal click chemistry to unambiguously profile the substrates of a specific PMT. The following protocols encapsulate the logic and methods of selectively profiling the substrates of a candidate PMT by (1) engineering the selected PMT to accommodate a bulky SAM analogue; (2) generating the proteome containing the engineered PMT; (3) visualizing the proteome-wide substrates of the designated PMT via bioorthogonal labeling with a fluorescent tag and finally (4) pulling down the proteome-wide substrates for mass spectrometric analysis.

Keywords

G9a; protein engineering; synthetic cofactor; click chemistry

INTRODUCTION

To maintain genome stability, human DNA is wrapped around the core histone octamer (H2A, H2B, H3 and H4) to form nucleosomes. These basic structural repeats can be further organized into the higher order architecture of chromatin and eventually chromosomes (Qiu, 2006). To program the chromatin for gene expression or repression, the histone tails undergo frequent posttranslational modifications, such as methylation, acetylation, phosphorylation, and ubiquitylation. The combinations of these modifications have been shown to modulate downstream signals and thus render meaningful biological outcomes (Kouzarides, 2007).

Histone methylation is catalyzed by more than 60 human protein methyltransferases (PMTs), which use *S*-adenosyl-*L*-methionine (SAM) (Fig. 1b) as a cofactor and deliver SAM's sulfonium methyl group to specific arginine or lysine residues on histones (Kouzarides, 2007). The site and extent (mono-, di- or tri-) of histone methylation are tightly associated with the biological readouts of gene transcription or silencing (Martin and Zhang,

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2005). For instance, the tri-methylation of histone 3 lysine 4 (H3K4) by multiple PMTs (*e.g.* MLL1–5 and SET1A/B) is linked to transcriptional activation, while the di/tri-methylation of histone 3 lysine 9 (H3K9) by G9a, GLP and SUV39H1/2 correspond to gene silencing (Lachner and Jenuwein, 2002; Martin and Zhang, 2005). Among 9 human protein arginine methyltransferases (PRMTs), PRMT1, CARM1/PRMT4, PRMT5 and PRMT6 have demonstrated *in vivo* methylation activities on histone substrates (Bedford and Clarke, 2009).

Accumulated evidence shows that PMTs also act on diverse non-histone targets. Among such examples are the methylation of TAF10, ER α , PCAF, NF- κ B, DNMT1, HIV transactivator Tat and FOXO3 by SET7/9 (Calnan et al., 2012; Ea and Baltimore, 2009; Esteve et al., 2009; Kouskouti et al., 2004; Masatsugu and Yamamoto, 2009; Mowen et al., 2001; Pagans et al., 2010; Subramanian et al., 2008; Yamagata et al., 2008; Zhao et al., 2008), transcription factors STAT1, RUNX1, and FOXO1 by PRMT1, and transcription coactivators p300 and CBP by CARM1 (Chevallard-Briet et al., 2002; Xu et al., 2001). Certain non-histone targets can be methylated by multiple PMTs (*e.g.* the tumor suppressor p53 by SET7/9, SET8, SMYD2, G9a, and GLP) (Chuikov et al., 2004; Huang et al., 2010; Huang et al., 2006; Shi et al., 2007). The collective actions of protein-methylating enzymes, or ‘writers’ of methylation, orchestrate physiological functions of the substrates in an epigenetic manner (Strahl and Allis, 2000). Aberrant methylation activities have been implicated in many human diseases including cancer (Bhaumik et al., 2007; Chi et al., 2010).

Elucidating methylation landscape (or methylome) of an individual PMT is essential in order to elucidate its physiological or pathological roles. To address the need, our laboratory recently formulated the concept of Bioorthogonal Profiling of Protein Methylation (BPPM). Here specific PMTs can be engineered to accommodate the SAM analogues and thus transfer a bioorthogonal azido or alkynyl moiety to respective substrates (Fig. 1a) (Islam et al., 2012; Islam et al., 2011; Wang et al., 2011b). Given that the synthetic SAM analogues are bulkier than native SAM, they are expected to be inert toward native PMTs and thus selectively label the substrates of designated (engineered) PMTs. In the previous work, we have demonstrated that the SAM-binding pockets of G9a and GLP can be tailored via site-directed mutagenesis. The exogenously expressed mutant proteins were screened against 4-azidobut-2-enyl SAM (Ab-SAM). The matched mutant-cofactor pairs were identified to modify the substrates of G9a and GLP. The resultant azido-labeled proteins were readily visualized with commercially available TAMRA-DIBO (“Click-iT”) fluorescent dye or subject to biotinylation with a “Click-iT” biotin probe for target pulldown and identification (Fig 1b) (Islam et al., 2012).

With G9a and Ab-SAM as a paradigm, the current protocols present the step-wise development of the BPPM methodology (Fig 1c). We first describe the procedure relying on matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) to identify an active PMT mutant toward Ab-SAM (**BASIC PROTOCOL 1**). We then describe the procedure of transiently expressing and harvesting full-length, active, PMT variant in its cellular context (**BASIC PROTOCOL 2**). The enzymatic transferring of the azido moiety from Ab-SAM was then carried out in the context of the cellular proteome containing the engineered PMT variant. The azido-labeled proteome was then visualized with TAMRA-DIBO via in-gel fluorescence (**BASIC PROTOCOL 3**). In parallel, the Ab-SAM-labeled cell lysates were treated with biotin-DIBO, followed by target pulldown with streptavidin beads and target identification with mass spectrometer (**BASIC PROTOCOL 4**). The overall approach is featured by its robustness and generality to profile the targets of designated PMTs via BPPM.

STRATEGIC PLANNING

Ab-SAM as a BPPM reagent

A set of bulky SAM derivatives containing terminal-alkyne and sulfonium- β -sp² substitutions have been reported as suitable cofactor surrogates of native or engineered PMTs (e.g. (*E*)-pent-2-en-4-ynyl, (*E*)-hex-2-en-5-ynyl, 4-propargyloxy-but-2-enyl SAM for native MLL4, engineered G9a and PRMT1, respectively). We recently expanded the cofactor repertoire by including 4-azido-but-2-enyl *S*-adenosyl-*L*-methionine (Ab-SAM) (Islam et al., 2012). While these SAM analogues all contain the catalytically essential sulfonium- β -sp² substitution and the terminal alkyne/azido groups for azide-alkyne cycloaddition (CuAAC or click chemistry), Ab-SAM solely stands out as a BPPM reagent on the basis of its distinct sulfonium- δ azido moiety, a labeling handle favored for less toxic copper-free click chemistry (Baskin et al., 2007). The distinct geometry, size and polarity of the azide group (a linear 3-atom dipolar) may also restrain this cofactor from being recognized by native PMTs and thus make it more suitable for BPPM even in the presence of irrelevant cellular PMTs. These features motivated us to use Ab-SAM as a demo to document the BPPM protocols for proteome-wide labeling of PMT substrates.

