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TITLE:

Quantitative methods to study protein arginine methyltransferase 1-9 activity in cells

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SUMMARY:

These protocols provide the methodology used to assess the enzymatic activity of individual members of the protein arginine methyltransferase (PRMT) family in cells. Detailed guidelines on assessing PRMT activity using endogenous and exogenous biomarkers, methyl-arginine recognizing antibodies, and inhibitor tool compounds are described.

ABSTRACT

Protein methyltransferases (PRMTs) catalyze the transfer of a methyl group to arginine residues of substrate proteins. The PRMT family consists of nine members that can monomethylate or symmetrically/asymmetrically dimethylate arginine residues. Several antibodies recognizing different types of arginine methylation of various proteins are available; thus, providing tools for the development of PRMT activity biomarker assays. PRMT antibody-based assays are challenging due to overlapping substrates and motif-based antibody specificities. These issues and the experimental setup to investigate the arginine methylation contributed by individual PRMTs are discussed. Through the careful selection of the representative substrates that are biomarkers for eight out of nine PRMTs, a panel of PRMT activity assays were designed. Here, the protocols for cellular assays quantitatively measuring the enzymatic activity of individual members of the PRMT family in cells are reported. The advantage of the described methods is their straightforward performance in any lab with cell culture and fluorescent western blot capabilities. The substrate specificity and chosen antibody reliability were fully validated with knockdown and overexpression approaches. In addition to detailed guidelines of the assay biomarkers and antibodies, information on the use of an inhibitor tool compound collection for PRMTs is also provided.

INTRODUCTION

Arginine methylation is an important post-translational modification that regulates protein-protein and protein-RNA interactions, thus playing an important role in various cellular processes such as pre-mRNA splicing, DNA damage, transcription response, and growth factor-mediated transduction[1,2]. Arginine is methylated by protein arginine methyltransferases (PRMTs) resulting in monomethyl arginine (Rme1), asymmetrical dimethylarginine (Rme2a), or symmetrical dimethylarginine (Rme2s)[3]. Based on the methylation type, PRMTs are classified into three

groups: Type I (PRMT1, 2, 3, 4, 6, and 8), which catalyze mono- and asymmetric dimethylation; Type II (PRMT5 and PRMT9), which catalyze mono- and symmetric dimethylation; and Type III (PRMT7), which can only monomethylate arginine[3].

Due to a growing number of commercially available arginine methylation-specific antibodies, PRMT activity can be measured using western blotting. Fluorescent-based western blot is the preferred technique over chemiluminescent detection due to a greater dynamic range and linearity, higher sensitivity, and allowing for multiplexing[4]. To quantify the protein methylation levels, normalization of the methylation signal to total protein levels is required. By choosing the antibodies for total and methylated protein raised in different host species (e.g., mouse and rabbit), secondary antibodies labeled with different fluorophores can be used and the signal for both antibodies can be determined in the same sample band. Methyl-arginine antibodies were developed to identify and characterize monomethylated, asymmetrically, or symmetrically dimethylated proteins where methyl-arginine is found in a specific context. Since the majority of PRMTs methylate glycine- and arginine-rich motifs within their substrates[5], several antibodies were raised for the peptides containing monomethyl or asymmetric, symmetric dimethyl-arginine-glycine repeats such as D5A12, ASYM 24, or ASYM 25, and SYM11, respectively. Other methyl-arginine antibodies were generated against a peptide library containing asymmetric, symmetric dimethyl- and monomethyl arginine in a repeat context facilitating the detection of methyl-arginine in these particular contexts[6]. There is also an increasing number of antibodies that recognize specific arginine mark on a single protein which enable selective detection of methylation such as histone H4R3me2a or BAF155-R1064me2a.

There are several commercially available PRMT inhibitors, which can be used as tools for PRMT cellular assays. However, not all of them are thoroughly characterized for selectivity and off-target effects and some should be used with caution. The Structural Genomic Consortium, in collaboration with academic labs and pharma partners, has developed well-characterized potent, selective, and cell-permeable PRMT inhibitors (chemical probes) that can be used with no restrictions by the scientific community. Information on these inhibitors can be found on https://www.thesgc.org/chemical-probes/epigenetics and https://www.chemicalprobes.org/. Chemical probes are small-molecule inhibitors with *in vitro* IC_{50} or K_d < 100 nM, over 30-fold selectivity over proteins in the same family, and significant cellular activity at 1 µM. Additionally, each chemical probe has a close chemical analog that is inactive against the intended target[7,8,9,10,11,12].

The goal of this protocol is to measure the cellular activity of individual PRMT family members using the fluorescent western blot method. Here detailed information on validated assay biomarkers, antibodies, and potent cell-active inhibitors as well as valuable strategies for successful assay implementation are provided.

PROTOCOL

1. Cell culturing and plating

NOTE: Culture cells with recommended media and test routinely for mycoplasma contamination. HEK293T, MCF7, and C2C12 cells were chosen as examples since these cell lines were successfully used in PRMT assays.

1.1. Culture HEK293T, MCF7, and C2C12 in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U mL^{-1}), and streptomycin (100 µg mL^{-1}) in 10 cm tissue-culture treated (TC) dishes.

1.2. For the PRMT8 assay, grow PRMT1 inducible knockdown HEK293T cells in media containing doxycycline (2 µg/mL) for 3 days before assay start.

1.3. To plate the cells, remove and discard media from the plate.

1.4. Add 10 mL of PBS (without Ca^{+2} and Mg^{+2} ions) to wash cells and discard the solution.

1.5. Add 1 mL of Trypsin-EDTA (0.25%), incubate for 1 min at RT, and then discard the solution. Incubate until cells become round and detach from the plate. Tap the plate to help detach cells, if needed. For hard-to-trypsinize cells, such as C2C12, incubate the plate for 1-2 min at 37 °C.

NOTE: Avoid cell exposure to trypsin solution for longer periods (>10 min) as it will reduce cell viability.

1.6. Add 1 mL of prewarmed media to the plate, and gently pipette cells up and down to break-up cell clumps. Transfer cells to a 15 mL tube, and add 3-5 mL of media.

1.7. To measure cell number, mix 10 µL of cells with 10 µL of Trypan blue and transfer 10 µL to hemocytometer or use any other cell counting method.

1.8. Dilute cells to recommended cell density and put 500 µL/well into 24-well TC plates (Ta**ble 1**). For endogenous assays (PRMT1, PRMT4, PRMT5, PRMT7, and PRMT9), move to Step 3.1.

2. Cell transfection

2.1. For exogenous assays (PRMT3, PRMT6, PRMT8) transfect HEK293T cells with the recommended amount of DNA (Table 2). HEK293T cells are easy to transfect so any transfection reagent can be used, following the manufacturer's instruction.

3. Compound treatment

NOTE: Do not exceed 0.1% final dimethyl sulfoxide (DMSO) concentration in culture media. Keep the same DMSO concentration in each well. The selective PRMT inhibitors (chemical probes) and their closely related inactive analogs can be found in **Table 3.**

3.1. For **endogenous assays** (PRMT1, PRMT4, PRMT5, PRMT7, and PRMT9), remove media from cells and replace with 500 µL of media with compound or DMSO alone (control).

NOTE: It usually takes 2 days to observe over 80% decrease in R methylation levels.

3.2. For **exogenous assays** (PRMT3, PRMT6, PRMT8), remove media 4 h after transfection, add 500 µL of media with compound or DMSO alone (control), and incubate for 20-24 h.

4. Cell lysate preparation

4.1. Remove all media from wells, wash with 100 µL of PBS to remove residual media, and add 60 µL of lysis buffer (20 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM $MgCl_2$, 0.5% Triton X-100, 12.5 U mL−1 benzonase, complete EDTA-free protease inhibitor cocktail) to each well.

4.1.1. Incubate for 1 min at RT, rocking the plate to distribute the lysis buffer over the cells. Then add 3 µL of 20% w/v sodium dodecyl sulfate (SDS), to a final 1 % concentration, and mix by gently shaking. Transfer lysate into microcentrifuge tubes and keep it on ice.

NOTE: Add benzonase and protein inhibitor cocktail fresh before use. The addition of benzonase rapidly hydrolyzes nucleic acids which reduces cell lysate viscosity.

4.2. Determine protein concentration of the samples using BCA Protein Assay Kit or use any other method which tolerates 1% SDS in solution.

4.2.1. Add 2 µL of lysate and protein standards (0, 1, 2, 4, and 8 µg/mL of BSA in lysis buffer) into the well of the 96-well clear plate.

4.2.2. Mix reagent A with reagent B at 50:1 ratio and add 200 µL per well. Incubate for 20 min at 37 °C and read the absorbance.

4.3. Adjust the protein concentration with lysis buffer to be equal across the samples.

4.4. Add 20 µL of 4x Loading Buffer to 60 µL of cell lysate and heat at 95 °C for 5 min. After heat denaturation, the lysates can be stored at -20 °C.

5. Western blot analysis

5.1. Load 5-20 µg of total cell lysate for analysis of histone proteins and 20-100 µg for other proteins into a 4–12% Bis-Tris protein gel.

5.2. Run the gel in MOPS SDS running buffer (50 mM MOPS, 50 mM Tris Base, 0.1% SDS w/v, 1 mM EDTA, pH 7.7) for about 2 h at 100 V or until the dye front reaches the bottom of the gel.

