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Profiling Substrates of Protein Arginine N-Methyltransferase 3 with S-Adenosyl-L-methionine Analogues

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Abstract

Protein arginine *N*-methyltransferase 3 (PRMT3) belongs to the family of type I PRMTs and harbors the activity to use *S*-adenosyl-*L*-methionine (SAM) as a methyl-donor cofactor for protein arginine labeling. However, PRMT3's functions remain elusive with the lacked knowledge of its target scope in cellular settings. Inspired by the emerging Bioorthogonal Profiling of Protein Methylation (BPPM) using engineered methyltransferases and SAM analogues for target identification, the current work documented the endeavor to systematically explore the SAM-binding pocket of PRMT3 and identify suitable PRMT3 variants for BPPM. The M233G single point mutation transforms PRMT3 into a promiscuous alkyltransferase using sp²-β-sulfonium-containing SAM analogues as cofactor surrogates. Here the conserved methionine was defined as a hot spot that can be engineered alone or in the combination with nearby residues to render cofactor promiscuity of multiple type I PRMTs. With this promiscuous variant and the matched 4-propargyloxy-but-2-enyl (Pob)-SAM analogue as the BPPM reagents, more than 80 novel proteins were readily uncovered as potential targets of PRMT3 in the cellular context. Subsequent target validation and functional analysis correlated the PRMT3 methylation to several biological processes such as cytoskeleton dynamics, whose roles might be compensated by other PRMTs. These BPPM-revealed substrates are primarily localized but not restricted in cytoplasm, the preferred site of PRMT3. The broad localization pattern may implicate the diverse roles of PRMT3 in the cellular setting. The revelation of PRMT3 targets and the transformative character of BPPM for other PRMTs present unprecedented pathways toward elucidating physiological and pathological roles of diverse PRMTs.

INTRODUCTION

Arginine methylation is a post-translational modification that is associated with many essential biological processes including transcriptional regulation (1), RNA processing (2), DNA repair (3) and signal transduction (4). In the past decade, the epigenetic roles of dysregulated PRMTs have caught the increased attention because of their association with

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Supporting Information Available: Supplementary Figure 1–4 and Supplementary Tables 1–2, materials and methods. This material is free of charge via the internet at <http://pubs.acs.org>.

multiple human diseases including cancers (5). Arginine methylation generally occurs on the guanidino nitrogens, which can be subject to monomethylation (MMA), symmetric dimethylation (sDMA) or asymmetric dimethylation (aDMA) (6). These events are catalyzed by protein arginine *N*-methyltransferases (PRMTs) with the cofactor *S*-adenosyl-*L*-methionine (SAM) as the methyl donor. So far, 9 human PRMTs (PRMT1–9) have been documented (5). These PRMTs can be annotated further into three subtypes according to their product specificity: type I PRMTs (*e.g.* PRMT1, 2, 3, 4, 6 and 8) that catalyze the formation of MMA and aDMA; type II PRMTs (*e.g.* PRMT5 and 9) that catalyze the formation of MMA and sDMA; type III PRMT (*e.g.* PRMT7) that can only catalyze the formation of MMA (7, 8).

PRMT3 was identified through yeast two-hybrid assay as a PRMT1-interacting partner, although the direct formation of PRMT3-PRMT1 complex has not been proved *in vivo* (9). This enzyme is primarily localized in cytoplasm and widely expressed in human tissues as well as in other eukaryotic organisms such as mouse, fruit fly and fission yeast (10). The elevated level of PRMT3 is also found in myocardial tissue from patients with coronary heart disease (11). PRMT3 contains a catalytic core that is conserved among type I PRMTs for arginine methylation and several distinct N-terminal regulatory subunits including a consensus sequence for tyrosine phosphorylation and a C2H2 zinc finger motif (12). The zinc finger motif can interact with a ribosomal protein 40S rpS2 and the formation of this complex enhances the methyltransferase activity of PRMT3 (13). In contrast, PRMT3 also binds the tumor suppressor DAL-1/4.1B (Differentially-expressed in Adenocarcinoma of the Lung) and this interaction inhibits PRMT3's enzymatic activity (14). Arginine methylation can reduce DAL-1/4.1B-induced apoptosis in MCF-7 breast cancer cells, implicating the antagonistic role of PRMT3 on DAL-1/4.1B-involved tumor suppression (15).

