PRMT5 glutathionylation affects methyltransferase activity

Glutathionylation decreases methyltransferase activity of PRMT5 and inhibits cell proliferation

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Abbreviations: PRMT5: protein arginine N-methyltransferase 5; Grx1: glutaredoxin-1; MEP50: methylosome protein-50; SAM: S-adenosylmethionine; H4R3me2: histone H4 arginine 3; H3R8me2: histone H3 arginine 8; H2AR3me2: histone H2A arginine 3; CDK: cyclin D1/cyclin-dependent kinase; ROS: reactive oxygen species; LC-MS/MS: liquid chromatography tandem mass spectrometry; H$_2$O$_2$: hydrogen peroxide; TIM: triosephosphate isomerase; IPA: Ingenuity Pathway Analysis.
ABSTRACT

Glutathionylation is an important posttranslational modification that protects proteins from further oxidative damage as well as influencing protein structure and activity. In the present study, we demonstrate that the cysteine-42 residue in protein arginine N-methyltransferase 5 (PRMT5) is glutathionylated in aged mice or in cells that have been exposed to oxidative stress. Deglutathionylation of this protein is catalyzed by glutaredoxin-1 (Grx1). Using mutagenesis and subsequent biochemical analyses, we show that glutathionylation decreased the binding affinity of PRMT5 with methylosome protein-50 (MEP50) and reduced the methyltransferase activity of PRMT5. Furthermore, overexpression of PRMT5-C42A mutant caused a significant increase in histone methylation in HEK293T and A549 cells and promoted cell growth, whereas overexpression of the PRMT5-C42D mutant, a mimic of glutathionylated PRMT5, inhibited cell proliferation. Taken together, our results demonstrate a new mechanism of regulation of PRMT5 methyltransferases activity and suggest that PRMT5 glutathionylation is partly responsible for reactive oxygen species-mediated cell growth inhibition.

Histone methylation alters the activity of the histone and regulates its interactions with other proteins, with important effects on the regulation of transcription. Dysregulation of histone methylation is associated with various diseases including cancer and neurodegenerative diseases (1). The methylation of histone arginine residues is catalyzed by the protein arginine N-methyltransferases (PRMTs) family, which require S-adenosylmethionine (SAM) as a methyl donor during the catalysis and alter the histones’ activity and interaction with other proteins, leading to changes in cell physiology (2-4). The PRMTs family belong to the SAM-dependent methyltransferases, which catalyze the transfer of methyl groups from SAM to arginine residues of various proteins (5). The PRMT family is classified into two subfamilies: namely, Type I and Type II PRMTs. The function of Type I PRMTs is to form monomethylation and asymmetric dimethylation of arginine residues, while Type II PRMTs is to catalyze monomethylation and symmetric dimethylation (6). Among the 9 members of the PRMT family, only PRMT5 acts as a Type II PRMT, catalyzing the symmetric dimethylation of histone H4 arginine 3 (H4R3me2), histone H3 arginine 8 (H3R8me2), and histone H2A arginine 3 (H2AR3me2) (7-10). Interaction with methylosome protein-50 (MEP50) is required for PRMT5 to form a fully functional holoenzyme in the form of a hetero-octameric complex (11). The hetero-octamer can interact with
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substrates and catalyze methyl transfer, resulting in symmetrical methylation of arginine residues (12, 13). This methyltransferase activity can be regulated by phosphorylation of either MEP50 or PRMT5. For instance, phosphorylation of threonine-5 of MEP50 by cyclin D1/cyclin-dependent kinase (CDK)-4 activates PRMT5 and promotes tumor cell growth (14).

Reactive oxygen species (ROS) are exogenous oxidants or active oxygenates produced during aerobic metabolism, which play important roles in physiological and pathological processes (15). ROS are mainly produced by the mitochondria, peroxisomes, NADPH oxidase on the cell membrane surface, through endoplasmic reticulum emergency responses, or oxidoreductase reactions (16). Under normal physiological conditions, the generation and elimination of ROS are balanced to maintain appropriate physiological functions of cells (17). When redox homeostasis is disrupted, increased ROS levels may result in irreversible damage to cellular components (18, 19).