5.3. If performing a wet transfer, assemble the transfer sandwich in ice-cold Tris-Glycine transfer buffer (25 mM Tris, 192 mM Glycine, 20% v/v methanol, and 0.05% w/v SDS).

5.3.1. Place sponges, filter paper, PVDF membrane, and gel according to manufacturer's instructions. Activate PVDF membrane by soaking in methanol and equilibrate gel in transfer buffer for 30 s before assembly.

NOTE: Use recommended PVDF western blotting membrane since it has low autofluorescence and suitability for low molecular weight proteins, such as histones (**Table of Materials**).

5.4. Transfer proteins from the gel to PVDF membrane in Tris-Glycine transfer buffer at 70 V for 1.5 h on ice.

5.5. Block membrane for 30 min in blocking buffer (5% w/v milk in phosphate-buffered saline, PBS). Rinse with wash buffer (PBST: 0.1% v/v Tween-20 in PBS), and incubate with primary antibodies in blocking buffer (5% BSA in PBST) overnight at 4 °C (**Table 3**).

NOTE: For longer storage, filter-sterilize BSA solution, add 0.02% w/v sodium azide, and keep at 4 °C.

5.6. Wash membrane 3 x 5 min with PBST. Then incubate with goat-anti-rabbit (IR800) and donkey anti-mouse (IR680) antibodies in blocking buffer (**Table of Materials, Table 3**) for 30 min at RT and wash 3 x 5 min with PBST.

5.7. Read the signal on a fluorescent western blot imager at 800 and 700 nm. Preferably use the instrument which allows imaging strong and faint bands clearly in a single image with high sensitivity and dynamic range, high signal-to-noise ratio, a warning when an image saturation is reached as well as multiplexing of two fluorescent colors in the same sample band.

5.8. Determine band intensities for western blot analysis using appropriate software for fluorescent western imaging.

REPRESENTATIVE RESULTS

Examples of western blot results for cellular assays of individual PRMTs are presented below. Assays details are also summarized in **Table 4**.

PRMT1 assay

PRMT1 is the main contributor to histone 4 arginine 3 asymmetric dimethylation (H4R3me2a) in cells[13]. Upon loss of PRMT1 activity, global Rme1 and Rme2s levels increase significantly[13]. As shown in **Figure 1A** and **1B**, several antibodies can be used to monitor global changes in Rme1, Rme2a, Rme2s, as well as asymmetric dimethylation of histone H4R3 (H4R3me2a). A significant decrease in global Rme2a and H4R3me2a levels and increases in Rme1 and Rme2s can be observed after 3 days of *PRMT1* knockdown (**Figure 1A, B**). Cell lines differ in basal H4R3me2a signal, therefore, to facilitate monitoring the loss of PRMT1 activity, cell lines such as MCF7 with

high basal methylation levels can be used (**Figure 1C**). The optimal time to observe the effect of PRMT1 inhibition, e.g. upon treatment with type I PRMT inhibitor MS023[8], is 2 days (**Figure 1D,1E**). Longer treatment results in reduced cell viability and growth.

PRMT3 assay

For the PRMT3 cellular assay, no selective biomarker proteins which methylation changes could be detected in western blot upon *PRMT3* knockdown or overexpression. PRMT3 was shown to asymmetrically dimethylate H4R3 *in vitro*[14], however, the mark is predominantly deposited by PRMT1, and therefore an exogenous assay with overexpressed PRMT3 was designed. Consistent with *in vitro* findings, overexpression of wild-type PRMT3 but not its catalytic mutant (E338Q) led to an increase in H4R3me2a levels (**Figure 2A**). HEK293T cells were used since they have low basal methylation of this mark (**Figure 1C**). The assay was further validated with PRMT3 selective inhibitor SGC707[7], which inhibited PRMT3-dependent H4R3 asymmetric methylation (**Figure 2B**).

PRMT4 assay

PRMT4 asymmetrically dimethylates BAF155 at arginine 1064[15]. Since the antibody detecting BAF165-R1064me2a is commercially available, the PRMT4 activity in cells can be monitored by western blot by detecting the changes in the R1064me2a mark levels. The loss of PRMT4 protein or inhibition of catalytic activity with the PRMT4 selective inhibitor, TP-064[10], results in a decrease in BAF165-R1064me2a levels (**Figure 3**). A 2-day treatment is usually sufficient to remove most of the methylation signal.

PRMT5 assay

PRMT5 is responsible for the majority of protein arginine symmetric dimethylation. It has been previously reported that the various SMN complex proteins, including SmBB', are PRMT5 substrates[16]. PRMT5 activity can be monitored by looking at changes in global levels of symmetric arginine dimethylation or symmetric dimethylation of SmBB' proteins. Knockdown of *PRMT5*, but not *PRMT1, 3, 4, 6,* and *7* results in a decrease in global Rme2s levels (**Figure 4A**). In most cell lines, the treatment of cells with PRMT5 selective inhibitors LLY-283[11] and GSK591 for 2-3 days suppressed most of the SmBB'Rme2s signal (**Figure 4B**). Most cells are sensitive to PRMT5 inhibition, which results in a decrease in cell proliferation and cell death with prolonged inhibitor exposure.

PRMT6 assay

It has been reported that PRMT6 is the main contributor to histone H3 arginine 2 asymmetric dimethylation (H3R2me2a) in cells[17]. In HEK293T cells, *PRMT6* knockdown for 3 days was not sufficient to observe a significant decrease in H3R2me2a levels. However, overexpression of wild type PRMT6 but not its catalytic mutant (V86K/D88A) increases levels of H3R2me2a, as well as H3R8me2a and H4R3me2a (**Figure 5A**). There are several inhibitors that inhibit PRMT6 activity with different potency and selectivity: selective, allosteric PRMT6 inhibitor SGC6870[18], PRMT type I inhibitor MS023[8], and PRMT4/6 inhibitor MS049[9]. All of these inhibited PRMT6 dependent H3R2 (**Figure 5B**), as well as H4R3 and H3R8 asymmetric dimethylation (data not shown).

PRMT7 assay

PRMT7 monomethylates arginine 469 in both constitutive and inducible forms of HSP70 (HSPA8 and HSPA1/6, respectively)[12]. Although there are no commercially available antibodies, which detect HSP70-R469me1 levels, the mark can be detected with pan monomethyl antibodies. The loss of PRMT7 protein or inhibition of catalytic activity with the PRMT7 selective inhibitor, SGC3027[12], results in decreased levels of HSP70-R469me1 (**Figure 6A, B**). SGC3027 is a cell-permeable prodrug, which in cells is converted by reductases to the PRMT7 selective inhibitor SGC8158, therefore cellular potency may differ between cell lines. Several cancer cell lines express inducible HSP70 isoforms at high levels, and methylation can be hard to detect due to an overlapping unspecific band of nuclear origin (**Figure 6C**). Therefore, for the PRMT7 cellular assay, cell lines that express mostly HSPA8 such as C2C12 are recommended, or since HSP70 localizes mainly in the cytoplasm, determine HSP70-R469me1 levels in the cytoplasmic fraction of preferred cell lines.

PRMT8 assay

PRMT8 is the only PRMT with a tissue-restricted expression pattern - largely expressed in the brain[19]. It shares 80% sequence similarity and has a similar substrate preference as PRMT1[19]. It differs from PRMT1 mainly at the N-terminus, where myristoylation results in the association of PRMT8 with the plasma membrane[20]. It has been reported that PRMT8 together with PRMT1 methylates RNA-binding protein EWS[21]. Since PRMT8 activity is low in non-neuronal cell lines and EWS can also be methylated by PRMT1, an assay in which PMRT8 is co-overexpressed together with EWS in *PRMT1* knockdown cells was developed. Since *PRMT1* is an essential gene and its long-term loss results in cell death, an inducible system in which *PRMT1* is knocked down for 3 days before use in the PRMT8 assay was utilized (**Figure 7A**). Co-expression of wild-type PRMT8, but not catalytically inactive mutant (E185Q), together with EWS resulted in increased levels of EWS asymmetric dimethylation (**Figure 7B**). Several asymmetric dimethylarginine antibodies were tested and the methylation was only detected with Asym25 antibody. The assay was further validated with a PRMT type I selective chemical probe, MS023[8], which decreased PRMT8-dependent asymmetric dimethylation of exogenous EWS (**Figure 7B**). Although MS023 is very potent in inhibiting PRMT8 in *in vitro* assays, in cells high concentrations of MS023 are required to see methylation inhibition[21].

PRMT9 assay

PRMT9 was shown to symmetrically dimethylate SAP145 at arginine 508[22]. Unfortunately, no commercially available antibodies can recognize the mark. For the PRMT9 assay, antibodies that were kindly gifted by Dr. Yanzhong Yang (Beckman Research Institute of City of Hope) were used. When overexpressed, wild type but not R508K mutant SAP145 is methylated by PRMT9 (**Figure 8A**). The assay was designed to monitor the levels of endogenous SAP145-R508me2s and was validated with Compound X, a prototype PRMT9 inhibitor (work in progress, not yet published), which potently inhibits PRMT9 *in vitro* with nanomolar potency. Compound X decreased SAP145-R508me2s levels in a dose-dependent manner (**Figure 8B**).