PRMT3 harbors *in vitro* methylation activity on the substrates of type I PRMTs such as high-mobility group A1 protein (HMGA1) (16) and nuclear poly(A)-binding protein (PABPN1) (17), both of which contain characteristic arginine- and glycine-rich motifs (9). However, the ribosomal protein 40S rpS2 was the prior well-characterized target of PRMT3 in cellular contexts (18, 19). Given that the enzymatic activity of PRMT3 is regulated by its other binding partners as exemplified above by 40S rpS2 and DAL-1/4.1B (13, 14), the presence of accurate cellular settings can be important to recapitulate biologically relevant methylation events of PRMT3. To meet this criterion upon profiling the substrates of PRMT3, we were intrigued by the emerging Bioorthogonal Profiling of Protein Methylation (BPPM) technology. In BPPM, designated methyltransferases are engineered to gain the function to process sulfonium-alkyl SAM analogues as alternative cofactors in the context of complex cellular components (20–22). The distinct sulfonium alkyl handles of the cofactor surrogates, such as those containing a terminal-alkyne for the azide-alkyne Huisgen cycloaddition (the click reaction), will then be transferred to the substrates for amenable target enrichment and characterization (21–23). Although the BPPM technology was successfully implemented to protein lysine methyltransferases, only the proof-of-principle effort has been made for developing the corresponding strategy for PRMTs (21, 22).

Here we reported a systematic approach to screen human PRMT3 mutants and identify its gain-of-function variant to process SAM analogues for substrate labeling (Figure 1). The M233 residue of PRMT3 was characterized as the hot spot that can be tailored for BPPM. Strikingly, the comparable methionine mutants of PRMT1, a PRMT3 homologue, showed resemblant but not identical characters toward SAM analogues, underscoring the difference among the closely-related PRMTs. With the single point M233G mutant and the matched 4-propargyloxy-but-2-enyl (Pob)-SAM analogue as the BPPM reagents, around 80 novel targets of PRMT3 were readily identified from the proteome of HEK293T cells with a panel of selected targets validated with native PRMT3 and SAM. Revealing the full spectrum of

PRMT3 targets is expected to be an unprecedented step toward elucidating the biological roles of PRMT3 in the cellular setting.

RESULTS AND DISCUSSION

Rationale of engineering PRMT3 toward promiscuous recognition of SAM analogues

The conserved catalytic cores of type I PRMTs (PRMT1, 2, 3, 4, 6 and 8) have two motifs: the substrate interacting motif featured by a double-Glu loop and a THW loop for substrate recognition and enzyme catalysis, and the SAM binding motif, which is typically occupied by *S*-adenosyl-*L*-homocysteine (SAH) in crystal structures (*e.g.* PDB: 2FYT of human PRMT3 in Figure 2). A prior proof-of-concept effort showed that the conserved Met48 and Tyr39 in PRMT1's SAM binding motif (equivalent to Met233 and Tyr224 in PRMT3; Figure 2A) could be engineered to accommodate bulky SAM analogues (22). Other conserved residues in PRMT3 – Ile229, His230, Tyr243 and Met340 – are also involved in the cofactor recognition as revealed by its structure in complex with SAH (Figure 2B). To explore these SAM-recognition residues for cofactor promiscuity, we systematically mutated PRMT3's Tyr224, Ile229, His230, Met233, Tyr243 and Met340 into smaller hydrophobic residues (Group I in Figure 3: *e.g.* Gly, Ala, Val), larger hydrophobic residues (Group II in Figure 3: *e.g.* Trp) and polar residues (Group III in Figure 3: *e.g.* Ser, Thr, Asn, Gln). This molecular editing is expected to alter PRMT3's SAM binding motif to the degree that allows certain variants to process bulky sulfonium-alkyl SAM analogues as alternative cofactors.