Under conditions of oxidative stress, cysteine residues in proteins are oxidized to form S-hydroxylation products which may be further oxidized to sulfinic acids, thiosulfinate ester sulfonamides, or sulfonic acids (20). These irreversible oxidative modifications of cysteine residues can lead to direct damage or degradation of the oxidized proteins (21, 22). Glutathione (GSH) is one of the most abundant molecules in cells, which can form disulfide bonds with the sulfhydryl groups of free cysteine residue, resulting in glutathionylation that can protect these residues from irreversible oxidation under oxidative stress (23-25). Deglutathionylation is carried out by glutaredoxins (26). Although glutathionylation prevents irreversible oxidation, studies have shown that it can induce protein degradation (27, 28). For example, glutathionylation of cytoskeletal proteins can lead to degradation of important structural proteins, thus affecting cytoskeletal organization and cell motility (29). Studies have demonstrated that glutathionylation can affect protein activity and thus is involved in the regulation of a number of physiological processes in cells (30). For instance, glutathionylation of transcription factors alters their binding with DNA and thereby regulates gene transcription, while glutathionylation of kinases and phosphatases regulates cell signal transduction (31, 32). Glutathionylation is regulated and tightly controlled in cells under normal physiological conditions, whereas high cellular ROS can disrupt this balance by promoting glutathionylation.

It has been reported that PRMT5 is upregulated in solid and hematological cancers, and its expression is positively correlated with disease progression (33). The enzymatic activity of PRMT5 has been found to be regulated by post-translational modifications such as phosphorylation or acetylation (34-36). It is important to identify other modifications that can regulate the activity
PRMT5 glutathionylation affects methyltransferase activity of PRMT5. In the present study, we found that Cys42 of PRMT5 is glutathionylated in aged mice and in response to oxidative stress with concomitant decreased methyltransferase activity.

Experimental procedures

Cell culture

Human embryonic kidney 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Wisent, Montreal, QC) and 1% streptomycin/penicillin (Wisent, Montreal, QC) at 37°C in a humidified incubator with 5% CO₂.

Cross-linking and pull-down assay

Equal amounts of proteins obtained from Grx1-overexpression and the control cell line were divided into two parts of which one was added with BS3. Then, all the cell lysates were incubated with Anti-FLAG Affinity Gel (Sigma) at 4°C overnight. After washing the beads with PBS five times, the beads were boiled with loading buffer for elution. After separating the protein mixtures on an SDS-PAGE gel, the bands were cut into pieces in 25 mM dithiothreitol and alkylation with 55 mM iodoacetamide in dark at 37°C for 16h followed by trypsin digestion. Then the digested peptides were extracted by the 1% formic acid in a 50% acetonitrile aqueous solution. The extractions were dried in Speedvac to reduce the volume and sent to LC-MS/MS for analysis. Proteins were searched against the homo sapiens database from UniProt using PD2.1 (Proteome Discoverer Searching Algorithm Version 2.1) and identified as binding partners of Grx1 by the label-free quantitation method. The experiments were carried out in three biological replicates to ensure accuracy.

Establishment of stable overexpression cells

The human PRMT5 cDNA was obtained from U87 cells. A Flag tag was added at the C-terminus of PRMT5 coding region, and the recombinant human PRMT5 DNA was cloned into the pLVX-IRESZsGreen1 vector. HEK 293T cells were transfected with pLVX-PRMT5-IRES-ZsGreen1 and other packing vectors to obtain lentiviral particles. After that, HEK 293T cells were infected with lentiviral particles and then sorted by flow cytometer when GFP is positive to generate stable cell lines.
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**MALDI-TOF/TOF detection of glutathionylation**

The 4800 MALDI-TOF/TOF analyzer obtained from Applied Biosystems was used to identify whether the mass spectra of the target peptide were with glutathionylation or not. The target peptide (QGFDLCMPVFHPR) was synthesized from BankPeptide and treated with 100mM GSSG at 37°C for 1h to be glutathionylated. The Grx1 selective reduction assay was used to undergo the deglutathionylation of the glutathionylated peptide. Then, the 4800 MALDI-TOF/TOF analyzer was used for acquiring mass spectra in the positive ion reflector mode of substrate peptides and its products. 10 mg CHCA was prepared to a final volume of 1 mL in 50% ACN (v/v), 0.1% TFA solution as CHCA matrix solution, and the prepared CHCA matrix solution was mixed with samples using 1:1 ratio (v/v) before spotting onto 1536-MTP AnchorChip targets. After air dry, MALDI-MS analysis was carried out to obtain the mass spectra of the target peptides either with or without glutathionylation.