FIGURE AND TABLE LEGENDS

Figure 1. PRMT1 cellular assay. (**A**) *PRMT1* knockdown results in a decrease of global asymmetric arginine dimethylation (Rme2a) and increased levels of symmetric arginine dimethylation

(Rme2s) and monomethylation (Rme1). The PRMT1 knockdown efficiency is presented in panel B. (**B**) PRMT1 knockdown decreases asymmetric dimethylation of histone H4R3 (H4R3me2a). (**C**) The basal H4R3me2a levels differ across different cell lines. (**D**) Type I PRMT inhibitor MS023 decreases H4R3me2a levels in a dose-dependent manner. MCF7 cells were treated with MS023 for 2 days. (**E**) The graph represents the nonlinear fit of H4R3me2a signal intensities normalized to total histone H4. MS023 IC50 = 8.3 nM (n=1).

Figure 2. PRMT3 cellular assay. (**A**) The overexpression of wild-type (WT) but not E338Q catalytic mutant of PRMT3 increases H4R3me2a levels in HEK293T cells. Cells were transfected with FLAG-tagged PRMT3 for 24 h. (**B**) PRMT3 selective inhibitor, SGC707, decreases ectopic PRMT3 dependent H4R3 asymmetric demethylation

Figure 3. PRMT4 cellular assay. (**A**) *PRMT4* knockdown results in a decrease of BAF155-R1064 asymmetric arginine dimethylation (HEK293T cells). (**B**) PRMT4 selective inhibitor, TP-064, decreases BAF155-R1064Rme2a levels. HEK293T cells were treated with compound for 2 days.

Figure 4. PRMT5 cellular assay. (**A**) *PRMT5* knockdown results in a decrease of global symmetric arginine dimethylation levels (MCF7 cells). (**B**) PRMT5 selective inhibitors GSK591 and LLY-283, decrease SmBB' symmetric arginine dimethylation (green), while total levels of SmBB' remain unchanged (red). MCF7 cells were treated with compounds for 2 days.

Figure 5. PRMT6 cellular assay. (**A**) The overexpression of wild type (WT) but not V86K/D88A catalytic mutant PRMT6 increases H4R3me2a, H3R2me2a, and H3R8me2a levels in HEK293T cells. Cells were transfected with FLAG-tagged PRMT6 for 24 h. (**B**) PRMT6 selective inhibitor (SGC6870), PRMT type I inhibitor (MS023), PRMT4/6 inhibitor (MS049), and PRMT6/1/8 inhibitor (MS117), decrease PRMT6 dependent H3R2me2a levels.

Figure 6. PRMT7 cellular assay. (**A**) *PRMT7* knockout results in a decrease of HSP70-R469 monomethylation (HCT116 cells). (**B**) PRMT7 selective inhibitors, SGC3027, decreases HSP70-R469 monomethylation in C2C12 cells. Cells were treated with compound for 2 days. (**C**) Detection of HSP70-R469 methylation of inducible HSP70 (HSPA1/6) with pan monomethyl arginine antibodies (Rme1) can be difficult due to an overlapping unspecific band of nuclear origin. It is recommended to measure HSP70 methylation levels in the cytoplasmic fraction.

Figure 7. PRMT8 cellular assay. (**A**) PRMT8 methylation of EWS can be detected when *PRMT1* activity is inhibited by knockdown. HEK293T cells were transduced with an inducible *PRMT1* knockdown vector. After 3 days of doxycycline treatment, PRMT1 levels were drastically reduced. (**B**) When *PRMT1* is knocked down, exogenous EWS is asymmetrically dimethylated by overexpressed wild type PRMT8 but not catalytic mutant (E185Q) of PRMT8. The methylation is decreased by a high concentration of PRMT type I inhibitor (MS023). HEK293T *PRMT1*KD cells were co-transfected with FLAG-tagged PRMT8 wild type or catalytic mutant and GFP-tagged EWS and treated with MS023 for 20 h.

Figure 8. PRMT9 cellular assay. (**A**) Wild type but not R508K mutant SAP145 is methylated by PRMT9. HEK293T cells were transfected with GFP-tagged SAP145 for 1 day. (**B**) The prototype PRMT9 inhibitor (Compound X) decreases PRMT9 dependent R508 symmetric dimethylation of SAP145 in a dose-dependent manner. HEK293T cells were treated with the compound for 2 days.

Table 1. Cell types and densities are recommended for PRMT assays.

Table 2. The DNA concentration for transfection experiment.

Table 3. Recommended antibodies and PRMT chemical probe/negative control tool compounds.

Table 4. PRMT assays summary.

DISCUSSION

Here, the detailed cellular assay protocols for members of the PRMT family are described that use fluorescent western blotting methods. Unique substrates for which the changes in arginine methylation can be easily detected upon individual PRMT loss or catalytic inhibition and cannot be compensated by other family members were selected. Some proteins are methylated by multiple PRMTs[21,23], suggesting an overlap in substrate specificity where some PRMTs contribute only a small amount of cellular mark in a given protein substrate[24,25,26,27], for example, both PRMT8 and PRMT1 contribute to methylation of EWS. Therefore, each assay required thorough validation of substrates and antibodies with knockdown and/or overexpression experiments and further validation with well-characterized selective inhibitors. PRMT specific substrates were identified for which methylation mark changes could be detected within 2-3 days post-PRMT loss/inhibition to avoid compounding effects of reduced cell viability and proliferation that may indirectly affect the methyl-arginine mark levels. Although it was possible to find unique substrates for PRMT1, 4, 5, 7, and 9; for PRMT3, 6, and 8 the gain of function approach had to be employed. Several arginine methyl-specific antibodies were tested for various cellular targets, but none were able to detect significant changes within 3 days of *PRMT3* and *PRMT6* knockdown; therefore, biomarker assays were developed using ectopically expressed enzymes together with catalytically inactive mutants, which served as a control for the baseline substrate methylation. PRMT8 is a close PRMT1 homolog and shares similar substrate preferences. As a PRMT8 selective biomarker could not be identified, an assay in P*RMT1* knockdown cells was developed, where PRMT8 was co-expressed together with EWS. PRMT1 is also a major enzyme responsible for H4R3 asymmetric methylation, therefore, to use H4R3me2a as a biomarker for PRMT3 and PRMT6 cellular assays, cells with low basal H4R3me2a levels were chosen as well as catalytic inactive mutants were used as a background control. Although endogenous assays are preferred, exogenous assays prove invaluable for testing the cellular potency of several selective PRMT inhibitors[7,8,9]. With growing knowledge of PRMT biology, we expect to improve the assays by finding more specific biomarker proteins for PRMT3, PRMT6, and PRMT8.

The use of validated antibodies and appropriate controls are critical for the PRMT assay performance. All antibodies recommended here have been thoroughly validated by knockdown

and overexpression experiments, however, batch-to-batch differences, especially in the case of polyclonal antibodies, may still influence their performance. Therefore, it is crucial to use genetic methods and chemical probes together with their closely related negative controls to confirm assay reliability. Additionally, for PRMT assays that require protein overexpression, it is crucial to use catalytically inactive mutants along with wild-type protein to determine the basal methylation levels.

This collection of quantitative assays for profiling the activity of PRMTs in cells can be broadly useful for the scientific community since it can be rapidly and easily implemented with minimal equipment and limited technical expertise, involving only basic cell culturing and fluorescent western blotting techniques. The recommended antibodies and chemical probes for PRMTs can also be utilized for activity-based protein profiling (ABPP) assays to establish the suitability of a given ABPP probe, monitor target engagement, and assess off-target effects by using the competitive ABPP format. The assay development approaches discussed here can also be extrapolated for other enzyme families such as protein lysine-methyltransferases and acetyltransferases.

DISCLOSURE:

The authors do not have any competing financial interests or other conflicting interests to declare.