To identify SAM derivatives as active cofactors of PRMT3 mutants, a collection of such candidates with varied sulfonium-alkyl substituents were synthesized as described previously (20, 22–24). These compounds include propyl, allyl, 2-butynyl, (*E*)-pent-2-en-4-ynyl (EnYn), and 4-propargyloxy-but-2-enyl (Pob)-SAM (compounds 2–6 in Figure 3,4). In particular, EnYn-SAM 5 and Pob-SAM 6 contain a terminal alkyne moiety that, after being transferred to substrates, is amenable for the conjugation with azido-containing fluorescent dyes or biotin probes for target characterization (22, 23).

Primary fluorogenic assay to screen active enzyme-cofactor pairs

To systematically examine the compatibility of the large collection of PRMT3 mutants toward SAM 1 and the SAM analogues, a previously established fluorogenic assay was implemented for the primary screening (25). Here, the two coupling enzymes, SAH hydrolase (SAHH) and adenosine deaminase (ADA), were used to irreversibly convert the commonly shared byproduct SAH of transalkylation reactions into inosine and homocysteine. The latter was then quantified by 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) with the increased fluorescence as readout. Using an Arg- and Gly-rich RGG peptide as a substrate, native PRMT3 and > 50 single point PRMT3 mutants were screened against SAM 1 and its analogues 2–4 in a combinatorial manner. The robustness of the current assay to detect the formation of the transalkylation byproduct SAH was confirmed by the strong fluorescent readout of native PRMT3 and SAM (Figure 3).

Identification of active enzyme-cofactor pairs

Among the > 50 examined PRMT3 mutants, the M233G variant from Group I displayed the highest activity toward the bulky SAM analogues (Figure 3). Except for propyl-SAM 2, which is inert to the whole set of PRMT3 mutants, the M233G variant can efficiently process allyl-SAM 3 and 2-butynyl-SAM 4, as evidenced by the strong signals in the fluorogenic assay. Although I229A, I229S, H230C, H230Q, M233L, M233T and M340F variants also displayed detectable activities – reflected by their modest fluorescence readout – toward either allyl-SAM 3 or 2-butynyl-SAM 4, none of them gave comparable

fluorescence signals as the M233G variant. Replacing the Gly with any other amino acid (*e.g.* small hydrophobic Ala, Val, Leu and Ile; bulky hydrophobic Phe and Trp; polar Ser, Cys and Thr) either dramatically decreases or completely abolishes the ability of the M233G variant to act on the bulky SAM analogues. In contrast with native PRMT3, the M233G variant, as well as the Y224F, I229V, and Y243F mutants, partially maintains the activity toward the native cofactor SAM **1**, indicating that these residues are not essential for PRMT3's methylation activity. Because native PRMT3 is barely active toward allyl-SAM **3** and inert to other examined bulky SAM analogues, the M233G single point mutation transforms PRMT3 into a promiscuous enzyme with a gained activity toward bulky SAM analogues.

Given that several variants of Y224, I229 and H230 displayed detectable promiscuity to recognize SAM analogues and these residues are adjacent to the key M233 residue, we generated a small collection of double point mutants by combining the key M233G site with one of these secondary sites (Group IV in Figure 3). Here the introduction of any second-site mutation either barely maintains (*e.g.* I229A/M233G and H230A/M233G) or abolishes the activity of the M233G variant toward SAM **1** and SAM analogues **2–4** (Group IV in Figure 3). Furthermore, among the examined SAM analogues, sp^2 - β -sulfonium allyl-SAM **3** was shown to be more active than propyl-SAM **2** and 2-butyryl-SAM **4** toward various PRMT3 mutants. This observation is consistent with the previous finding that the β -sulfonium sp^2 substituent is essential for the transalkylation activity, likely through stabilizing the tentative S_N2 transition state (20, 22, 23). Collectively, PRMT3 M233G mutant was identified as the most suitable variant to utilize sp^2 - β -sulfonium-containing SAM analogues as cofactor surrogates for target labeling.