**PRMT5 activity detection assay**

The Epigenase™ PRMT Methyltransferase (Type II-Specific) Activity/Inhibition Assay Kit (Colorimetric) was used to detect the PRMT5 activity. Because PRMT5 belongs to type II PRMTs, it could catalyze symmetric dimethylation of histone arginine residues using S-adenosyl-methionine (SAM) as a methyl donor. In this assay, a peptide of H4 is stably coated in microplate wells. Active PRMT5 was added to bind to the substrate and transfer a methyl group from SAM to the substrate. The products can be recognized with a specific antibody, and the amount of products can then be calorimetrically measured by reading Optical density (OD) at 450 nm so as to calculate the activity of PRMT5.

**Western blot analysis**

Cells were lysed at 4°C in lysis buffer (Solarbio, Beijing, China) and then mixed with 1% Protease Inhibitor Cocktail (Thermo-Pierce Biotechnology, Rockford, IL). The BCA protein assay kit (Solarbio, Beijing, China) was used to determine protein concentrations. Equal amounts of protein were separated by 12% or 15% SDS-PAGE gel and transferred to PVDF (polyvinylidene difluoride) membranes. The target protein was probed with the appropriate primary antibody and then detected by secondary antibodies with horseradish peroxidase. Immunoblots were processed on films through the chemiluminescence technique. The values were normalized based on the expression level of actin. Anti-Grx1 antibody was from Abcam (ab187507); anti-actin antibody...
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was from Cell Signaling Technology (4970); anti-PRMT5 antibody was from SantaCruz (sc-376937); against H3-R8me2s, H3-R2me2s, H4-R3me2s, HIST3H3, Histone H4 were from Abclonal (A2374, A2373, A3159, A2348, A1131).

Experimental design and statistical rationale

TMT labeling was used to perform quantitative proteomics on three independent biological replicates to minimize the variability between samples. In results of quantitative analysis, we used at least 2 unique peptides to identify proteins and the abundances from each channel were used to calculate the mean ratios of identified proteins. Significantly changed proteins were screened by volcano plot analysis which is detected by Student’s t-test. Histograms of protein fold-changes in proteomic data were used to demonstrate normal data distribution. Pearson correlation was calculated to test the reproducibility of protein quantification. The target modified peptides including methylated and glutathionylated peptides were confirmed manually in three independent biological replicates.

Quantitative proteomics analysis

Proteins (200 μg) were extracted from cells in which PRMT5 and its mutants were overexpressed with a buffer containing 8 M urea/PBS and 1% protease inhibitor. The proteins were reduced by adding 5 mM Tris (2-carboxyethyl) phosphine (TCEP) for 10 min and then alkylated by incubating with 10 mM iodoacetamide for 30 min at room temperature in the dark. After the urea concentration was diluted below 1.5 M with PBS, protein samples were digested with the sequencing-grade trypsin for 16 h at 37 °C at a substrate/enzyme ratio of 50:1 (w:w). The resulting peptides were desalted with a Sep-Pak C18 Cartridge (Waters, 186004619) followed by vacuum drying, and labeled with Tandem Mass Tag reagent (TMT, Thermo, Waltham, MA). TMT-labeling peptides were separately pooled and were fractionated using the high-pH reversed phase chromatography.

For LC-MS/MS analysis, the labeled peptides were mixed and loaded onto a trap column, and then separated with a reverse-phase analytical column using a Thermo-Dionex Ultimate 3000 HPLC system which was directly coupled with a Thermo Orbitrap Fusion Lumos mass spectrometer. The 135 min reverse-phase separation was used with mobile phase A (0.1% FA) and mobile phase B (100% ACN, 0.1% FA). The mass spectrometer was operated in the data-dependent acquisition mode and MS1 spectra were acquired in the Orbitrap at a mass range of 300–1500 m/z
with a resolution of 120,000. For MS2 scans, the top 20 most intense precursor ions were fragmented in the HCD collision cell at a normalized collision energy of 35% using a 0.7 Da isolation window and the dynamic exclusion duration was 15 s. The AGC target is 20,000 while the maximum injection time is 60 ms.