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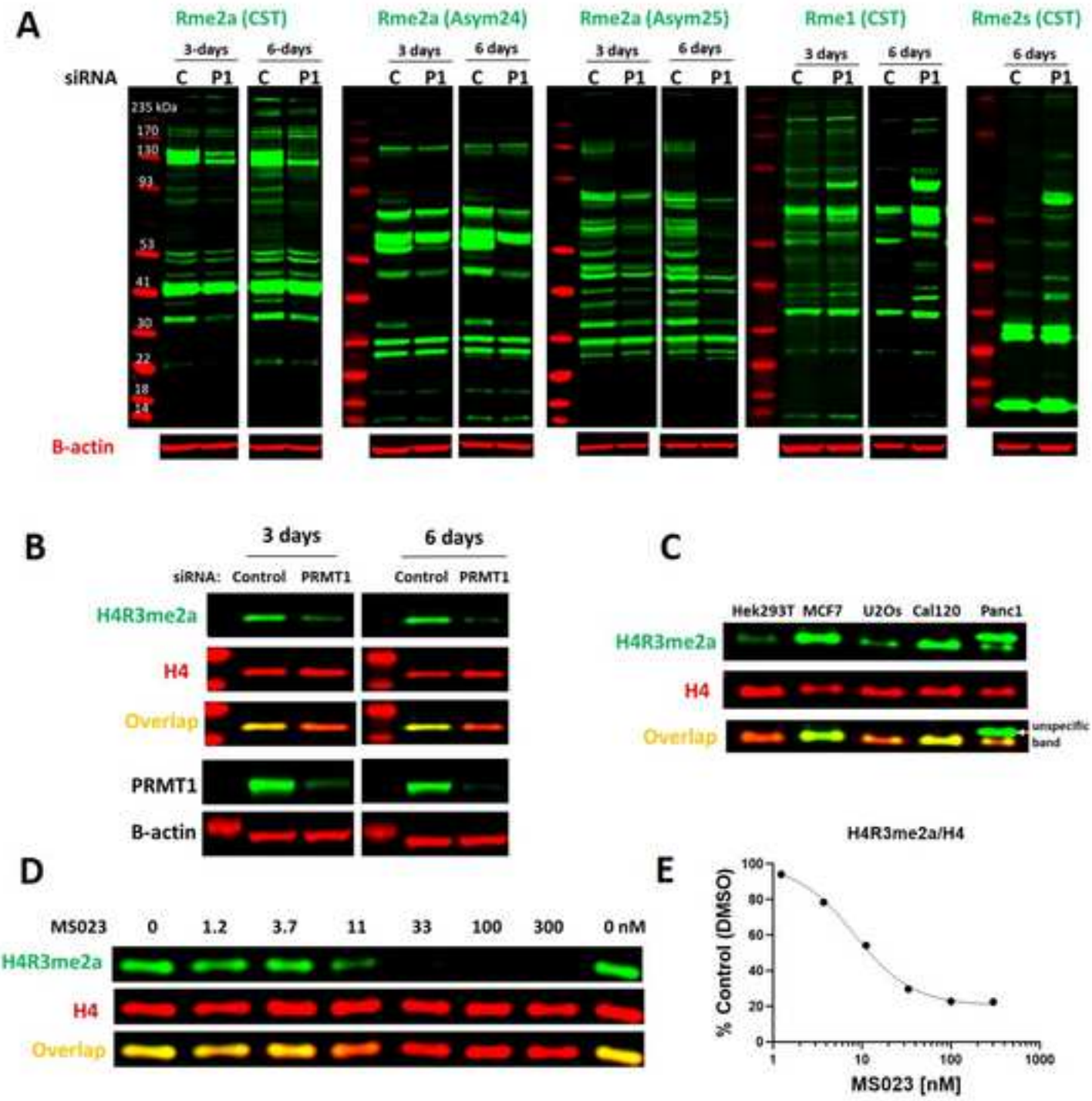