Validate active enzyme-cofactor pairs through secondary MS and in-gel fluorescence assays

To further validate the cofactor promiscuity of the M233G variant, we implemented a secondary MALDI-TOF MS assay to examine its ability to process two bulkier SAM analogues (EnYn-SAM **5** and Pob-SAM **6**) that contain the transferable terminal alkyne for the click reaction as well as the sp^2 - β -sulfonium for transalkylation activation (Figure 4, S1). The anticipated modifications of EnYn-SAM **5** and Pob-SAM **6** on the RGG peptide were readily detected (Figure 4, S1A and S1B). Compared to allyl-SAM **3**, 2-butyryl-SAM **4** and EnYn-SAM **5**, Pob-SAM **6** is a more active SAM analogue as evidenced by both its predominant mono- and di-alkylation products (likely on two Arg residues for the latter, Figure S1B) and the least inhibition by the transalkylation byproduct SAH (Figure 4, S1). Replacing M233 with either Ala or Leu or adding a secondary point mutation such as Y224A and Y224F (a double-point mutant of PRMT1 at the equivalent sites is active as will be discussed later) significantly hindered the ability of PRMT3 M233G variant to process the two bulky SAM analogues (Figure 4). A similar trend was also observed for PRMT3 M233G mutant to process less bulky SAM analogues allyl-SAM **3** and 2-butyryl-SAM **4** (Figure 3).

Given that PRMT3 M233G variant maintains residual activity towards native SAM **1** (Figure 3), a competition assay between native SAM **1** and Pob-SAM **6** was performed to compare directly their activities as the cofactors of the M233G variant. In the presence of the equivalent amount of **1** and **6**, the 4-propargyloxy-but-2-enylated peptide derived from **6** was the dominant product (Figure S2). To compare the activities of the M233G variant and native PRMT3 to process Pob-SAM **6** and SAM **1**, respectively, 1:1 ratio of the M233G and native PRMT3 were incubated with the physiologically relevant concentration of SAM (27) and Pob-SAM (50 μ M). Consistent with the existence of multiple Arg sites of the RGG peptide for modification, the majority of the peptide products carried both methylation and

4-propargyloxy-but-2-enylation with their overall ratio of 1:1.3 (Figure S2). This observation indicated that the M233G mutant can act on Pob-SAM **6** in a comparable efficiency with native PRMT3 on the native cofactor SAM. These findings therefore highlighted the M233G variant and Pob-SAM **6** as the efficient enzyme-cofactor pair for target labeling even in the presence of native PRMT3 and SAM.

With the M233G variant and Pob-SAM **6** as potential BPPM reagents, we then examined their activity on a known protein substrate of PRMT3, GST-GAR (the Arg- and Gly-rich domain of human fibrillarin) (9). After incubating GST-GAR with the enzyme-cofactor pair, followed by the click conjugation with tetramethylrhodamine (TAMRA) azide fluorescent dye (Figure 5A), TAMRA-labeled GST-GAR product was readily visualized via in-gel fluorescence (Figure 5B). The absence of Pob-SAM **6** gave undetectable in-gel fluorescence signal, indicating that the nonspecific labeling of the click chemistry is negligible under the current setting (Figure 5B). In contrast, a slight background labeling arises from Pob-SAM **6** even in the absence of PRMT3 M233G mutant (Figure 5B). Here we reasoned that the nonspecific background labeling originates from thiol residues of certain reactive cysteines, as evidenced by its disappearance if GST-GAR was pre-treated with the Cys blocking reagent iodoacetamide (28) (Figure S3A). The labeling of GST-GAR by the enzyme-cofactor pair is time-dependent and reaches the saturation after 8-h incubation under the current setting (Figure S3B). The Pob-SAM **6**-labeled GST-GAR was also subject to tandem MS analysis (Figure 5A). The characteristic b_3 ion is consistent with the anticipated modification on an Arg of the GST-GAR substrate (Figure 5C). Taken together, the abilities to act on the peptide and protein substrates confirmed the robust bioorthogonality and labeling efficiency of the PRMT3 M233G variant and Pob-SAM **6**.