Identification of Active PMT Variants for BPPM

As described previously, our lab has been successful in engineering multiple PMTs to process SAM analogue cofactors for BPPM (Islam et al., 2012; Islam et al., 2011; Wang et al., 2011b). A common strategy in these cases is to generate and screen a collection of PMT mutants as BPPM candidates. Although the choices of the PMT mutants vary case by case, our previous experiences were to focus on tailoring the residues that are adjacent to the binding site of SAM's methyl group according to available PMT structures. For the PMTs whose structures have not been reported, we relied on sequence alignment to identify the comparable SAM-recognizing residues. In **BASIC PROTOCOL 1**, G9a was chosen to exemplify the selection and screening process of identifying active PMT variants for BPPM. X-ray structures and sequence alignments of G9a with other SET-domain-containing PMTs revealed that a set of conserved aromatic residues are located at the interface between the channel for substrate lysine binding and the pocket for SAM interaction (Fig 2a, 2b). We anticipated that mutating the bulky hydrophobic residue(s) may vacant space in the active site allowing the accommodation of the bulky Ab-SAM analogue, while maintaining the transalkylation activity of native G9a (Islam et al., 2012), we carried out the screening with a single Ala mutation in this defined region (Y1067, Y1085, F1152, Y1154 and F1158 in Fig 2b). These residues directly contact with the substrate or SAM cofactor as revealed by G9a's structures (Islam et al., 2012; Islam et al., 2011) and contain no posttranslational modification as reported so far. The corresponding G9a mutants were expressed in *E. coli* and purified with conventional methodologies (Islam et al., 2012). The G9a variants identified here for BPPM application are expected to be stable under the conditions of expression and purification, and thus enable the subsequent activity assay (**STRATEGIC PLANNING** below & **BASIC PROTOCOL 1**). We were able to identify the active G9a mutant Y1154A from the small, rational collection of G9a variants (**BASIC PROTOCOL 1**). As shown for the non-SET-domain arginine methyltransferase PRMT1 (Wang et al., 2011b), the concept of PMT engineering can be general for other PMTs and SAM analogues.

In vitro Activity Assay with H3K9 Peptide Substrate

Many PMTs can recognize synthetic peptides as well as full-length proteins as substrates. Given the availability of synthetic peptides through solid-phase peptide synthesis and their ready characterization with mass spectrometer, we preferred peptides as substrates for initial *in vitro* screening of matched enzyme-cofactor pairs. G9a di-methylates a H3K9 peptide derived from its *in vivo* substrate histone H3 [ARTKQTARKSTGGKAPRKQLA(amino

acids 1–21)] (Rathert et al., 2008). This peptide can be readily prepared with a solid-phase peptide synthesizer, purified to > 95% with HPLC and characterized by MALDI-TOF mass spectrometry. MALDI-TOF mass spectrometry was then applied to monitor the ability of the G9a mutants to modify the peptide substrate with Ab-SAM. The reaction with native G9a and SAM was carried in parallel as a positive control to avoid the false negatives, which may arise from the assay buffer or instrument failure. Our previous work has shown that H3K9 alkylation does not affect the ionization efficiency of the peptide and thus allows a direct quantification of the alkylation according to the peak ratios of mass spectrometric signals (Islam et al., 2011). Native G9a modifies the H3K9 21-aa peptide substrate with a high efficiency. If it were not the case, the optimization of peptide length (generally longer peptide) or the use of alternative peptide substrates should be considered at this stage. Prior to the assay, the reaction conditions with native PMTs and SAM must be optimized for buffer components (*e.g.* pH, detergents and salts), reaction time, and the concentrations of enzymes, peptide substrates and cofactors. If a large panel of enzyme-cofactor pairs (*e.g.* a 100 × 30 panel) needs to be screened and the capacity of MALDI-TOF is insufficient, an alternative high throughput, fluorogenic PMT assay can be used as detailed previously (Wang et al., 2011a).

Additional Parameters for Efficient Substrate Labeling with Ab-SAM in Cellular Contexts

For the proteome-wide BPPM with Ab-SAM, the engineered PMTs can either be exogenously expressed and then mixed with cell lysates or expressed within cells of interest via transient transfection. We noticed that the labeling efficiency of Ab-SAM often correlates to the amount of the exogenously-added or transiently-expressed PMT variants in the cell lysates. In addition, the constructs of PMT variants affect Ab-SAM's efficiency to label targets. For instance, although the engineered methyltransferase catalytic domain of G9a is active *in vitro*, the substrate labeling of the G9a construct is barely detectable in the presence of complex cellular proteome. In contrast, the transiently-expressed full-length G9a robustly labels its substrates in the same cellular context (Islam et al., 2012). Although the cause of this difference remains to be explored, it is likely that other coactivators in the cellular context play the role to promote the activity of the full-length G9a variant via the interaction with G9a's non-catalytic regions. In the current protocols, after identifying the active G9a variant for Ab-SAM, we relied on the transient transfection approach to overexpress the corresponding full-length G9a variant in HEK293T cells for BPPM.

To further enhance the substrate labeling efficiency of engineered PMTs and Ab-SAM, it will be desirable to inhibit the methylation activities of endogenous PMTs of interest resulting in a hypomethylated proteome as the substrate candidates for BPPM. To achieve this goal, the cells can be pre-treated with pan- or target-specific PMT inhibitors (Herrmann et al., 2005; Vedadi et al., 2011). In the current protocol with engineered G9a and Ab-SAM, HEK293T cells were treated with adenosine-2',3'-dialdehyde (Adox, Sigma A7154) to generate the hypomethylated proteome. Adox is a potent inhibitor of *S*-adenosylhomocysteine hydrolase, which is responsible for the intracellular degradation of *S*-adenosylhomocysteine (SAH), a pan-PMT inhibitor. The Adox treatment elevates SAH's intercellular concentration and thus reduces proteome-wide methylation (Herrmann et al., 2005).

BASIC PROTOCOL 1

IDENTIFYING ACTIVE G9a MUTANTS FOR Ab-SAM

The following is a standard protocol for the identification of pairs of active PMT mutants and SAM analogue cofactor, exemplified with Ab-SAM and G9a (aa 913 – 1193). Here the exogenously expressed and purified native G9a and G9a variants were incubated with the aa

1–21 H3K9 peptide and Ab-SAM. The extent of Ab-SAM-mediated transalkylation on the peptide was quantified by MALDI-MS. The synthesis of Ab-SAM (Fig 1c) is described in **SUPPORTING PROTOCOL 4**. This protocol is generally applicable to other PMTs and SAM analogue cofactors of interest.

Materials

Tris buffer, 50 mM Tris-HCl (pH = 8.0). Tris-HCl rather than Tris sodium salt was used to reduce the final concentration of buffer salt. The former was neutralized with NaOH.

H3 (aa 1–21) peptide substrate, 100 μ M stock in 0.1% (v/v) TFA and deionized distilled water (DD water). This material can be stored at -80°C for > 6 months.

SAM (Sigma) stock solution, 1 mM stock in 0.01% (v/v) TFA and DD water. With this buffer, this material can be stored at -80°C for < 3 months.

Ab-SAM stock solution, 1 mM stock in 0.01% (v/v) TFA and DD water (Islam et al., 2012). The acidity of the storage solution is important for Ab-SAM's stability. With this buffer, this material can be stored at -80°C for < 3 months. The degradation of Ab-SAM is often observed after a long-term storage.

Exogenously expressed and purified native G9a (aa 913–1193) and corresponding G9a variants (Y1067A, Y1085A, R1109A, F1152A, Y1154A and F1158A) in 25 mM Tris-HCl (pH = 8), 200 mM NaCl and 15% (v/v) glycerol (Islam et al., 2012). This G9a construct was chosen because of the ready expression of its protein products in *E. Coli* and the robust *in vitro* methylation activity of the native construct. We recommended preparing the stock solutions to the highest concentrations that can be reached or at least above 20 μ M. With this buffer and > 20 μ M stock concentration, this material can be stored at -80°C for > 6 months.