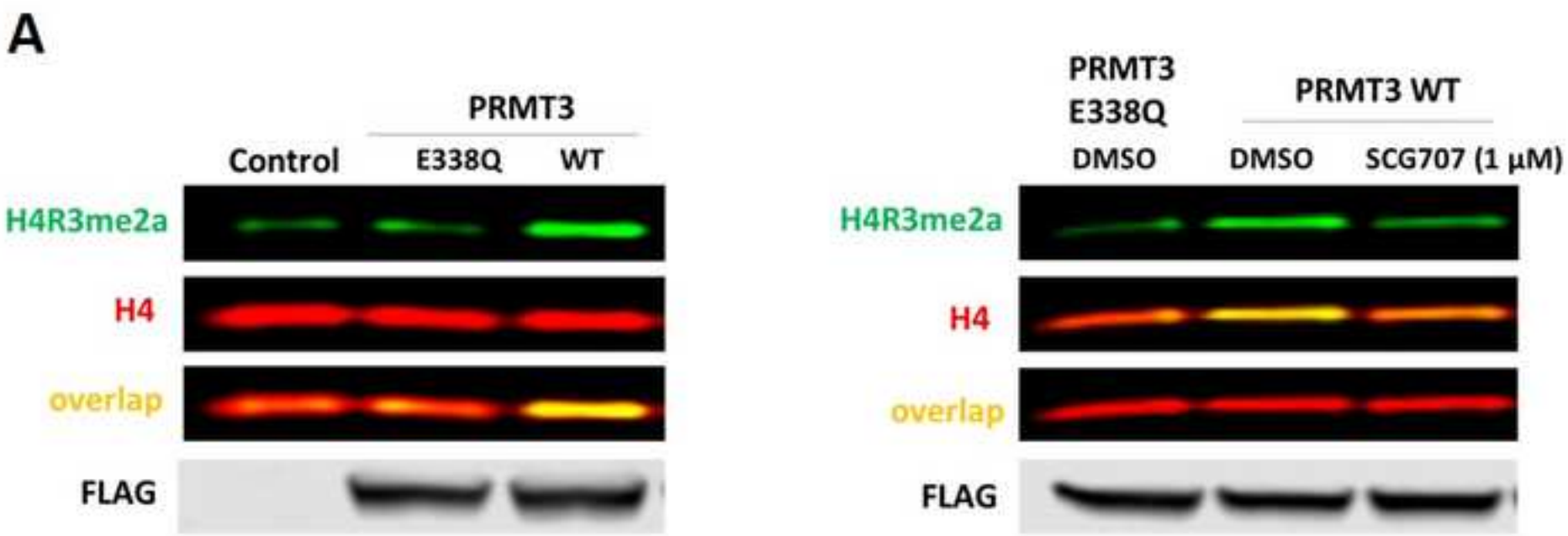

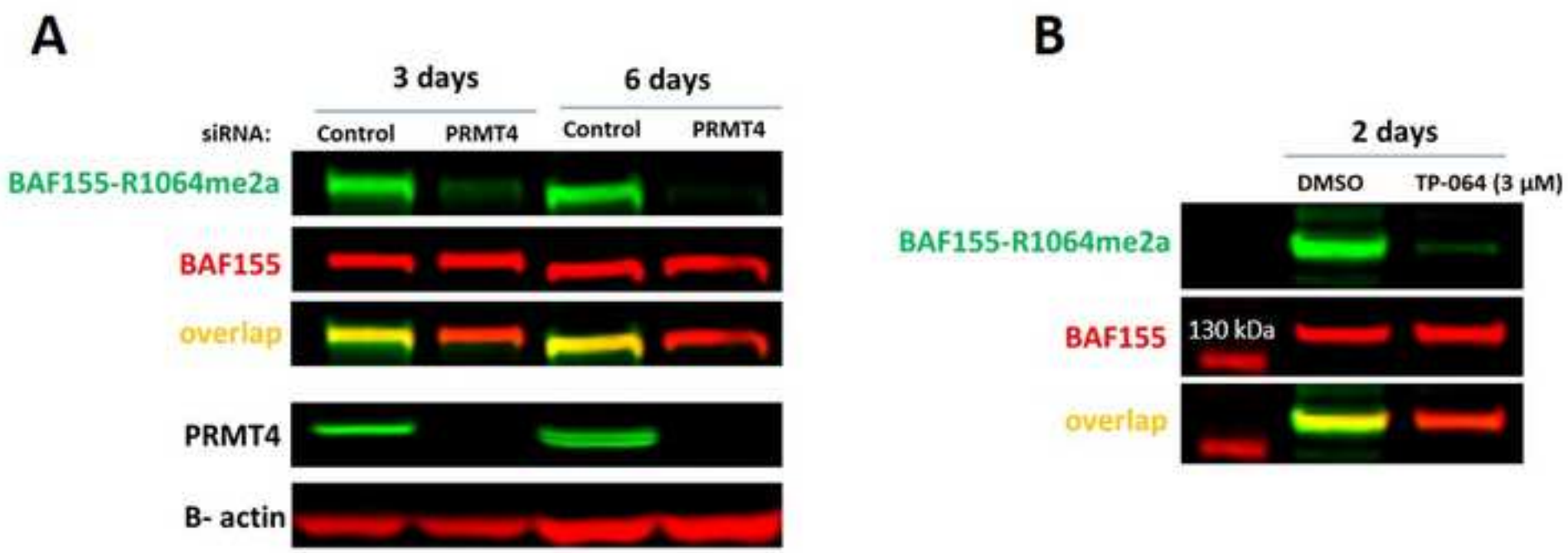

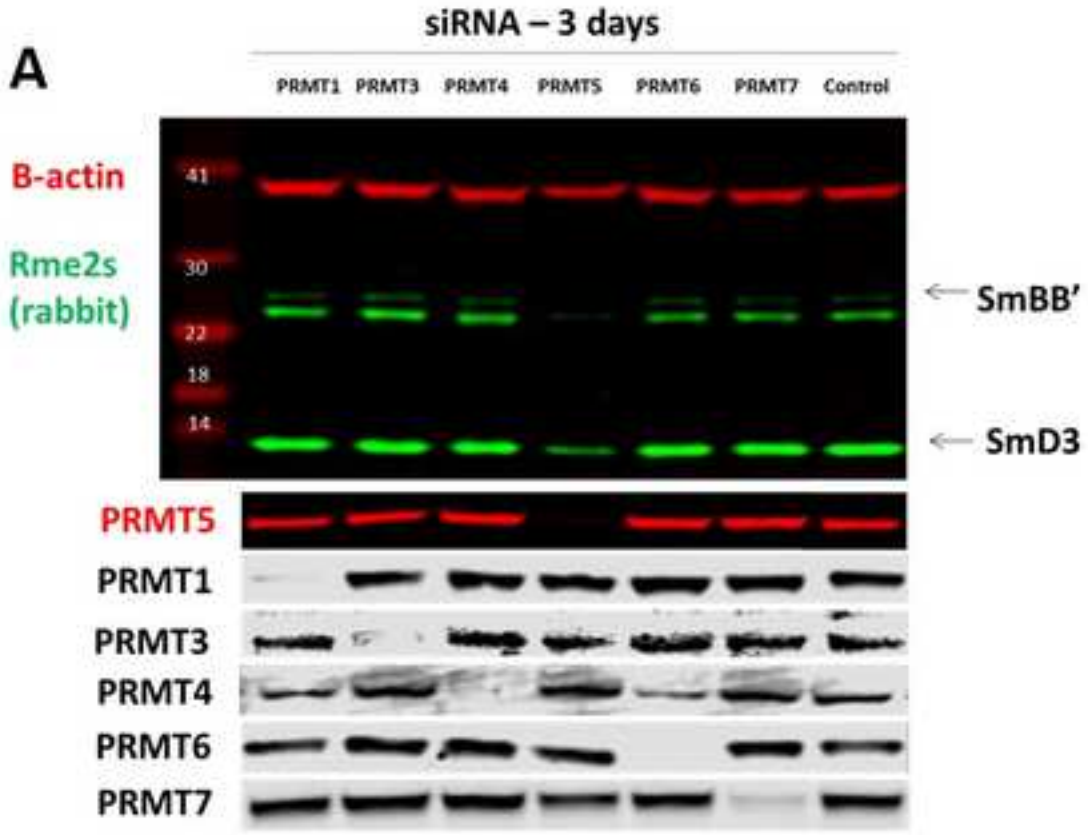

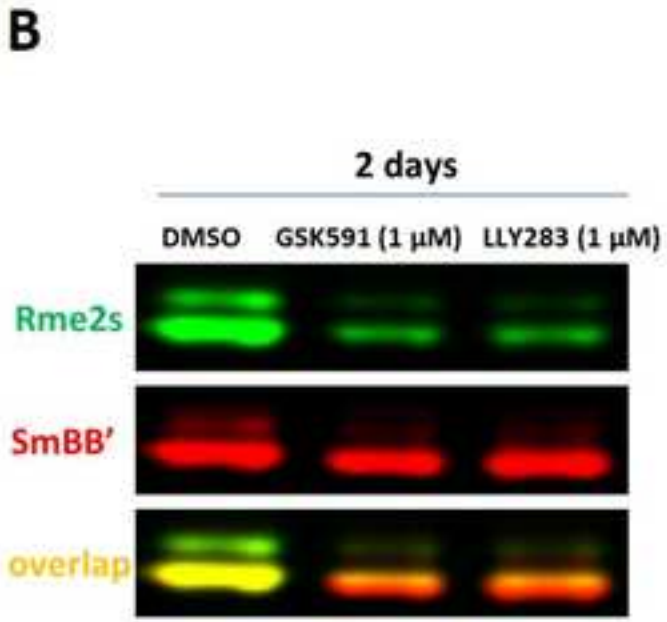

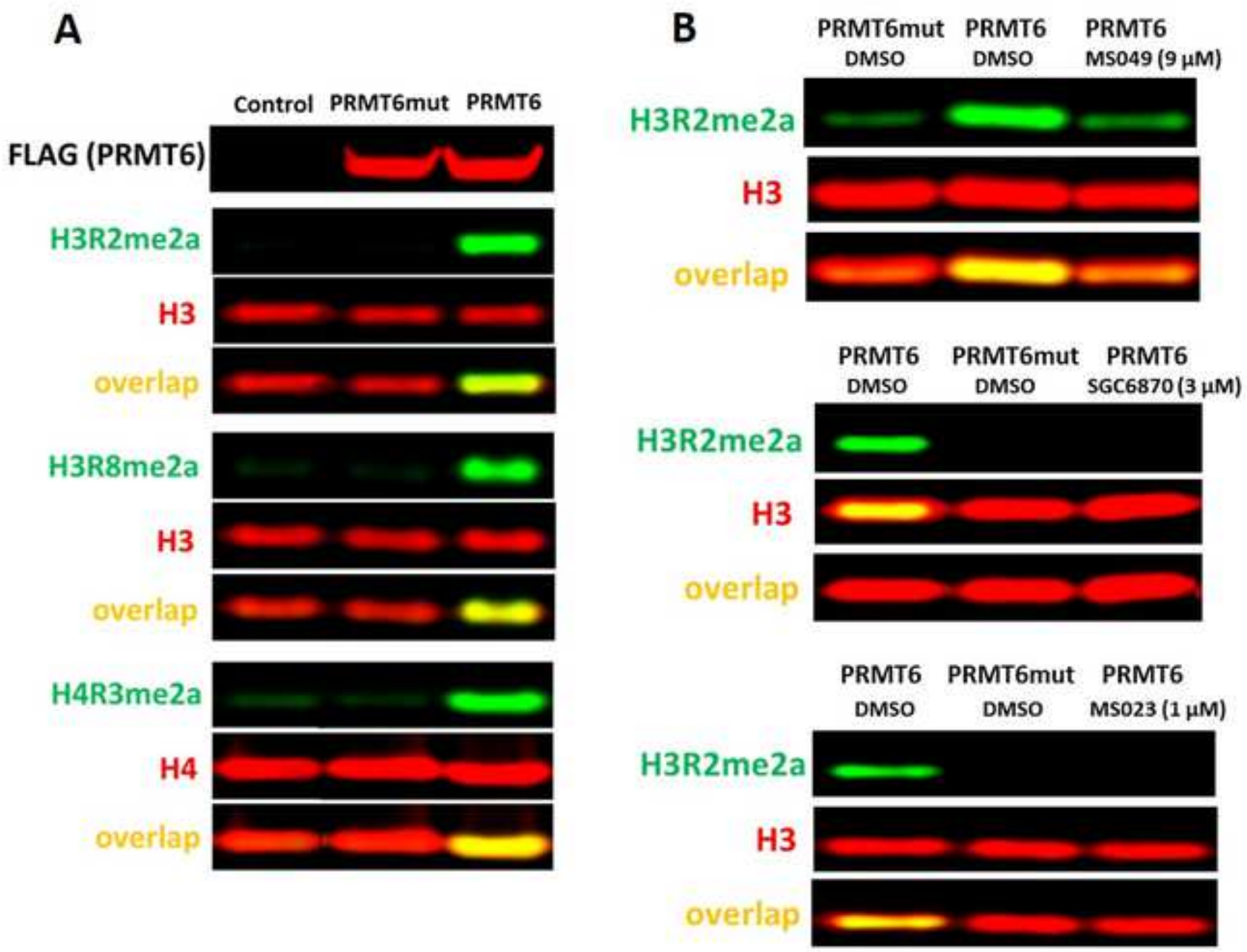

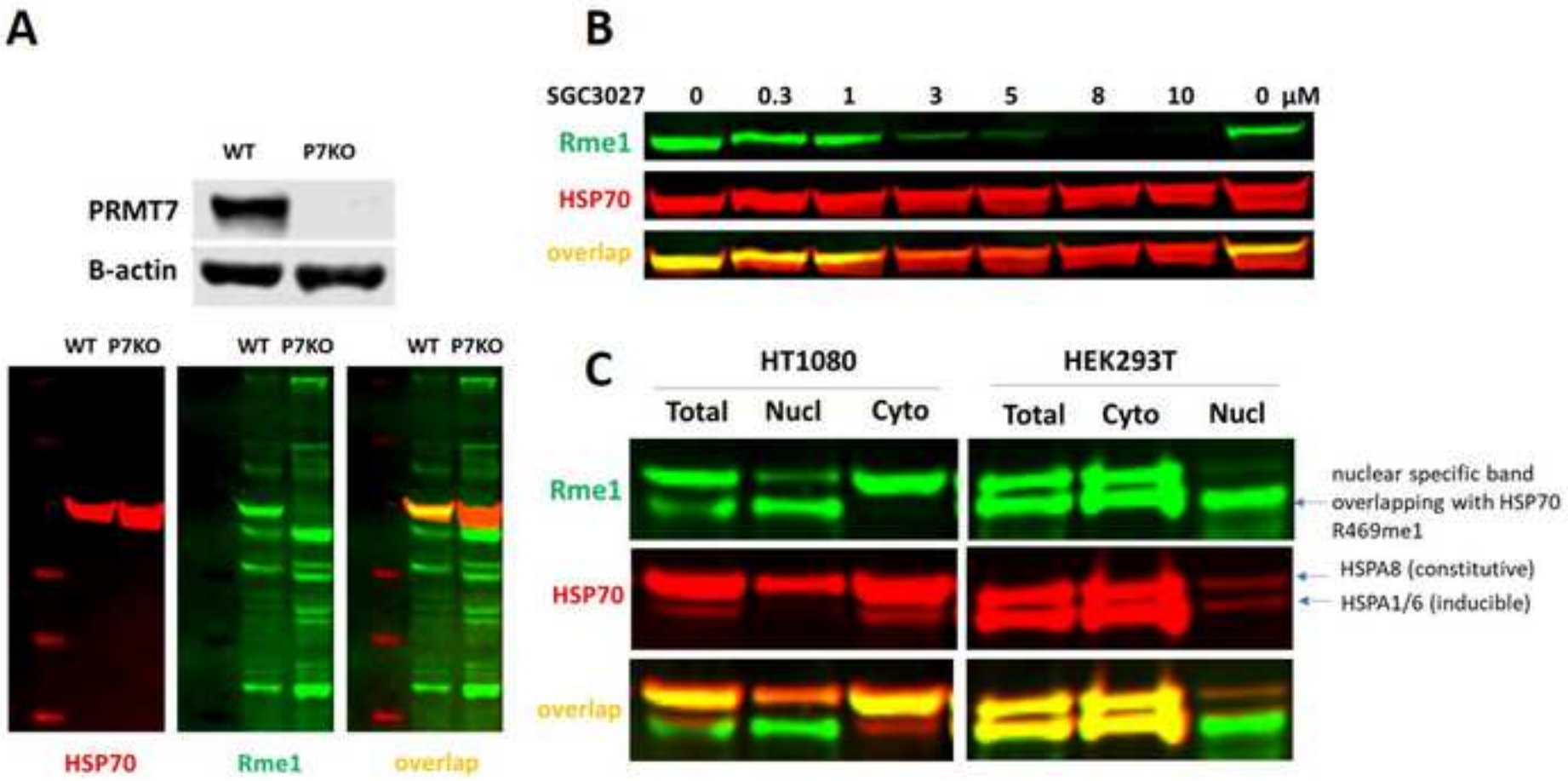

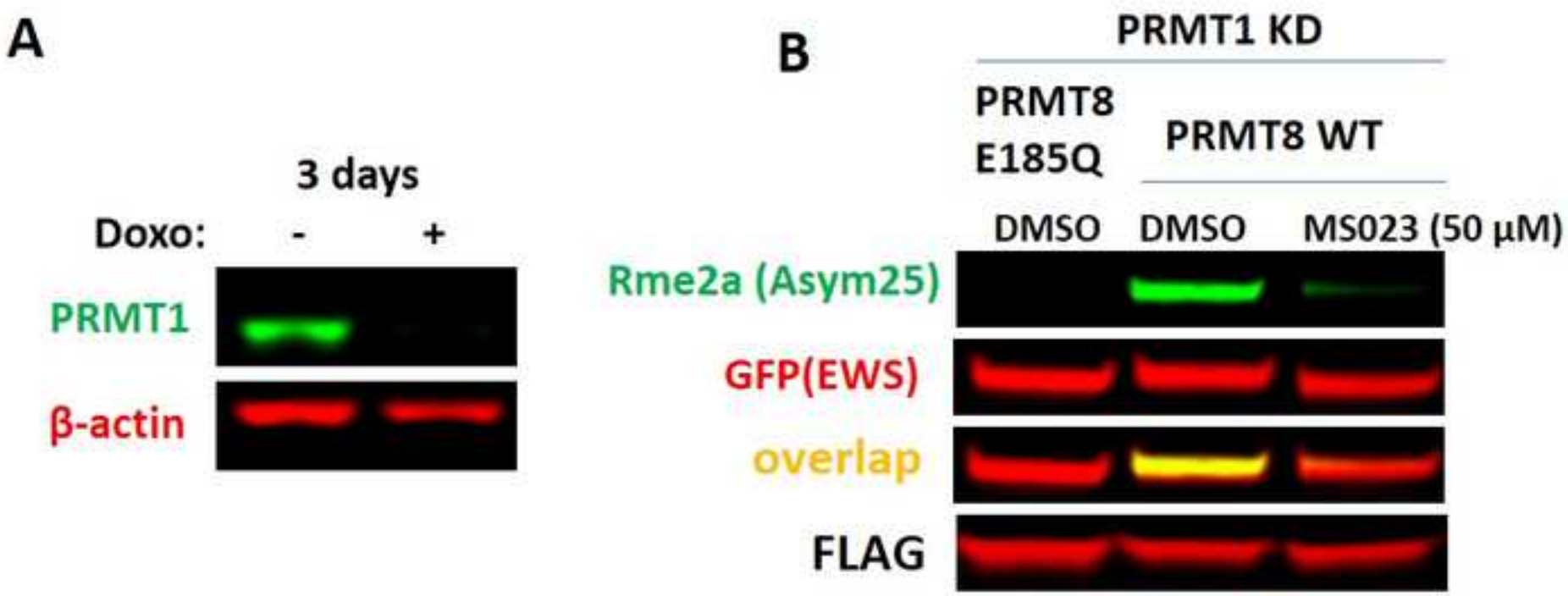

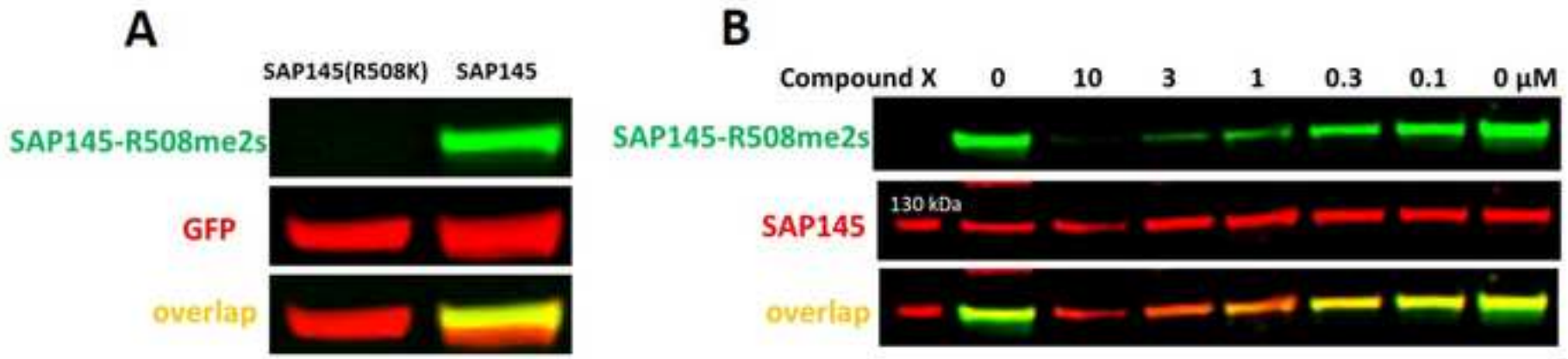

PRMT	Cells	Density per ml
PRMT1	MCF7	1×10^5
PRMT3	HEK293T	2×10^5
PRMT4	HEK293T	1×10^5
PRMT5	MCF7	1×10^5
PRMT6	HEK293T	2×10^5
PRMT7	C2C12	1×10^5
PRMT8	HEK293T (PRMT1 KD)*	2×10^5
PRMT9	HEK293T	2×10^5

*treat cells with doxycycline (2 µg/mL) 3 days before plating for PRMT8 assay

PRMT	µg DNA/24-well	Addgene #
PRMT3	0.5 FLAG-PRMT3	164695
	or 0.5 FLAG-PRMT3 (E338Q)	164696
PRMT6	0.5 FLAG-PRMT6	164697
	or 0.5 FLAG-PRMT6(V86K/D88A)	164698
PRMT8	0.05 EWS-GFP	164701
	0.45 PRMT8-FLAG	164699
	or 0.45 PRMT8(E185Q)-FLAG	164700
PRMT9	0.05 SAP145-GFP	NA
	or 0.05 SAP145-R508K-GFP	
	0.45 empty vector	