BPPM of PRMT3 with engineered enzyme-cofactor pair

With the validated PRMT3 M233G variant and Pob-SAM **6** as the active enzyme-cofactor pair, we envisioned BPPM to reveal PRMT3 substrates (Figure 6). Here HEK293T cells were transfected with the vector of the full-length PRMT3 harboring the single point M233G mutation. Its expression level was confirmed by western blot (Figure S4A). The cells were then treated with adenosine-2',3',-dialdehyde (AdOx) to induce hypomethylated proteome (29). The hypomethylated cell lysates were processed with RNase (see discussion below), a step that was shown to be beneficial to uncover cellular substrates of PRMT3 (12). The resultant cell lysates containing the full-length engineered PRMT3 were incubated with Pob-SAM **6**, followed by the treatment of a chemically-cleavable azido-azo-biotin probe (Figure 6) (30). After the affinity enrichment with streptavidin and the selective cleavage of azo-biotin-immobilized proteins with sodium dithionite, the identities of PRMT3 substrates were readily revealed by MS analysis. Here empty-vector-transfected cells were processed similarly to reveal the background labeling, likely arising from the nonspecific reaction between Pob-SAM and certain reactive Cys (Figure S3A, S4B).

With a cut-off threshold of 10 for mass spectral scores derived from the numbers of recovered peptides and overall peptide coverage of individual identified proteins (Supporting Information), 79 distinct proteins were identified from the cell lysates containing PRMT3 M233G variant (Sample) but absent from the control containing the empty vector. Additional 4 proteins (score 10) were also designated as the BPPM-revealed substrates of PRMT3 because of their significantly higher scores than those in the control (50% increase). Among the total 83 protein targets revealed by BPPM is 40S rpS2, a known *in vivo* substrate of PRMT3 (Table S1) (13, 18). In addition, two proteins – TCOF1 and MYO18B – had their modified Arg sites identified (Table S2). The little recovery of the peptide sequences harboring modified Arg sites can be attributed to either their susceptibility to workup conditions (*e.g.* protease digestion) prior to MS or low ionization

efficiency thereafter. For the two recovered modification sequences, neither of the Arg sites are located in the canonical Arg- and Gly-rich domain, which was known to be recognized by PRMT3 (9, 26). This observation suggests that PRMT3 can recognize other Arg-containing sequence motifs.

To maximally uncover PRMT3's cellular substrates, several steps were incorporated in the current BPPM protocol, including the overexpression of PRMT3 M233G variant for amplified activity (Figure S4A), the production of hypomethylated proteome as suitable substrate candidates (22), and the treatment of RNase to release RNA-PRMT3 complex for more efficient target labeling (12). Here we reasoned that adding these steps, though could also bring false positives, enable the amplification of less abundant methylation events. The resultant revealed targets can be subject to vigorous validation in native contexts as will be exemplified later. In the situation of RNase treatment (Table S1), since PRMT3 contains a C2H2 zinc finger motif that is known to interact with RNAs and some of its substrates could be components of protein-RNA complexes (31, 32), RNase treatment is expected to better expose the enzyme and substrates for BPPM labeling. Indeed, among the BPPM-revealed PRMT3 substrate candidates are several heterogeneous nuclear ribonucleoprotein (hnRNP) proteins (*e.g.* hnRNP D0, A3, H3, and F; Table S1) that are known to complex with RNA (33).