The protein expression and purification were reported before and described as the following. Plasmids containing N-terminal His6-tagged methyltransferase SET domain of human G9a (aa 913–1193) were obtained from Dr. Jingrong Min's lab at the University of Toronto. Mutants were generated by the QuickChange site-directed mutagenesis (Stratagene) following manufacturer's protocol. 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 μ M ZnSO_4 at 17°C overnight with 0.5 mM IPTG. Protein was purified first using Ni-NTA agarose resin (Qiagen) and then by gel exclusion chromatography (Superdex-75, GE Healthcare). The concentrated protein was stored at -80°C before use.

1.5 ml microcentrifuge tubes

Vortex

Refrigerated microcentrifuge (4°C)

1. Calculate the amount of the materials needed to prepare a reaction mixture of 20 μ l containing: 10 μ M peptide substrate, 100 μ M Ab-SAM, and 1 μ M G9a variant. The rest of the volume will be balanced with 50 mM Tris (pH = 8.0) to reach the final volume of 20 μ l.

Keep all reagents on ice to avoid decomposition.

2. Add 50 mM Tris buffer to balance, (calculated above) into a 1.5 ml microcentrifuge tube.
3. Add 2 μ l peptide for the final concentration of 10 μ M.

4. Add 2 μ l SAM or Ab-SAM for the final concentration of 100 μ M.
5. Add the calculated volumes of the stock solution of native G9a or G9a mutants for the final concentration of 1 μ M. These volumes may vary according to the concentrations of protein stocks.
6. Gently vortex the reaction mixture and then centrifuge for 1 min at 5,000 g (-4°C).
7. Incubate the mixture at ambient temperature (23°C) for 45 min.
8. Process the sample for MALDI-MS analysis (see **SUPPORTING PROTOCOL 1**).

An example of expected results is shown in Fig. 3a, 3b.

BASIC PROTOCOL 2

CELL CULTURE AND CELL LYSATES

The cells of interest were transiently transfected with the identified active G9a variant (**BASIC PROTOCOL 1**). The cells are then cultured in the presence of Adox to generate hypomethylation proteome, harvested and lysed for BPPM.

The optimal growth, transfection conditions (media type, confluency of cells, the amount of plasmids etc.) and the required amount of protein for BPPM analysis depend on the types of examined cells. These parameters need to be optimized prior to the following experiments. The parameters of HEK 293T cells used in BASIC PROTOCOL 3, 4 are provided here.

Materials

Human embryonic kidney (HEK) 293T cells propagated in Dulbecco modified Eagle medium (DMEM, GIBCO) supplemented with 10 % (v/v) fetal calf serum (FCS)

Lipofectamin 2000 (Invitrogen)

Opti-MEM reduced serum media (GIBCO)

Mock pCDNA3 or the pCDNA3-FLAG plasmid vectors carrying engineered full-length G9a variants (Islam et al., 2012)

Adox (Sigma, A7154) stock solution, 1.5 mM in deionized distilled water (DD water)

1 $\times$ PBS (GIBCO)

Modified RIPA lysis buffer supplemented with EDTA-free protease inhibitors (see **Recipe**)

Tris-HCl buffer (see **Recipe**)

Bradford Assay reagents (Bio-Rad)

1.5 ml microcentrifuge tubes

37°C , 5% CO_2 cell culture incubator

Refrigerated centrifuge (4°C)

Sonicator (Misonix Ultrasonic Liquid Processor)

Refrigerated high speed centrifuge (4°C , 21,000 g capacity)

2 ml (small scale) or 4 ml (large scale) detergent removal, spin columns (Pierce, cat. No: 87778, 87779)

Cell culturing and transfection

The size of cell culture flask is determined by the amount of protein required for BPPM. T-75 and T-25 flasks (353108/353136 - BD Falcon™ 25/75 cm² Cell Culture Flask) are used for in-gel fluorescence imaging and proteomic pulldown, respectively (we routinely transfect mutants directly in flasks). The amounts of reagents such as Lipofectamine, Opti-MEM, or individual plasmids are proportional to the amount of treated cells. These parameters should be optimized prior to reach maximal transfection efficiency confirmed by Western blot with proper antibodies. The following protocol was described for a T-25 flask.

- 1 HEK293T cells are grown in DMEM (10% FCS) to reach 40% confluency.
- 2 Add 8 µl of Lipofectamin 2000 to 400 µl Opti-MEM, shake thoroughly, and suspend for 5 min. Into the mixture, add 4 µg of plasmids, shake thoroughly, and suspend for 20 min.
- 3 Exchange growth media, and then add the plasmid mixture prepared above dropwise, into the flask.
- 4 Incubate the treated HEK293T cells for 8 – 12 h.

A media exchange step after 8 -12 h incubation is required for cells with high sensitivity towards Lipofectamin, although the step is not necessary for HEK293T cells.

- 5 Add 15 µM Adox to growth media (the final concentration from 1.5 mM stock solution), and incubate for an additional 48 h (around 2 × doubling time of HEK 293T cells).
- 6 Harvest cells by repetitive pipetting and then spinning down at 1,500 g at 4 °C for 10 min.

Use of trypsin to harvest cells at this step is not recommended because the treatment may degrade cell surface proteins.

- 7 Remove the supernatant, wash the cells with shelf-stored PBS (50 × volume of cell pellet), and then centrifuge at 1,500 g at 4 °C for 10 min.

The purpose of the step is to remove serum proteins.

- 8 Remove supernatant and transfer the cell pellets into a 1.5 ml microcentrifuge tube.

*The cell pellets at this stage can be stored as the pellet at –80 °C for < 2 months. Allocate the samples for Western blot to confirm transfection efficiency and **BASIC PROTOCOL 3** and **4** for proteome-wide BPPM at this step.*

Cell Lysis

- 9 Suspend the cell pellets in 200 µl (2×10^6 cells from T-25 flask for in-gel fluorescence) or 1 ml (1×10^7 cells from T-75 flask for proteomic profiling) of the modified RIPA buffer, gently tap the tube, and then incubate the cell suspension on ice for 20 min.

When 1 ml RIPA is used, the samples should be split into several tubes with < 400 µl of RIPA mixture in each tube.

- 10 Sonicate the RIPA cell suspension on an ice bath with a single 5 min pulse at 60% amplitude.

The fully lysed cells should be translucent. In some cases, adding extra modified RIPA buffer (25% v/v) and another cycle of sonication are required. Keep the samples on ice bath during the sonication to prevent heat-causing denaturing or protein degradation.

- 11 Centrifuge the lysed cells at 4 °C at 21,000 g for 30 min and recover the supernatant.
- 12 Pass the supernatant through the detergent removal spin column and elute the protein sample with Tris-HCl buffer (follow the manufacturer's protocols).
This step is necessary for removal of the detergents in the RIPA, which otherwise inhibit subsequent enzymatic labeling reaction.
- 13 Quantify protein concentration harvested above using Bradford assay.
- 14 Dilute the sample to a final concentration of 2 mg protein/ml with the Tris-HCl buffer.

*Transfection efficiency can be confirmed by Western blot at this stage (Gallagher et al., 2008). Once the cells are lysed, the resultant samples should be used immediately. Otherwise, stop at Step 8 in **BASIC PROTOCOL 2**. For the large scale preparation, we recommend first performing the lysis and Western blot with a small scale of 15 µl RIPA buffer and 1.5×10^5 cells to confirm transfection efficiency.*

BASIC PROTOCOL 3

PROTEOME-WIDE BPPM USING IN-GEL FLUORESCENCE

Upon the transfection of engineered full-length G9a, the resultant cell lysates were treated with Ab-SAM as described below. The proteome-wide substrates of G9a are expected to carry the 'clickable' 4-azidobut-2-enyl moiety. These labeled targets were subject to mild copper-free, strain promoted azide-alkyne cycloaddition (SPAAC) with commercially available fluorescent probe TAMRA-DIBO (Invitrogen). The TAMRA-DIBO-conjugated G9a substrates can then be resolved by SDS-PAGE and visualized with in-gel fluorescence.