The raw MS data were analyzed by the Proteome Discoverer 2.1 software and the generated MS/MS spectra were searched against homo sapiens database (downloaded from Uniprot, released on October 25, 2017, containing 20,168 entries). The following parameters were used for database searching: fixed modifications of TMT 6-plex on lysine or peptide N-terminus and carbamidomethylation on cysteine; variable modification of oxidation on methionine; two trypsin missed cleavages were permitted; the tolerances of precursor and fragment mass were 10 ppm and 0.02 Da; at least two unique peptides for identification of proteins; and 1% FDR (false discovery rate) at peptide-spectrum match (PSM) level.

Cell cycle

Cells were harvested and washed by ice-cold PBS three times. The cell pellet was resuspended by 70% ethanol for 2h on ice to fix cell states. And then, the fixed cells were stained with PI at the present of RNase A. Stained cells were used to identify cell-cycle by BD FACS Aria Flow Cytometer (Becton Dickinson, NJ). The cell cycle analysis was based on the percentage of cells in different cell-cycle phase to analyze the cell states.

Cell proliferation assay with CCK8

Cell proliferation was determined using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) by accounting the activity of dehydrogenase in cells which was proportional to the number of living cells. Cells were counted by TC20™ Automated Cell Counter (BIO-RAD) and plated at 2000 cells/well on 96-well plates in three replicates. Absorbance at 450 nm was measured 2h after CCK-8 addition at each time point of 0, 12, 24, 36, 48, 60, 72, 84, and 96 hours to represent relative cell numbers.

Statistic method

GraphPad Prism software version 6.0 was used for statistical analysis and graph preparation. Data are shown as the mean ± SEM, and significance was determined with Student’s t-test. P values of < 0.05 were considered significant (*, p < 0.05; **, p < 0.01; ***, p < 0.001), while ‘n’ stands
PRMT5 glutathionylation affects methyltransferase activity for independent experimental replications. The signaling pathways of protein differentially expressed were analyzed using Ingenuity Pathway Analysis (IPA, QIAGEN), and Database for Annotation, Visualization, and Integrated Discovery (DAVID). The proteins enriched by different methods were classified with functional protein association networks (STRING).

Results

PRMT5 is glutathionylated in aged mice or in conditions of oxidative stress

As age increases, aging-related oxidative damage accumulate and aggravate DNA and protein damages (37). Cysteine sulfhydryl groups are sensitive to changes in the intracellular redox state and can be specifically and reversibly modified. We analyzed kidney samples from 96- and 8-week-old mice to identify differences in glutathionylation between these two groups. Based on liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis, the glutathionylation level of a peptide containing a cysteine residue (C42) of PRMT5 was higher in old mice compared to the young as represented by the intensity ratio of the glutathionylated vs. unmodified peptide (Fig. 1a).

To confirm whether oxidative stress drove PRMT5 glutathionylation, we constructed a FLAG-tagged PRMT5-overexpression cell line and treated cells with hydrogen peroxide (H2O2) for 12 h. After lysis, PRMT5 was enriched using M2 resin and analyzed by LC-MS/MS. Following H2O2 treatment, we observed that a peptide at m/z 565.27 corresponding to the triply-charged peptide (QGFDLCMPVFHPR) was converted into a peptide at m/z 667.30 corresponding to the triply-charged glutathionylated peptide (Fig. 1b). To further confirm the credibility of the peptides, peptide fragmentation resulted in fragmented ions which matched the predicted b and y ions, confirming glutathionylation of Cys42 (Fig. 1c-1d). By evaluating the ion intensity, we determined that 1 mM H2O2 treatment caused a 4-fold increase in PRMT5 glutathionylation (Fig. 1e, Table S1).

Glutaredoxin-1 removes glutathione from glutathionylated PRMT5

Grx1 is the deglutathionylation enzyme in mammalian cells, which regulates ROS levels (38). To evaluate whether glutathionylated PRMT5 is a substrate of Grx1, we performed immunoprecipitation to verify the interaction between the two proteins. To capture transient interactions, we added the BS3 as a cross linker into the cell lysate. Immunoprecipitation and in-
gel digestion followed by LC-MS/MS analysis revealed that PRMT5 was significantly enriched in the cross-linked samples. Western blotting also confirmed the interaction between Grx1 and PRMT5 (Fig. 2a), indicating that PRMT5 is a substrate of Grx1.