Among the total 83 BPPM-revealed unique substrate candidates of PRMT3, only 40S rpS2 was validated before (18, 19). We therefore selected a small panel of these candidates (TUBA1C, TUBB4A, TPI1, KRT6A, and PRMT3) on the basis of their availability for further validation. TUBA1C, TUBB4A, TPI1, and KRT6A were reacted with native PRMT3 and SAM. For PRMT3 automethylation, the reaction was carried out with native PRMT3 and SAM. The methylation was then confirmed by western blot using the antibodies that recognize MMA or aDMA (34, 35). Four of the examined proteins – TUBA1C, TPI1, KRT6A and PRMT3 – were asymmetrically dimethylated by PRMT3 (Figure 7A). In the case of PRMT3, a weak methylation was detected in the absence of SAM treatment, which is probably due to PRMT3's automethylation by the endogenous SAM in bacteria cells that were used for protein expression (Figure 7A) (36). PRMT3 thus joins PRMT1 (37), PRMT4 (38), PRMT6 (39) and PRMT8 (40) as the type I PRMTs that harbor automethylation activity. Besides the observed aDMA, TUBA1C and TUBB4A were monomethylated by PRMT3, suggesting that PRMT3 can alter its product specificity (MMA versus aDMA of different substrates, Figure 7A). The validation of these PRMT3 substrates further proves the robustness of the BPPM approach to reveal PRMT3's targets in the cellular context.

Functional analysis of BPPM-revealed PRMT3 substrates

IPA (Ingenuity Systems, www.ingenuity.com) was implemented to further annotate the functions of the 83 targets of PRMT3 revealed by the BPPM technology (Figure 7A and Table S1). The majority (70%) of the targets localize in the cytoplasm, which is consistent with the subcellular preference of PRMT3 (9, 41) (Figure 7B and Table S1). The identification of nuclear protein substrates (23%) may claim broader roles of PRMT3 in the cellular context (Figure 7B and Table S1). Other 7% BPPM-revealed PRMT3 targets are plasma membrane and extracellular proteins. The biological functions of these noncanonical targets remained to be examined.

KRT6A was revealed by the BPPM approach and validated *in vitro* with the native enzyme-cofactor pair as PRMT3's substrate. KRT6A belongs to the family of keratins, which are intermediate filament proteins expressed in various types of epithelia (42). It is known that an Asp deletion in KRT6A's central rod domain was found in patients with pachyonychia congenita (43). However, the effect of this deletion on the protein level and how KRT6A is dysregulated in pachyonychia congenita are not clear. KRT6A methylation by PRMT3 may

project a new avenue to address this question because many Args are localized in the central rod domain. Two other validated substrates of PRMT3 – TUBA1C and TUBB4A – belong to the microtubule-forming α - and β -tubulin families, respectively. The α -tubulin TUBA8 and the β -tubulin TUBB2B were also revealed by the BPPM approach as potential targets of PRMT3 (Table S1). Except for TUBA1C, other three tubulins are expressed in a tissue-specific manner with TUBB4A and TUBB2B in brain (44) and TUBA8 in skeletal muscle (45). Several Arg sites on these tubulins are located at the interface between α - and β -tubulin dimers (46), implicating the potential role of Arg methylation on dimerization of tubulins and thus cytoskeleton dynamics. Recently, the brain-specific β -tubulin TUBB2A was reported as the substrate of PRMT6 (47), suggesting the involvement of multiple PRMTs in cytoskeleton dynamics. Furthermore, TPI1 was also identified as a cytoplasmic target of PRMT3, which was reported to be also methylated by PRMT5 and the resultant methylation is expected to affect TPI1's dimerization (48). These scenarios are consistent with the finding of an abundant set of proteins can be recognized by multiple PRMTs (35). It is known that PRMT3 knock-out mice are viable and only show benign phenotypes, which gradually disappear at adulthood (19). The lack of the phenotype in PRMT3 knock-out mice could be associated with the functional compensation of PRMT3 by other PRMTs through acting on the same set of targets.