Materials

Freshly prepared 2 mg/ml cell lysates (2×10^6 cells from T-25 flask obtained from **BASIC PROTOCOL 2**)

Ab-SAM stock solution, 8 mM stock in 0.01% (v/v) TFA and DD water (Islam et al., 2012). The acidity of the storage solution is important for Ab-SAM's stability. With this buffer, this material can be stored at -80°C for < 3 months. The degradation of Ab-SAM is often observed after a long-term storage.

Tris-HCl buffer (see **Recipe**)

5 mM stock solution of TAMRA-DIBO alkyne (Invitrogen, C10410) in DMSO

1 × protein loading buffer (see **Recipe**)

Fluorescent protein molecular weight ladder (15 – 250 kDa, Bio-Rad)

12-well 4% to 12% Tris-HCl protein gel (Criterion XT Precast Gel, Bio-Rad)

1 × electrophoresis buffer MOPS (Bio-Rad)

Coomassie Blue staining reagent (Bio-Rad)

Fluorescent gel destaining solution: 4:1:5 (v/v/v) methanol/acetic acid/DD water

1.5 ml microcentrifuge tubes
2 ml detergent removal spin columns (Pierce, cat. No: 87778)
Refrigerated microcentrifuge (4 °C)
70 °C heating block
SDS-PAGE Electrophoresis gel dock for Criterion XT Precast Gel (Bio-Rad)
Fluorescence gel scanner equipped with the excitation wavelength at 532 nm, < 580 nm cut-off filter and 30 nm band-pass (Amersham Biosciences Typhoon 9400)

Whole cell lysate enzymatic labeling

The concentration of Ab-SAM and the reaction time described below were customized for the full-length G9a Y1154A mutant in the context of HEK293T cells. These parameters should be optimized for individual PMTs and cell lines of interest. The concentrations of Ab-SAM and the reaction time were evaluated systematically. In general, start with low concentration of cofactors and short reaction time, and gradually increase until desirable signal-to-noise ratios are reached. For certain PMTs with low activities, in-gel fluorescence is not a good choice of BPPM to reveal targets. BASIC PROTOCOL 4 is more suitable for these scenarios.

- 1 Dispense 40 µg cell lysates of each sample (20 µl of 2 mg/ml stock) into 1.5 ml microcentrifuge tubes. Treat the samples with 250 µM Ab-SAM (0.625 µl of the 8 mM stock).

Using less than 40 µg protein can make subsequent precipitation steps difficult. Steps 1–5 should be carried out with minimal time intervals, otherwise causing the gradual loss of labeling efficiency for unknown reasons.

- 2 Incubate the samples at ambient temperature (23 °C) for 2 h.
- 3 Pass the reaction mixture through the 2 ml detergent removal spin column and elute the proteins with Tris-HCl buffer according to the manufacturer's protocol.

The purpose of this step is to remove excess cofactor.

Click reaction with TAMRA-DIBO probe

- 4 Add 100 µM TAMRA-DIBO probe (0.4 µl of the 5 mM stock) and incubate the reaction mixtures in the dark at ambient temperature (23 °C) for 1 h.

TAMRA dye is light sensitive. Keep the samples minimally exposed to light thereafter.

- 5 Quench the 'click' reaction and precipitate the proteins (see **SUPPORTING PROTOCOL 2**).

Sample preparation for SDS-PAGE

- 6 Dissolved the precipitated protein pellets in 50 µl freshly-prepared 1 × loading buffer by vortexing the samples for 10 s, followed by centrifugation at 5,000 g for 1 min.
- 7 Denature the protein samples using a 70 °C heat block for 10 min.

Cover the sample tubes with tin-foil during the heating process to avoid degradation of TAMRA dye. Keep the temperature of the heat block at 70 °C in the denaturing step due to dye degradation at higher temperatures.

- 8 Vortex the samples for 10 s and centrifuge at 5,000 g for 1 min.
- 9 Load 25 µl samples and the 15–250 kDa fluorescent protein ladders onto SDS-PAGE gel submerged in 1 × MOPS buffer.

Dilute the commercial fluorescent protein ladder at least 10-fold and load the minimal amount for visualization. We also recommend leaving an empty lane between the protein ladder and protein samples to minimize the undesired effect of overflow.

- 10 Run the gel at 200 V for 55 min.

Place the gel box in the dark and cover it with tin-foil to minimize light-mediated dye decomposition.

Scan and destain gel

- 11 Scan the gel obtained above using a fluorescent gel scanner.

Use the mode with the excitation at 532 nm, < 580 nm cut-off filter and 30 nm band-pass. Amersham Biosciences Typhoon 9400 was used successfully for this purpose. In general, the background of the gel is high at this stage because of the non-specific labeling from the dye. The background labeling can be significantly reduced through the destaining step.

- 12 Destain the gel with the destaining solution consisting of 4:1:5 (v/v/v) methanol/acetic acid/DD water at ambient temperature (23 °C) for 4 h.
- 13 Repeat the scanning process of Step 11 above.

Steps 11–13 can be repeated multiple times until the images with desired signal-to-noise ratios were obtained.

- 14 After the desired images were obtained, the whole gel was stained with Coomassie Blue (Bio-Rad) to confirm that equal amounts of protein were loaded on each lane.

In case of protein misloading, repeat Step 9 and thereafter after adjusting the protein concentrations

BASIC PROTOCOL 4

SAMPLE PREPARATION OF PROTEOME-WIDE BPPM FOR MS ANALYSIS

After incubating the cell lysates containing the full-length engineered G9a with Ab-SAM, the substrates of G9a are expected to be labeled enzymatically with the ‘clickable’ 4-azidobut-2-enyl moiety. The samples can be treated with TAMRA-DIBO (Invitrogen) as described in **BASIC PROTOCOL 3** for in-gel fluorescence visualization or Click-iT biotin-DIBO (Invitrogen) as described in the current protocol for streptavidin pulldown followed by MS analysis. We consistently observed that MS analysis of BPPM samples reveals more targets than in-gel fluorescence. More importantly, the MS analysis allows the release of protein identities for biological follow-up.

Materials

Freshly prepared 2 mg/ml cell lysates (1×10^7 cells from T-75 flask obtained from **BASIC PROTOCOL 2**)

Ab-SAM stock solution, 8 mM stock in 0.01% (v/v) TFA and DD water (Islam et al., 2012). The acidity of the storage solution is important for Ab-SAM's stability. With this buffer, this material can be stored at -80°C for < 3 months. The degradation of Ab-SAM is often observed after a long-term storage.