To verify that the glutathionylated PRMT5 was a substrate of Grx1 both in vitro and in vivo, we synthesized a peptide (QGFDFLCMPVFHPR) from the PRMT5 sequence which was then glutathionylated by glutathione disulfide (GSSG), while the mass of the glutathionylated peptide is 1,997.57. Deglutathionylation of the modified peptide was catalyzed by Grx1 in the presence of glutathione reductase (GR), reduced nicotinamide adenine dinucleotide (NADH), and glutathione (GSH), resulting in a product at m/z 1,692.55 (Fig. 2b, Fig. S1). The mass difference matched that of GSH, confirming that Grx1 catalyzed the deglutathionylation reaction. Moreover, PRMT5 glutathionylation was decreased when GRX1 was overexpressed in the PRMT5 overexpression cells and increased when GRX1 was knocked down (Fig. 2c, Table S1). This further confirms that glutathionylated PRMT5 is a substrate of Grx1.

**Glutathionylation leads to decreased PRMT5 activity**

Preliminary studies have shown that PRMT5 interacts with MEP50 to form a hetero-octamer and functions as a histone methyltransferase (39). To determine the effect of glutathionylation on the methyltransferase activity of PRMT5, we used GSSG to react with PRMT5 in vitro to generate the glutathionylated PRMT5 and examined its enzyme activity on methylation of a H4 peptide. The activity of PRMT5 alone was about 50% of PRMT in complex with MEP50. However, the prior treatment of PRMT5 with GSSG greatly decreased its activity and resulted in an approximately 70% decrease in methyltransferase activity of PRMT5, indicating that glutathionylation decreased the PRMT5 activity (Fig. 3a).

Furthermore, we examined whether glutathionylation inhibited PRMT5 activities in vivo. We constructed 293T cells in which PRMT5-WT and its mutant (PRMT5-C42A, PRMT5-C42D) were overexpressed with the C42D mutant mimics the glutathionylated form of PRMT5 and the C42A mimics the un-glutathionylated form of PRMT5. The expression levels of PRMT5-WT and its mutants in 293T cells was assessed by real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and western blotting, confirming that mRNA expression level of PRMT5 was 12-fold higher than in control (pLVX) cells while the expression levels of PRMT5-WT and its mutants were almost consistent (P < 0.001, Student’s t-test) (Fig. 3b). To explore changes in the stability of C42D mutant, we purified the PRMT5-WT and PRMT5-C42D proteins and confirmed
that no obvious changes appeared in structural alterations caused by mutations via circular dichroism spectroscopy (Fig. S2). As expected, examination of di-methylation on H4R3, H3R2, and H3R8 by western blotting revealed that histone methylation increased when either PRMT5-WT or PRMT5-C42A was overexpressed while decreased when PRMT5-C42D was overexpressed. This confirms that glutathionylation decreases the methyltransferase activity of PRMT5 in vivo (Fig. 3c–d). To quantify di-methylation of H4R3, H3R2, and H3R8 under different overexpression conditions, we extracted nuclear proteins from these cells and performed semi-quantification of the di-methylation ratio by LC-MS/MS (Fig. 3e–g). We found that MS results were consistent with western blot data, indicating that glutathionylation indeed decreases the methyltransferase activity of PRMT5 in vivo.

**Glutathionylation of PRMT5 disrupts the interaction with MEP50**

Previous studies have reported that human PRMT5 contains an N-terminal triosephosphate isomerase (TIM) barrel and a β-barrel with a dimerization domain on the C-terminus (40). The human PRMT5 methyltransferase complex is composed of four PRMT5 proteins and four MEP50 proteins (41). The PRMT5 molecule forms two dimers in a typical head-to-tail arrangement, which then form a tetramer via hydrogen bonds. The PRMT5 tetramer forms the core of the complex, and MEP50 interacts through the N-terminal TIM barrel domain. The methylation substrates of PRMT5 and SAM bind to the C-terminal β-barrel of PRMT5 to enable transfer of the methyl group. Methyltransferase activity of the PRMT5-MEP50 complex is higher than that of PRMT5 alone. This may be due to positive allosteric effects of MEP50 on the binding of cofactors, SAM or substrates. The essential role of MEP50 in transporting protein substrates to PRMT5 has also been verified.