Different cofactor promiscuity of engineered type I PRMTs

The novel PRMT3 targets uncovered here prove BPPM as a valuable tool to profile substrates of SAM-dependent methyltransferases. Here we showed that the M233G variant of PRMT3 is active toward multiple sp^2 - β -sulfonium-containing SAM analogues including Pob-SAM **6**. A prior work on PRMT1, a closely related PRMT3 homologue, showed that the Y39FM48G double mutant of PRMT1 (equivalent to the Y224FM233G of PRMT3) renders the higher activity toward Pob-SAM **6** than its M48G single mutant (22). Surprisingly, this pattern was not observed for PRMT3. Although the M233G variant of PRMT3, like the M48G of PRMT1, is promiscuous to sp^2 - β -sulfonium-containing SAM analogues, the corresponding Y224FM233G mutant is completely inert. Furthermore, none of the M233G double mutants displayed higher efficiency to process the SAM analogues than the M233G single mutant (Figure 3). Such difference argues that PRMT3 and PRMT1, though containing similar SAM-binding pockets, recognize the transalkylation cofactors in a similar but not identical manner. These results further suggest that the conserved methionines of type I PRMTs (M48 in PRMT1 and M233 in PRMT3) as well as its adjacent residues are hot spots to be engineered to process bulky SAM analogues.

CONCLUSION

Through the primary enzyme-coupled fluorogenic assay, around 60 PRMT3 mutants were screened against sulfonium-alkyl SAM analogues to identify active enzyme-cofactor pairs for BPPM. The activity of potential enzyme-cofactor pairs was validated with the secondary MS and in-gel fluorescence assays. The M233G single mutant was the most promiscuous PRMT3 variant to process the sp^2 - β -sulfonium-containing SAM analogues for substrate labeling. With the enzyme-cofactor pair as the desirable BPPM reagents, > 80 proteins were uncovered as the potential targets of PRMT3 in a cellular context and with a panel of selected candidates (KRT6A, TUBA1C, TUBB4A, TPI1, and PRMT3) validated under the native setting. Most BPPM-revealed targets of PRMT3 are localized in cytoplasm (70%), consistent with its primary localization site. However, a decent percentage of the targets (23%) are nuclear, plasma membrane and extracellular proteins. The broader localization pattern of the targets could implicate the diverse roles of PRMT3 in cellular contexts. Upon comparing engineered PRMT1 and PRMT3, we identified the conserved hot spot of type I PRMTs (M48 in PRMT1 and M233 in PRMT3) that can be tailored for cofactor

promiscuity. However, the difference between PRMT1/3 mutants also argues that even closely related PRMTs need to be engineered separately to recognize SAM analogues. The current work is featured by the capacity of the BPPM technology to profile the targets of PRMT3 and serves as the starting point to apply the BPPM technology to many other PMTs to profile proteome-wide nonhistone targets and map genome-wide histone modification sites (49–54). Revealing these PMT substrates and loci are expected to be the key step towards elucidating their functions under various biological settings.

MATERIALS AND METHODS

See Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Bioorthogonal Profiling of Protein Methylation (BPPM) technology for labeling substrates of PRMT3. Here the designated enzyme PRMT3 will be engineered to recognize an otherwise-inert SAM analogue in which SAM's methyl group is replaced with other chemical moieties. The methyltransferase is thus transformed into an alkyltransferase for bioorthogonal target labeling.

Figure 2.

A) Sequence comparison of the SAM binding motifs of human PRMT3 and PRMT1. Here the residues of PRMT3 for mutagenesis (Tyr224, Ile229, His230, Met233, Tyr243, and Met340, highlighted in red) are clustered within the SAM binding motif conserved among type I PRMTs. B) Structural comparison of the SAM binding pockets of human PRMT3 (blue; PDB Code: 2FYT) and rat PRMT1 (grey; PDB: 1ORI), the latter of which is different from human PRMT1 by a single amino acid remote from the SAM-binding site. Labeled are the distances (Å) between the highlighted residue and the sulfur atom of the cofactor analogue SAH. A prior study showed that the Tyr39/Met48 double-point mutation of PRMT1 (equivalent to Tyr224/Met233 in PRMT3) can act on bulky SAM analogues as cofactor surrogates (22).