Additional notes
gift from Dr. Yanzhong Yang, Beckman Research Institute of City of Hope

Table 3

Click here to access/download;Table;Table 3 19032021_FINAL.xlsx

PRMT	Antibody
PRMT1	H4R3me2a (1:2000)
	Rme1 (1:1000)
	Rme2s (1:2000)
	Rme2a (1:2000)
	Rme2a (ASYM24, 1:3000)
	Rme2a (ASYM25, 1:2000)
	H4 (1:2000)
	B-actin (1:500)
PRMT3	H4 (1:2000)
	H4R3me2a (1:2000)
	FLAG (1:5000)
PRMT4	BAF155 (1:200)
	BAF155-R1064me2a (1:3000)
PRMT5	anti-SmBB' (1:100)
	Rme2s (#13222, 1:2000)
PRMT6	H4R3me2a (1:2000)
	H4 (1:2000)
	H3R2me2a (1:2000)
	H3R8me2a (1:2000)
	H3 (1:5000)
	FLAG (1:5000)
PRMT7	Rme1 (1:1000)
	Hsp/Hsc70 (1:2000)*
PRMT8	GFP (1:3000)
	Rme2a (ASYM25,1:2000)
	FLAG (1:5000)
PRMT9	SAP145 (1:1000)
	SAP145-R508me2s -kind gift from Dr. Yanzhong Yang, Beckman Research Institute of City of Hope (1:1000) (PIMID: 25737013)
Secondary antibodies	goat-anti-rabbit IgG-IR800 (1:5000)
	donkey anti-mouse IgG-IR680 (1:5000)