Tris-HCl buffer (see **Recipe**)

Bradford Assay reagents (Bio-Rad)

5 mM stock solution of Click-iT biotin-DIBO alkyne (Invitrogen, C10412) in DMSO

Ice-cold 0.5 % SDS (w/v) dilution buffer supplemented with EDTA-free Roche protease inhibitors (see **Recipe**)

Streptavidin agarose beads (GE healthcare, 17-5113-01)

Ice-cold 0.2 % SDS (w/v) PBS buffer (see **Recipe**)

Ice-cold 250 mM ABC buffer (ammonium bicarbonate)

Ice-cold PBS buffer (GIBCO)

8 M Urea

Freshly prepared 200 mM TCEP in DD water

Freshly prepared 400 mM idoacetamide in DD water

1 $\times$ loading buffer (see **Recipe**)

Fluorescent protein molecular weight ladder (15 – 250 kDa, Bio-Rad)

12-well 4% to 12% Tris-HCl protein gel (Criterion XT Precast Gel, Bio-Rad)

1 $\times$ electrophoresis buffer MOPS (Bio-Rad)

Coomassie Blue staining reagent (Bio-Rad)

15 ml and 50 ml conical polypropylene tubes

Turn-over-turn mixer

1.5 ml microcentrifuge tubes

Refrigerated centrifuge (4°C)

100°C heating block

SDS-PAGE Electrophoresis gel dock for Criterion XT Precast Gel (Bio-Rad)

Whole cell lysate enzymatic labeling

The following conditions have been optimized via in-gel fluorescence visualization as described in **BASIC PROTOCOL 3**)

- 1 Dispense 10 mg cell lysates for each sample (5 ml of 2 mg/ml stock) into 50 ml conical tubes. Treat the sample with 200 μM Ab-SAM (125 μl of the 8 mM stock).
- 2 Incubate the samples at ambient temperature (23°C) for 2 h.

- 3 Quench the reactions and precipitate the proteins with cold MeOH (see **SUPPORTING PROTOCOL 3**)

This step can also remove excessive cofactor. Steps 1–6 should be carried out with minimal time intervals, otherwise causing the gradual loss of labeling efficiency for unknown reasons.

Click reaction with biotin-DIBO probe

- 4 Dissolved the precipitated protein pellets in 5 ml freshly prepared 0.5 % SDS (w/v) dilution buffer (see **Recipe**).

Solubilizing pellets can be facilitated by repetitive pipetting and brief sonication.

- 5 Add 200 μ M biotin-DIBO alkyne (200 μ l of the 5 mM stock) and then gently shake the reaction mixture at ambient temperature (23 °C) for 1 h.
- 6 Quench the reactions and precipitate the proteins with cold MeOH (see **SUPPORTING PROTOCOL 3**)

This step can remove excess biotin-DIBO probe and thus avoid saturating streptavidin beads in a subsequent step.

Protein pulldown with streptavidin beads

- 7 Resolubilize the precipitated protein pellets in 3 ml freshly prepared 0.5 % SDS (w/v) dilution buffer (see **Recipe**).
- 8 Quantify protein concentrations using the Bradford assay.
Protein samples cannot be fully recovered after the precipitation steps.
- 9 Into a 50 ml conical tube, add 100 μ l packed streptavidin agarose beads for every 5 mg protein sample.
- 10 Wash the beads with 15 ml PBS buffer by inverting the tube several times, centrifuge the beads at 4,000 g at ambient temperature (23 °C) for 2 min, and then remove the supernatant. Repeat this step for 3 times.
- 11 Resuspend the beads in 100 μ l 0.5 % SDS (w/v) dilution buffer for every 100 μ l of beads.
- 12 Mix 200 μ l of the bead suspension with each protein sample with a turn-over-turn mixer for 1 h.
- 13 Centrifuge the beads at 4,000 g for 2 min and remove the supernatant.

Wash streptavidin beads

- 14 Resuspend the beads in 10 ml 0.2 % SDS (w/v) buffer, invert-mix the sample for several times and centrifuge them at 4,000 g at 4 °C for 2 min.
- 15 Discard the supernatant, wash the beads with 10 ml PBS, centrifuge the mixture at 4 °C at 4,000 g for 2 min and discard the supernatant. Repeat the step for 3 times.
- 16 Wash the beads with 10 ml ABC buffer, centrifuge the mixture at 4 °C at 4,000 g for 2 min, and discard the supernatant. Repeat the step for 3 times.

The extensive washing steps reduce the amount of proteins non-specifically bound to the beads.

Reduction and iodoacetamide capping of cysteine

- 17 Mix the beads with freshly prepared reduction buffer containing 500 μ l 8 M Urea, 25 μ l of 200 mM TCEP (freshly prepared) and 25 μ l of 400 mM iodoacetamide (freshly prepared). Incubate in the dark at ambient temperature (23 °C) for 40 min.

The purpose of this step is to reduce disulfide bonds of cysteines and cap them with the alkylation reagent iodoacetamide to prevent protein aggregation.

- 18 Add 10 ml ABC buffer and centrifuge the sample at 4 °C at 4,000 g for 2 min.
- 19 Remove the supernatant and resuspend the beads in 1 ml ABC buffer, transfer the suspension to a 2 ml Dolphin tube, and then centrifuge at 4 °C at 4,000 g for 2 min.

Protein elution from streptavidin beads

- 20 Remove the supernatant and resuspend the beads in 100 μ l 1 $\times$ loading buffer.
- 21 Place the tube on 100 °C heating block for 10 min.
- 22 Centrifuge the beads at ambient temperature (23 °C) at 4,000 g for 2 min.
- 23 Collect the supernatant, load 10 μ l elution protein on SDS-PAGE gel, followed by electrophoresis at 200 V for 55 min.
- 24 Stain gel with Coomassie Blue (Bio-Rad) according to the manufacturer's protocol.

We generally use small portion of the samples to run the gel to estimate the amount of the proteins pulled down. Following the confirmation step, we use the remaining sample for proteomic analysis.

- 25 Process the eluted protein for proteomic analysis according to standard protocols (e.g., (Islam et al., 2012)).

SUPPORTING PROTOCOL 1

SAMPLE PREPARATION FOR MALDI-MS

We used the following standard protocol to prepare peptide samples for MALDI-MS analysis. The sensitivity of the samples can vary according to the composition of the assay buffer. The presence of detergents in the assay buffer will interfere with subsequent MALDI-MS analysis. If detergent(s) has to be included for the optimal activities of examined enzymes, an alternative protocol should be used to process the samples for MALDI-MS analysis (see **ALTERNATIVE PROTOCOL 1**).

Materials

20 μ l *in-vitro* enzymatic reaction mixture containing peptide substrate in detergent-free buffer (**BASIC PROTOCOL 1**)

Saturated solution of α -Cyano-hydroxy-cinnamic acid (Protea Biosciences) in DD water

MALDI sample plate (ABSciex)

MALDI-MS (Time-of-flight, Voyager-DE STR, Applied Biosystems, Framingham, MA, USA with a 2.0-m flight tube)

1. Add 1 μ l of the enzymatic reaction mixture onto the MALDI sample plate.
2. Mix 1 μ l the saturated α -cyano-hydroxy-cinnamic acid with the enzymatic reaction mixture on the MALDI sample plate, and pipet the mixture up and down for 5 times.
3. Air-dry the sample completely at ambient temperature (23 °C).
4. Measure the MS signals of the sample with MALDI-MS.

In our case, desorption/ionization was obtained by using a 337-nm nitrogen laser with a 3-ns pulse width. Laser power was adjusted to slightly above threshold to obtain good resolution and signal/noise ratios. External standard Calibration Mix 2 (Applied Biosystems) was used for mass calibration. Each measurement was obtained by accumulating three spectra collected at ten different positions with 500 shots per position.

SUPPORTING PROTOCOL 2

PROTEIN PRECIPITATION WITH METHANOL/CHLOROFORM/WATER

The following is a standard protocol for protein precipitation with a mixture of 4:1:5 (v/v/v) methanol/chloroform/DD water. This precipitation protocol was implemented after click chemistry to remove recessive TAMRA-DIBO probe.