Figure 3.

Heat map analysis of the activities of PRMT3 variants with SAM analogues as cofactor candidates. PRMT3 mutants are divided into four groups: Group I with the target sites replaced with small hydrophobic residues; Group II with hydrophobic residues; Group III with polar residues; Group IV for double-point mutants. A previously developed fluorogenic assay (25) was implemented to screen native and mutated PRMT3 with SAM **1** and SAM analogues (propyl-SAM **2**, allyl-SAM **3** and 2-butyryl-SAM **4**) as potential cofactors. The relative degree of the modifications on the peptide substrate was quantified with the fluorescent readout of native PRMT3-SAM pair referenced as 100%.

Figure 4. MALDI-TOF MS analysis of PRMT3 mutants with clickable SAM analogues as cofactor surrogates. Promiscuous PRMT3 variants were further tested by MALDI-TOF MS with RGG peptide as the substrate and terminal-alkyne-containing EnYn-SAM **5** and Pob-SAM **6** as cofactors. The pie chart represents the percentage of distribution of native and modified (mono- and di-) peptide. Here the RGG peptide contains multiple Arg sites (26) that can be modified.

Figure 5.

A) Schematic representation of GST-GAR labeled by PRMT3 M233G with Pob-SAM 6 as a cofactor. GST-GAR was incubated with PRMT3 M233G mutant and Pob SAM 6. The product was then conjugated with TAMRA azide fluorescent dye and analyzed by in-gel fluorescence. The product was also subject to tandem MS to analyze the sites of Arg modification. B) In-gel fluorescence of GST-GAR labeled by PRMT3 M233G variant with Pob-SAM 6, followed by the TAMRA azide dye. GST-GAR was labeled by PRMT3 M233G mutant only in the presence of Pob-SAM 6. The background labeling by Pob-SAM 6 in the absence of the enzyme likely arises from GST-GAR's reactive Cys residues (Figure S3). C) Tandem mass spectrum of a representative GST-GAR fragment labeled by PRMT3 M233G and Pob-SAM 6. CB, Coomassie Brilliant Blue stain as the protein-loading control.

Figure 6.

Workflow of substrate identification with PRMT3 M233G mutant and Pob-SAM 6 as BPPM reagents. HEK293T cells were transfected with either empty vector or the vector carrying the PRMT3 M233G variant. The resultant transfected cells were then treated with AdOx to induce hypomethylation. These cells were lysed, followed by the treatment of RNase to release the substrates of PRMT3 as reported previously (12). The cell lysates were incubated with Pob-SAM 6. The labeled targets were conjugated with the cleavable azido-azo-biotin probe, followed by streptavidin enrichment, sodium dithionite elution and mass spectrometry analysis (28).

Figure 7.

BPPM-revealed substrates of PRMT3 and their function annotation. A) Western blots of methylated KRT6A, TUBA1C, TUBB4A, PRMT3 and TPI1 by native PRMT3 and SAM. The selected targets revealed by BPPM – KRT6A, TUBA1C, TUBB4A, PRMT3 and TPI1 – were reacted with native PRMT3 and SAM. The methylated protein substrates were confirmed by western blot using antibodies that recognize monomethylated (MMA) or asymmetrically dimethylated arginines (aDMA) (34, 35). Ponceau stain was used as loading control. B) Analysis of subcellular localizations of the BPPM-revealed targets of PRMT3. IPA analysis (Ingenuity Systems, www.ingenuity.com) showed that 70% of the 83 potential PRMT3 targets are localized in cytoplasm, which is consistent with its preferred subcellular site (9, 41). However, BPPM-revealed PRMT3 substrates were also found in nucleus (23%) and plasma membrane (5%), suggesting broad roles of PRMT3 in a cellular context. The complete list of the BPPM-revealed targets and Arg modification sites are listed in Table S1 and S2, respectively.