*- antibody recognizes HSPA8, HSPA1 and HSPA6 (t

Chemical probe (Cell activity IC50)	Negative control
MS023 -PRMT type I	MS094
(PRMT1, PRMT6, PRMT3, PRMT4 IC50 = 9, 56, 1000, 5000 nM, respectively)	
SGC707 (IC50 = 91 nM)	XY-1
TP-064 (IC50 = 43 nM)	TP064N
SKI-73 (IC50 = 540 nM)*	SKI-73N*
LLY-283 (IC50 = 30 nM)	LLY-284
GSK591 (IC50 = 56 nM)	SGC2096
SGC6870 (IC50 = 0.9 μM)	SGC6870N
MS023 -PRMT type I	MS094
(PRMT1, PRMT6, PRMT3, PRMT4 IC50 = 9, 56, 1000, 5000 nM, respectively)	
MS049 (PRMT 4, 6 IC50 = 970, 1400 nM, respectively)	MS049N
SGC3027 (IC50 = 1300 nM) *	SGC3027N *
MS023 (50 μM)	MS094

ested with overexpressed GFP-tagged proteins), *prodrug – the IC50 may differ between var

ious cell lines

PRMT	Biomarker
PRMT1	H4R3me2a, Rme1, Rme2s, Rme2a
PRMT3	H4R3me2a
PRMT4	BAF155-R1064me2a
PRMT5	SmBB'-Rme2s
PRMT6	H4R3me2a
	H3R2me2a
	H3R8me2a
PRMT7	HSP70-R469me1
PRMT8	EWS-Rme2a
PRMT9	SAP145-R508me2s

Assay readout
H4R3me2a levels normalized to total H4
global Rme1, Rme2a or Rme2s levels normalized to B-actin.
H4R3me2a methylation levels caused by exogenous FLAG-tagged PRMT3 WT or catalytic E338Q mutant (background) normalized to total histone H4
BAF155-R1064me2a levels normalized to total BAF155
SmBB'-Rme2s levels detected with pan Rme2s antibodies (CST) normalized to total SmBB'
H4R3me2a, H3R2me2a or H3R8me2a methylation levels are increased by exogenous FLAG-tagged PRMT6 WT but not catalytic V86K,D88A mutant (background) normalized to total histone H4 or H3, respectively
HSP70-Rme1 methylation levels normalized to total HSP70
Exogenous GFP-tagged EWS methylation levels caused by exogenous FLAG-tagged PRMT8 WT or E185Q catalytic mutant (background), normalized to total GFP signal in PRMT1 KO cells.
PRMT9 dependent SAP145 symmetric dimethylation at R508 normalized to SAP145

Assay validation
Knockdown of *PRMT1* decreased basal H4R3me2a and global Rme2a levels and increased global Rme1 and Rme2s levels in cells (Fig.1A, B). PRMT Type I chemical probe MS023 decreased the levels of H4R3me2a in a dose-dependent manner (Fig. 1D).
Overexpression of wild type PRMT3 but not its catalytic mutant (E338Q) increased H4R3me2a (Fig. 2A). PRMT3 selective inhibitor SGC707 decreased PRMT3 dependent increase in H4R3me2a levels (Fig. 2B)
PRMT4 knockdown decreased asymmetric dimethylation of BAF155 (Fig. 3A). 2 day treatment with PRMT4 selective chemical probe (TP-064) decreased asymmetric dimethylation of BAF155 (Fig. 3B).
Knockdown of *PRMT5* resulted in decreased SmBB' symmetric dimethylation levels (Fig. 4A). 2 day treatment with PRMT5 selective chemical probes, GSK591 and LLY285, decreased SmBB'-Rme2s levels (Fig. 4B).
Overexpression of wild type PRMT6 but not its catalytic mutant (V86K,D88A) increased H3R2me2a, H3R8me2a and H4R3me2a levels (Fig. 5A). Allosteric PRMT6 inhibitor (SGC6870), PRMT type I inhibitor MS023 , PRMT4/6 inhibitor MS049 decreased PRMT6 dependent increase in H3R2me2a levels (Fig. 5B).
PRMT7 knockout or knockdown reduced HSP70 monomethylation (Fig. 6A). 2 day treatment with PRMT7 selective chemical probe SGC3027 decreased PRMT7 dependent HSP70 monomethylation in a dose-dependent manner (Fig. 6B).
Overexpression of the wild type PRMT8 but not catalytic E185Q mutant methylated ectopic EWS only in PRMT1 KD cells (Fig. 7A). PRMT type I chemical probe MS023 inhibited asymmetric dimethylation of exogenous EWS by PRMT8 (Fig. 8B).
The loss PRMT9 but not PRMT5 lead to decreased symmetric dimethylation of SAP145. GFP-tagged SAP145 WT but not SAP145mut (R508K) was methylated by PRMT9 (Fig. 8A). 2-day treatment with Copound X, the prototype PRMT9 inhibitor, decreased SAP145-R508me2s levels in a dose-dependent manner Fig 8B).

Recommended cell line	Ref.
Cells differ in basal H4R3me2a levels (Fig. 1C). MCF7 cells have high basal H4R3me2a levels which makes it preferable for assays monitoring the decrease in PRMT1 activity.	8
HEK293T cells have low basal H4R3me2a levels (Fig 1C), which is preferable for monitoring exogenous PRMT3 activity	7
Any cell line	10
Any cell line	11
HEK293T cells have low basal H4R3me2a, H3R2me2a and H3R8me2a levels, which is preferable for monitoring exogenous PRMT6 activity	8,9
C2C12, HT180 Several cancer cell lines express an inducible form of HSP70 whose methylation signal overlaps with an unspecific protein of nuclear origin (Fig. 6C). In this case, we recommend analyzing HSP70 methylation levels in the cytoplasmic fraction.	12
HEK293T *PRMT1* KD (inducible). *PRMT1* knockdown results in cell death therefore we recommend using an inducible system.	8
Any cell line	21

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10 cm TC dishes	Greiner bio-one	664160	
24-well TC plates	Greiner bio-one	662160	
4–12% Bis-Tris Protein Gels	ThermoFisher Scientiffic	NP0323BOX, NP0322BOX,NP0321BOX	
Amersham Hybond P PVDF membrane	Millipore-Sigma	10600021	
anti-Asym 24	Millipore-Sigma	07-414	
anti-Asym 25	Millipore-Sigma	09-814	
anti-B-actin	Santa Cruz Biotechnologies	sc-47778	
anti-BAF155	Santa Cruz Biotechnologies	sc-32763	
anti-BAF155-R1064me2a	Millipore-Sigma	ABE1339	
anti-FLAG (#, 1:5000)	Millipore-Sigma	F4799	
anti-GFP	Clontech	632381	
anti-H3	Abcam	ab10799	
anti-H3R2me2a	Millipore-Sigma	04-848	
anti-H3R8me2a	Rockland	600-401-I67	
anti-H4	Abcam	ab174628	
anti-H4R3me2a	Active Motif	39705	
anti-Hsp/Hsc70	Enzo	ADI-SPA-820	
anti-PRMT1	Millipore-Sigma	07-404	
anti-PRMT3	Abcam	ab191562	
anti-PRMT4	Bethyl	#A300-421A	
anti-PRMT5	Abcam	ab109451	
anti-PRMT6	Abcam	ab47244	
anti-PRMT7	Abcam	ab179822	
anti-Rme1	CST	8015	
anti-Rme2a	CST	13522	
anti-Rme2s	CST	13222	
anti-Rme2s (ASYM25), Millipore, , 1:2000)		09-814	
anti-SAP145 (Abcam, #, 1:1000)	Abcam	ab56800	
anti-SAP145-R508me2s			kind gift from Dr. Yanzhong Yang, Beckman Research Institute of City of Hope
anti-SmBB'	Santa Cruz Biotechnologies	sc-130670	
benzonase			PRODUCED IN-HOUSE
BSA	Millipore-Sigma	A7906	
C2C12			gift from Dr. Stephane Richard, McGill University
cOmplete, EDTA-free Protease Inhibitor Cocktail	Millipore-Sigma	11873580001	
DMEM	Wisent	319-005-CL	
DMSO	Bioshop	DMS666.100	
donkey anti-mouse IgG-IR680	Licor	926-68072	
doxycycline	Millipore-Sigma	D9891	
EDTA	Bioshop	EDT111.500	
FBS	Wisent	80150	
glycine	Bioshop	GLN002.5	
goat-anti-rabbit IgG-IR800	Licor	926-32211	
HEK293T			gift from Dr. Sam Benchimol, York University
Image Studio Software ver 5.2	Licor		
Loading buffer: NuPAGE LDS Sample Buffer (4x)	ThermoFisher Scientiffic	NP0007	
MCF7		ATCC® HTB-22™	
NaCl	Bioshop	SOD001.1	
NuPAGE MOPS SDS Running Buffer	ThermoFisher Scientiffic	NP0001	
Odyssey Blocking Buffer (dilute 4 x with PBST)	Licor	927-40000	Intercept (PBS) Blocking Buffer can also be used # 927-70001
Odyssey CLX Imaging System	Licor	model number 9140	
PBS (tissue culture)	Wisent	311-010-CL	
PBS (western blot)	Bioshop	PBS405.4	
penstrep	Wisent	450-201-EL	
Pierce™ BCA Protein Assay Kit	ThermoFisher Scientiffic	23225	
SDS	Bioshop	SDS001.1	
skim milk powder	Bioshop	SKI400.500	
TC20 automated cell counter	Biorad	1450102	
Tripsin-EDTA (0.25%)	Wisent	325-043-EL	
Tris	Bioshop	TRS003.5	
Tritton X-100	Bioshop	TRX506	
trypan blue	GIBCO	15250-061	
Tween-20	Bioshop	TWN510.500	

Dear Editor and production team,
Thank you for the helpful comments and suggestions. We have implemented the changes as outlined below.
Kind regards,
Magda

Editorial and production comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please use American English throughout.
Done, please see tracked changes

2. Please provide an email address for each author.
Done

3. Please make the title concise.
Changed, as per reviewer 1 suggestion.

4. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
Done

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Millipore-Sigma, Cell Signalling Technologies, Falcone, JetPRIME buffer, NuPAGE, Pierce™ BCA Protein Assay Kit, Amersham Hybond P, Odyssey Blocking Buffer, Odyssey CLX Imaging System from Licor, Image Studio Software, etc.
Done

We have removed details regarding the transfection protocol because protocols differ between transfection reagents. Without giving a name it is impossible to include the details.
We had to leave the name for the protein assay kit we use (BCA Protein Assay KIT), otherwise, we would have to remove the protocol description as well.