Materials

20 μ l cell lysates in a 1.5 ml microcentrifuge tube after the 'click' reaction (**BASIC PROTOCOL 3**)

An ice-cold mixture of 4:1:5 (v/v/v) methanol/chloroform/DD water

Ice-cold methanol

Refrigerated centrifuge (4 °C)

1. Add an ice-cold mixture of 600 μ l methanol, 200 μ l chloroform and 400 μ l water into the 20 μ l cell lysate.
2. Vortex the sample for 10 s and centrifuge at 4 °C at 21,000 g for 10 min.
3. Discard the upper aqueous layer carefully.

Discard as much of the aqueous layer as possible without disturbing the interface layer, where the precipitated proteins are located. A thin-layer, pink-colored precipitate should be visible between the upper aqueous phase and the lower chloroform phase.

4. Add another 450 μ l ice-cold methanol, vortex briefly and centrifuge at 4 °C at 21,000 g for 10 min.
5. Carefully remove the supernatant without disturbing the protein pellet.
6. Repeat Steps 4 and 5 twice.
7. Air-dry the precipitated pellet at ambient temperature (23 °C) in the dark for 25 min.

SUPPORTING PROTOCOL 3

PROTEIN PRECIPITATION WITH METHANOL

The following is a standard protocol for protein precipitation with methanol. This protocol was used to remove the residual Ab-SAM cofactor after the enzymatic reaction and the excessive Click-iT biotin-DIBO alkyne probe after the click reaction (**BASIC PROTOCOL 4**). This protocol was developed for large scale protein samples (> 5 mg).

Materials

5 ml reaction mixture (**BASIC PROTOCOL 4**)

Ice-cold methanol

Refrigerated centrifuge (4 °C)

1. Add 25 ml ice-cold methanol into a 50 ml conical tube containing the 5 ml reaction mixture.
2. Allow the proteins to precipitate at –80 °C overnight.
3. Collect the precipitated protein pellet by centrifuging the tube at 4 °C at 4000 g for 30 min.
4. Remove the supernatant carefully without disturbing the precipitated protein pellet.
5. Add an additional 25 ml ice-cold methanol into the tube and gently vortex for 10 s.
6. Centrifuge the tube at 4 °C at 4000 g for 30 min, and then remove supernatant carefully without disturbing the protein precipitated pellet.
7. Repeat Steps 5 and 6 twice.
8. Air-dry the protein pellet at ambient temperature (23 °C) for 20 min.

It is important to avoid drying the pellet longer than 20 min, because it is difficult to redissolve fully-dried protein pellet in the subsequent procedures.

SUPPORTING PROTOCOL 4

The following protocol provides a detailed synthesis, purification, and characterization of Ab-SAM and its precursors. The similar synthesis of Ab-SAM was also described in the previous work (Islam et al., 2011).

Synthesis of (E)-1-azido-4-bromobut-2-ene

In a round-bottom flask, 2 g of (E)-1,4-dibromobut-2-ene (9.34 mM) was placed and dissolved with THF (10 mL). 800 mg of sodium azide (12.2 mM) was dissolved in 1.5 mL water and added to the above mentioned solution of (E)-1,4-dibromobut-2-ene. Reaction was carried out for overnight at ambient temperature (23 °C). Diethyl ether of 100 mL was then added to the reaction and successively washed with 15 mL water and 15 mL saturated NaCl solution. Organic layer was dried with anhydrous Na₂SO₄ overnight and then evaporated under reduced pressure. Residue was purified by silica gel column chromatography using gradient petroleum ether-dichloromethane (DCM) (1~5%) solvent system to furnish 800 mg of (E)-1-azido-4-bromobut-2-ene (4.6 mM, 49%) and 250 mg of (E)-1,4-diazidobut-2-ene (1.8 mM, 19%) as colorless liquids. 165 mg of starting material (E)-1,4-dibromobut-2-ene (0.77 mM) was also recovered. *R_f*: 0.25 (5% DCM in hexane) ¹H-NMR (500 MHz, CDCl₃): δ 6.03-5.97 (m, 1H), 5.85-5.79 (m, 1H), 3.97 (d, 2H,

$J=7.45$ Hz), 3.81 (d, 2H, $J=5.85$ Hz). $^{13}\text{C-NMR}$ (125 MHz, CDCl_3): 130.9, 128.1, 51.6, 30.9.

Synthesis of 4-Azidobut-2-enyl S-Adenosyl-L-methionine (Ab-SAM)

12 mg of *S*-adenosyl-*L*-homocysteine (SAH) (0.031 mM) was placed in a capped 4 mL glass vial and dissolved into a freshly prepared 1 mL mixture of formic and acetic acids (1:1) and placed on an ice bath. 538 mg of (*E*)-1-azido-4-bromobut-2-ene (3.1 mM) and 5.4 mg of AgClO_4 (0.031 mM) were added to the acidic solution of SAH. Ice bath was removed after 5 min and the reaction was allowed to warm to ambient temperature (23 °C). Reaction progress was monitored by analytical reversed-phase HPLC (XBridge C18 5 μm 4.6 $\times$ 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. To drive the reaction to completion, 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 approximately after 5 h. The reaction was quenched by adding 20 mL of distilled water containing 0.01% TFA (v/v). The aqueous phase was washed with diethyl ether (3 $\times$ 15 mL) and then filtered through a Nalgene 0.2 μm syringe filter. Ab-SAM **2** was subsequently purified with preparative reversed-phase HPLC (XBridge Prep C18 5 μm OBD 19 $\times$ 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%). The diastereomeric mixture of Ab-SAM was collected and concentrated by SpeedVac for 2 h to remove acetonitrile, followed by overnight lyophilization. Ab-SAM **2** was redissolved in water containing 0.01% TFA (v/v) and stored at -80°C before use. Concentration of Ab-SAM was determined by UV absorption with $\epsilon_{260} = 15,400 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$. The compound as isolated in 40% yield. $T_R = 10\text{min}$. ^1H NMR (600 MHz, D_2O): δ 8.41 (s, 0.5H), 8.39 (s, 1H), 8.38 (s, 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 $\text{C}_{18}\text{H}_{26}\text{N}_9\text{O}_5\text{S}$: 480.1778; found: 480.1759.

ALTERNATIVE PROTOCOL 1

MALDI-MS ASSAY WITH PEPTIDE SAMPLE CONTAINING DETERGENT(S)

Although the catalytic domain of G9a and its Y1154A mutant are active in the reaction buffer containing no detergent (**BASIC PROTOCOL 1**), a small amount of detergent(s) in the reaction buffer can be essential for certain PMTs to be fully active (unpublished results of Luo group). Given that the detergent(s) may interfere with subsequent MALDI-MS analysis, the following protocol was developed to remove the detergent(s).

Materials

20 μL *in vitro* enzymatic reaction mixture containing peptide substrate (**BASIC PROTOCOL 1**)

Saturated solution of α -cyano-hydroxy-cinnamic acid (Protea Biosciences) in DD water: acetonitrile (1:1)

1:1 (v/v) mixture of acetonitrile and 0.1 % (v/v) TFA/DD water

C18 cartridge pipette tips (Waters)

MALDI sample plate (ABSciex)

MALDI-MS (Time-of-flight, Voyager-DE STR, Applied Biosystems, Framingham, MA, USA with a 2.0-m flight tube)

1. Pipet 20 μ l enzymatic reaction mixture through a C18 cartridge pipette tip for 10 times. Elute the peptide from the C18 cartridge with 1:1 (v/v) mixture of acetonitrile and 0.1 % (v/v) TFA/DD water.
2. Add 1 μ l of the peptide elute onto the MALDI sample plate.
3. Mix 1 μ l of the saturated α -cyano-hydroxy-cinnamic acid with the peptide elute on the MALDI sample plate, and pipet the mixture up and down for 10 times.
4. Air-dry the sample completely at ambient temperature (23 °C).
5. Measure the MS signals of the sample with MALDI-MS.