6. Line 64: Please move the hyperlinks to the reference section and use in text citations in the text.
Done

7. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

Done

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.
Done

9. 2: For how long do you perform the transfection? Do you change the medium in between?
It is indicated in the text that media should be replaced after 4 h after transfection, and the lysates are collected after 20-24h post-transfection.

10. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Most of the methodology described here was previously published in detail. The novel information is mainly focused on the reagents (e.g. antibodies, chemical probes) and biomarker proteins. We modified the protocol as per suggestion, where possible.

11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
None of the figures were previously published.

12. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
The discussion was revised according to suggestions, where possible.

13. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.
Done

14. Please do not abbreviate the journal titles in the reference section.
Done

15. Table 3: Please remove the commercial terms from the table and include it in the table of materials.

Done

Changes to be made by the Author(s) regarding the video:
1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.
We added a figure to the manuscript and used it for the results section.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.
We changed the narration to be consistent with the manuscript, where possible.

3. Please ensure that section titles for the protocol are the same in the text and the video.
Done

4. Please ensure that the narration matches with the video being shown.
Done

5. 2:55 – please remove the commercial term Pierce Tm from the video and from the narration. We cannot have commercial terms in the video and in the text.
Done

6. 3:27: Please show changing of the tips at this point when moving from +B to -B tube.
We have a new recording now incorporating this suggestion.

7. 3:44: Please include real video clips to show the gel electrophoresis, western transfer, and antibody incubations.
Done

8. 3:55- Please do not mention Odyssey Imaging system form Licor. We cannot have commercial terms in the video.
Done

9. Please include results section with data and findings presented after the protocol and before the conclusion section.
Done

10. Video edits
• Fade up from black at the start of the video.
Done

• Fade to black at end of the video.
Done

• 01:09 - Try to condense this section by editing out non-critical moments, such as when well plates are being re-arranged or when pipette tips are being changed. This could reduce the "dead air" between VO clips.
Done

• 03:02 - Give a second of pause between the end of section 2 and section 3 title card, or consider delaying the section 3 VO by a second to give a pause.
Done

11. Audio edits
• 00:49 - Edit out mouth click sound, consider starting this VO clip a few seconds earlier
• 04:18 - Edit out mouth click sound
• 04:39 - Edit out knocking sound
We made significant changes to the video and made sure to remove all noticeable mouth-click sounds.

Once done, please ensure that the revised video is no more than 15 min in length. Please upload the revised video at:
https://www.dropbox.com/request/6y0sUOoYrXNBmaFUtrOI?oref=e

Dear Reviewers,
We thank for your time and insightful suggestions and comments. We have incorporated the changes into the manuscript and provided additional details requested. We hope that in the present form the manuscript is suitable for the publication in JoVE.
Kind regards,
Magda Szewczyk

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this JOVE article titled "Methods to study protein arginine methyltransferase activity in cells", the authors describe a creative method for detecting activity of individual PRMT isozymes. This required a review of literature to ensure that the substrates being tested were only substrates for a particular isozyme or that the activity was measured by using biomarker assays with ectopically expressed enzymes along with the inactive mutants as a set of controls. These cell based assays can measure the enzymatic activity of PRMT isozymes quantitatively.

Given this article provides a complete overview of the methodology and explanation of the techniques required to conduct these cell-based assays, this reviewer believes this manuscript is acceptable for publication in JOVE after the following minor concerns are addressed.

Major Concerns:
None

Minor Concerns:
1. Given this article details the methods for quantitatively detecting the enzymatic activity of PRMT 1 - 9, the authors should consider changing the title to "Quantitative Methods to study Protein Arginine Methyltransferase 1 - 9 Activity in Cells"
We thank the reviewer for this suggestion, the title was changed accordingly

2. The authors could mention how these biomarker assays could be utilized/incorporate competitive ABPP assays.
We added the sentence to the Discussion.
"The recommended antibodies and chemical probes for PRMTs can also be utilized for activity-based protein profiling (ABPP) assays to establish the suitability of a given ABPP probe, monitor target engagement and assess off target effects by using the competitive ABPP format (Ref PMID: 29686618)."

3. The authors should highlight that the assays require minimal equipment except for cell culture capabilities and Western Blotting.

"The advantage of the described methods is their straightforward performance in any lab with cell culture and fluorescent western blot capabilities." – sentence added to abstract

"This collection of quantitative assays for profiling the activity of PMRTs in cells can be broadly useful for the scientific community since it can be rapidly and easily implemented with minimal equipment and limited technical expertise, involving only basic cell culturing and fluorescent western blotting techniques." – sentence added to the Discussion section.

Reviewer #2:
Magdalena et al. described a very good method for investigating the enzymatic activity of Protein arginine methyltransferases (PRMTs) in the cells. Importantly, they have covered eight out nine well-studied PRMTs in this article. They have detailed all the reagents and protocol fully. The video associated with the article is also well done. The manuscript is suitable for publication in the Journal of Visualized Experiments. In the discussion section, the authors may wish to discuss that this method can be easily extrapolated to the related Protein lysine methyltransferases.
We thank the reviewer for the suggestion. The following sentence was added to the Discussion. "The assay development approaches discussed here can also be extrapolated for other enzyme families such as protein lysine-methyltransferases and acetyltransferases."

Reviewer #3:
Methods to study protein arginine methyltransferase activity in cells

With growing interest on protein methyltransferases in diverse biological processes, this manuscript tries to provide guidelines to assay individual PRMT activity with common or unique substrate, modification-specific antibodies and specific inhibitors for each PRMT member. Experimental procedure is well-designed, performed and materials are appropriately selected. The details of assays for each PRMT are well-illustrated with representative data in the manuscript.

With few minor revisions, this manuscript is suitable for publication in JOVE.

1. Authors intend to show the best method for assaying each PRMT activity. However, it will be more beneficial to the readers if the reason for choosing LOF (loss of function) or GOF (gain of function) for each PRMT assay is provided. For example, overexpression rather than knockdown strategy was used in assaying PRMT3, or PRMT6 activity, while knockdown was used for assaying PRMT1 activity.
We thank the reviewer for bringing up this issue. The gain of function approach, although not preferable, was used for those PRMTs for which we could not detect significant changes in biomarker methylation upon knockdown in the timeframe of 3 days. We always start with knockdown experiments, however, if this approach is not working, we develop assays using an overexpression system. This challenge is explained and now clarified in the Discussion section.

2. H4R3me3a was shared in some PRMTs (PRMT1, PRMT3 and PRMT6). Authors need to show how much PRMT1 KD overrides the effect from PRMT3 or PRMT6 KD.

We thank the reviewer for identifying the lack of clarify in the use of H4R3me2a biomarker. PRMT1 is a major H4R3 methylator, therefore we used cells with lower basal H4R3me2a levels and by transfecting exogenous wild-type or catalytic mutant PRMT3 and PRMT6 we were able to distinguish the activity of these PRMTs on H4R3 that aligns well with previously reported in vitro data. Overexpression systems using wild-type and catalytic PRMT mutant enable subtracting the basal methylation levels (mostly dependent on PRMT1 activity). These challenges are explained and clarified in the Discussion section.

3. Figure 1A needs to include data for the efficiency of PRMT1 KD.
The sentence was added to Fig. 1A legend. "The PRMT1 knockdown efficiency is presented in panel B."

4. Please clarify whether the bands indicated as smBB' in Figure 4A correspond to Ariginine-methylated or total protein. If those are Arg-methylated smBB', then blot for total smBB' needs to be shown.
The sentence was added to Fig4 legend. "PRMT5 selective inhibitors, GSK591 and LLY-283, decrease SmBB' symmetric arginine dimethylation (green), while total levels of SmBB' remain unchanged (red)."

5. Knockdown efficiency of PRMTs (1, 3, 4, 6 and 7) in Figure 4A needs to be provided.
Thank you. The knockdown data is now provided in Fig. 4A.

6. The information about PRMT6 expressed is missing in the middle panel of Figure 5B. The legend about MS117, yet, it is missing in the figure.
Figure 5 and its legend were corrected.

7. Shouldn't PRMT E185Q in Figure 7B be PRMT8 E185Q?
Thank you. It is now corrected.