REAGENTS AND SOLUTIONS (RECIPES)

Deionized distilled water (DD water) was used unless mentioned otherwise.

Modified RIPA lysis buffer supplemented with EDTA-free protease inhibitors

This buffer was prepared by adding 5 mM freshly-prepared TCEP (the final concentration) and 1 $\times$ EDTA-free Roche protease inhibitor cocktail into RIPA buffer (Sigma) prior to use.

Tris-HCl Buffer

This buffer contains 50 mM Tris-HCl, prepared from 1 M stock (pH = 8.0), and 10% (v/v) glycerol. This mixture can be stored at ambient temperature (23 °C) up to 6 months. 2 mM freshly-prepared TCEP (the final concentration) and 1 $\times$ EDTA-free Roche protease inhibitor cocktail are added immediately prior to its use.

1 $\times$ loading buffer

This buffer containing 40 mM Tris-HCl (pH = 6.8), 69 mM sodium dodecyl sulfate (SDS). 10% (v/v) β -mercaptoanol (the final concentration), and 10 % (v/v) glycerol (the final concentration).

0.5 % SDS (w/v) dilution buffer supplemented EDTA-free Roche protease inhibitors

This buffer contain 50 mM triethanolamine, prepared from 1 M stock (pH = 7.4), 150 mM NaCl, and 0.5% (w/v) sodium dodecyl sulfate (SDS). This mixture can be stored at ambient temperature (23 °C) up to 12 months. 1 $\times$ EDTA-free Roche protease inhibitor cocktail is added immediately prior to its use according to the manufacturer's instructions.

0.2 % SDS (w/v) PBS buffer

This buffer was prepared by supplementing GIBCO's PBS with 0.2 % (w/v) SDS.

COMMENTARY

Background Information

Small-molecule chemical reporters coupled with bioorthogonal ligation reactions have recently demonstrated broad application in identifying the substrates of protein posttranslation modifiers (*e.g.* glycosylation, myristoylation and palmitoylation) (Charron et al., 2009; Prescher and Bertozzi, 2006; Yount et al., 2010). Meanwhile, the active-site engineering for identifying substrates of a specific posttranslation modifier has been

successfully documented for various kinases (Bishop et al., 2001; Kapoor and Mitchison, 1999; Lin et al., 2001). The two concepts were merged to develop Bioorthogonal Profiling of Protein Methylation (BPPM) to dissect substrate profiles of designated PMTs. Several previous attempts were made for global profiling of methylation substrates of a specific PMT with radiolabeled SAM as the cofactor and arrayed peptides/proteins as substrate candidates (Levy et al., 2011; Rathert et al., 2008). PMT-knockout proteome was also used as substrate candidates upon profiling PMT substrates (Rathert et al., 2008). However, because of the lack of enrichment strategy after target labeling, these methods are not suitable to detect less abundant methylation targets in biologically-relevant cellular contexts (Luo, 2012). This situation then triggered the development of the SAM analogue cofactors that contain terminal azido/alkynyl moieties (Binda et al., 2011; Dalhoff et al., 2006; Lee et al., 2010; Osborne et al., 2008; Peters et al., 2010; Stecher et al., 2009). These SAM analogues were shown to be active toward certain native PMTs and thus label the PMT substrates with the terminal azido/alkynyl groups. Such labeling provides suitable handles for target enrichment by attaching alkyl/azido-containing biotin probes via the azide-alkyne cycloaddition reaction (CuAAC or click chemistry) (Rostovtsev et al., 2002; Tornøe et al., 2002). Our BPPM technology significantly expands generality and efficiency of the SAM analogues as cofactor surrogates to profile PMT targets in various cellular contexts.

Critical Parameters and Troubleshooting

In order to engineer a designated PMT to process SAM analogue cofactors, the PMT construct should be readily expressed and purified exogenously. Native construct of the PMT should show decent activity *in vitro* toward known peptide substrates, a situation that allows rapid screening by simply replacing the native PMT and SAM cofactor with engineered PMTs and SAM analogues under the same conditions. Optimizing reaction conditions *in vitro* is critical for establishing the assay parameters to assure the maximal signal when less reactive enzyme-cofactors are used.

The experimental parameters of transient transfection such as cell confluency and the amount of plasmid used can alter significantly between cell types and among PMTs of interest. These parameters need to be optimized for each PMT construct of interest. Overexpression of designated PMTs in certain cell lines could be toxic. In that case, multiple cell lines should be examined to assure optimal transfection efficiency and the balanced toxicity. When working on large scale transfection (using multiple T-75 flasks), we strongly recommend examining a small aliquot of cells to confirm the transfection efficiency with Western blot before proceeding with the protocol(s). The cell lysates should not be stored and need be used immediately. We also recommend using freshly harvested cells for the proposed experiment and often observe the gradual loss of enzymatic activities if the cells are frozen for a long period of time. In general, such cells should not be stored for > 2 months.

It is essential using repetitive gel destaining steps to improve signal-to-noise ratios and thus better visualize the labeled proteins via in-gel fluorescence (**BASIC PROTOCOL 3**). Residual nonspecifically-bound TAMRA dye in gel lanes leads to high background, which can be removed by multiple washing steps. High quality reagents should be used to prepare the destaining solution to avoid general contaminants. In addition, SDS gels should be handled with great caution only at the edge areas. Physical manipulation of SDS gels leaves fingerprints on the gel, which will become an issue for in-gel fluorescence. Using the recommended amount of proteins is also crucial. Insufficient loading or overloading can cause weak signals or abnormal gel bands, respectively.

For proteome-wide BPPM with MS (**BASIC PROTOCOL 4**), 5 to 10 mg of Ab-SAM-treated cell lysates are recommended. Performing the protocol with a smaller amount than

recommended may limit the detection of low abundant proteins. It is essential to include multiple methanol washing steps after each precipitation step to remove undesired components. After the treatment with Ab-SAM, the washing steps remove the unreacted cofactor, which would otherwise consume the biotin-DIBO probe in the subsequent click reaction. After the click reaction, the methanol washing removes the unreacted biotin-DIBO, which would otherwise compete with biotinylated proteins for the streptavidin beads in the subsequent step of streptavidin pulldown. Fully dissolving precipitated protein pellets is essential and can be achieved by repetitive pipetting with SDS-containing buffers. If this step were an issue, mild sonication could be included. All the steps should be carried out without significant interval unless mentioned otherwise. We noticed that the prolonged intervals from Steps 1–5 (**BASIC PROTOCOL 3, 4**) cause the gradual loss of labeling efficiency for unknown reasons.

Anticipated Results

For **BASIC PROTOCOL 1**, the active enzyme-cofactor pairs are expected to convert the peptide substrate into the corresponding alkylated product (Fig. 3a). The pair of the unmodified and modified peptides will be reflected in MS. To be considered a positive signal, the intensity of the modified peptide needs to account for at least 10 % of the unmodified peptide. For near to 100 % modification, the unmodified peak will disappear completely as shown for G9a Y1154A mutant paired with Ab-SAM (Fig. 3b). The peptide peaks should show the characteristic isotopic distribution, which allows differentiating peptides from noise signals. Magnitude of modification can depend on the amount of cofactor and peptide used. The signal increase correlated with these parameters can be used to further confirm the positive hits.

For the ideal visualization via in-gel fluorescence (**BASIC PROTOCOL 3**), the control lanes (cells transfected with empty vectors) should show negligible amount of fluorescence labeling in contrast with the sample lanes (cells transfected with the active G9a variant), while the Coomassie staining should be comparable to show that the equal amount of total proteins were loaded (Fig 3c). If both control and sample lanes show weak fluorescence labeling, increasing the amount of loaded proteins in both lanes may result in more pronounced signal. However, it is possible that both control and sample lanes show comparable fluorescence labeling with no distinct band shown in the sample lane. Such situation would indicate either that the engineered PMT constructs are inactive in cellular contexts or that the targets of PMTs in cellular contexts cannot be readily detected via in-gel fluorescence. For either situation, it is worth performing proteome-wide BPPM with MS for further validation, given the higher sensitivity of MS (**BASIC PROTOCOL 4**). In an ideal situation, the signal lane in contrast to the control should display more labeling bands with Coomassie staining (Fig 3d). In certain situation, such differences cannot be distinguished by Coomassie staining. We therefore recommended performing the proteomic analysis regardless of the results of in-gel fluorescence and Coomassie staining. With the current protocol, we were able to identified > 100 targets, more than what are shown by in-gel fluorescence and Coomassie staining.

Time Considerations

BASIC PROTOCOL 1 can be completed within 1 h (depending on the number of samples tested). It takes 3 d to complete the cell culturing and harvest sessions in **BASIC PROTOCOL 2**. At this stage, the cells can be stored at –80 °C for a few weeks. Preparing resultant cell lysates can be completed in 2 h and must be immediately followed by **BASIC PROTOCOL 3** and **BASIC PROTOCOL 4**. The control experiments using Western blot to confirm the level of the expression can be performed in the same day after harvesting cells. It takes 3.5 h to reach the step of methanol/chloroform/water precipitation and washing, and

1.5 h to resolubilize the protein precipitates and run the SDS-PAGE gel in **BASIC PROTOCOL 3**. The precipitated proteins can be stored at -80°C for a few days before use. Destaining gel can be performed overnight. It takes 3 d to go through **BASIC PROTOCOL 4**. Briefly, the enzymatic reaction can be completed within 2 h followed by methanol precipitation. This step can be performed in the same day as the experiment of in-gel visualization in **BASIC PROTOCOL 3**. Each precipitation step needs to be carried on overnight to assure the high recovery of the proteins. The precipitated proteins can be stored at -80°C for a few days before use. Subsequent washing step takes around 1.5 h, followed by 1 h click reaction. After redissolving the biotinylated protein pellet, the following steps in **BASIC PROTOCOL 4** need to be carried out continuously. Streptavidin pulldown, washing and elution of labeled PMT substrates take 6 h. The resultant proteins can be stored at -80°C before MS-based proteomic analysis.

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Figure 1.

General principle of BPPM and corresponding workflow chart. **(a)** Schematic representation of BPPM by engineering a native PMT to utilize a synthetic SAM analogue cofactor. **(b)** Chemical entities used for BPPM. Compound 1, *S*-adenosyl-*L*-methionine (SAM); Compound 2, 4-azido-2-enyl *S*-adenosyl-*L*-methionine (Ab-SAM) with its transferrable azido moiety highlighted in blue; Compound 3, TAMRA-DIBO alkyne (Invitrogen) for the click conjugation and in-gel fluorescence; Compound 4, biotin-DIBO alkyne (Invitrogen) for the click conjugation and streptavidin pulldown. **(c)** Workflow chart for BPPM via in-gel fluorescence or proteomic analysis. **BASIC PROTOCOL 1** for the screening of PMT variants against SAM analogues; **BASIC PROTOCOL 2** for transient transfection of active variants construct and the production of corresponding cell lysates. **BASIC PROTOCOL 3** and **BASIC PROTOCOL 4** describe enzymatic labeling of the cell lysates with the clickable Ab-SAM, followed by copper free, bioorthogonal ligation for in-gel fluorescence imaging or target enrichment.

a

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GLP      --MGWGVRS LQDIPPGTFVCEYV GELISDSEADV R-----EEDS--YLFDDL NKGDEVY 1155
G9a      --MGWGVRLQTIPQGT FICEYV GELISDAEADV R-----EDDS--YLFDDL NKGDEVY 1098
SUV39H1  G-RGWGVRTLEKIRKNSFVMEYV GELITSEEAE RRGQIYDRQGAT--YLFDDL DYVED-VY 308
SET8     --KRGVVIATKQFSRGDFVVEYH GDLIEITDAKKREALYAQDPSTGCYMYFYQYLS-KTY 282
SET 7/9  SSAGEGLFSKVAVGPNVTVMSEFYNGVRITHQEVD SRDWALNGNTLS---LDEETVIDVPEP 280

GLP      CIDARFYGN-VSRFINHHCEPNLVPVRVFM AHQDLRFPRIAFFSTR LIEAGEQLGFDYGERFWDIKG 1220
G9a      CIDARYYGN-ISRFINHLCDPNLIPVRVFM LHQDLRFPRIAFFSSRD IRTGEELGFDYGDRFWDIKS 1163
SUV39H1  TVDAAYYGN-ISHFVNHS CDPNLQVYNVFIDN LDERLPRIAFFATRTIR AGEELTFDYNMLEQSR LA 374
SET8     CVDATRETNRLGR L INH SKCGNCQTK---LHDIDGVPH LILIASRDIA AGEELLYDYGDRSKAS IE 345
SET 7/9  YNHVSKYCASLGH KANHSFTPNCIYDMFVHP RFG---PIKCIRTLRAVEADEELTVAYGYDHSPPGK 344

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b **Figure 2.**

Rational engineering of G9a for bulky SAM analogue cofactors. **(a)** Sequence alignment of SET domain PMTs with the conserved active sites highlighted. **(b)** Schematic ball-and-stick representation of the active site of G9a (PDB 2O8J) bound with the reaction product dimethylated H3K9 substrate and the reaction byproduct SAH. The hydrophobic residues adjacent to SAM are highlighted.

Figure 3.

Anticipated results of BPPM with G9a and Ab-SAM as a paradigm (Islam et al., 2012). **(a)** Heat map analysis of % alkylation of H3K9 peptide substrate by G9a variants with Ab-SAM as a cofactor. **(b)** Representative MALDI-MS of H3K9 alkylated by G9a Y1154A mutant with Ab-SAM as the cofactor. **(c)** Representative in-gel fluorescence upon completing **BASIC PROTOCOL 3**. Here HEK293T cells were treated with pCDNA-FLAG full-length G9a Y1154A mutant or mock pCDNA3, followed by Ab-SAM and TAMRA-DIBO alkyne dye. The resultant labeled fluorescent proteins were resolved by SDS and visualized with in-gel fluorescence. **(d)** Coomassie staining of pulled down proteins upon completing **BASIC**

PROTOCOL 4. Here HEK293T cells were transfected with pCDNA-FLAG full-length G9a Y1154A mutant or the mock pCDNA3, followed by Ab-SAM and biotin-DIBO for 5 mg of 2 mg/ml cell lysates.