Glutathionylation of C42 of PRMT5 occurs on the N-terminal domain, which is far from the region where the C-terminus binds the substrate, so glutathionylation has no direct effect on substrate recognition. The structural organization of PRMT5/MEP50 complex illustrated that the C42 residue is located at the interface between PRMT5 and MEP50 in which the glutathionylation on Cys42 was coded by red color in Fig. 4a (PDB:4GQB). To explore the effects of glutathionylation on the binding affinity of PRMT5 with MEP50, we performed immunoprecipitation of PRMT5 in cells in which PRMT5-WT and its mutants (PRMT5-C42A, PRMT5-C42D) were overexpressed. The binding affinity between PRMT5-C42D and MEP50 was significantly weaker than that between PRMT5-WT and MEP50, while the interaction between
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PRMT5-C42A and MEP50 had the highest affinity (Fig. 4b). These results indicate that glutathionylation of C42 of PRMT5 decreases the strength of the interaction between PRMT5 and MEP50. The affinity of PRMT5 for kinase RIO 1, which has a similar function to MEP50, exhibited a similar trend (Fig. 4c, Table S2).

The PRMT-C42D mutation induces cell-cycle arrest at G2 phase and suppresses cell proliferation

To understand the effect of glutathionylation of C42 of PRMT5 on cellular processes, quantitative proteomic analysis was carried out to determine differentially expressed proteins between PRMT5-WT and PRMT5-C42D overexpression 293T cells. In total, we identified 9,953 proteins from the three biological replicates, 6,956 of which were identified in all three repeats (Fig. 5a). The results of the three independent biological replicates exhibited a tight correlation, indicating that these data were highly reproducible (Fig. 5b). The obtained quantitative ratios were then filtered by population statistics, which indicated the significant threshold cutoff of differentially expressed proteins to be 50% variation (Fig. S3). The volcano diagram showed that 117 proteins were upregulated (PRMT5-C42D/PRMT5-WT ratio > 1.5 and FDR-adjusted $P < 0.05$) and 137 proteins were downregulated (PRMT5-C42D/PRMT5-WT ratio < 0.67 and FDR-adjusted $P < 0.05$) (Fig. 5c, Table S3). This indicates that the C42D mutant, which mimics glutathionylation, affects cellular proteostasis. We performed Ingenuity Pathway Analysis (IPA) to cluster the differentially expressed proteins in the PRMT5-C42D overexpression cells (Fig. 5d). The top 20 canonical pathways enriched by IPA revealed that glutathionylation on C42 of PRMT5 was associated with four major biological processes: the cell cycle, epithelial-mesenchymal transition, NRF2-mediated oxidative stress response and EIF2 signaling. The proteins involved in the cell cycle, epithelial-mesenchymal transition, sirtuin signaling pathway and EIF2 signaling were mostly downregulated (Fig. 5e), whereas those involved in the NRF2-mediated oxidative stress response, aryl-hydrocarbon receptor signaling and the apoptosis pathway were upregulated (Fig. 5f). Quantitative proteomic and IPA analysis of PRMT5-WT and PRMT5-C42D-overexpressing A549 cells revealed similar enrichment patterns, suggesting that this effect is not specific to 293T cells (Fig. S4, Table S4).

It has been suggested that PRMT5 is a proto-oncogene in cancer and neurodegenerative disease (1). To understand how mutation of C42 might affect the role of PRMT5 in regulating cell proliferation, we focused on cell cycle regulation-related features in proteomic analysis and found...
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that proteins associated with the G2/M DNA-damage-checkpoint regulation were downregulated in PRMT5-C42D overexpression cells compared with PRMT5-WT overexpression cells (Fig. 6a). This indicates that overexpression of PRMT5-C42D induces G2/M arrest. Indeed, the growth rate of the PRMT5-C42D overexpression cells was significantly reduced compared with that of cells overexpressing PRMT5-WT (Fig. 6b-c). The G2-M arrest was further confirmed by flow cytometry (Fig. S5), in which PRMT5-C42D overexpression cells were found to have a significantly higher percentage of G2-M cells (Fig. 6d-e). This may be due to decreased methylation of selected transcription factors and histones, which could result in significant changes in growth rates.

Discussion

Previous studies have reported that post-translational modifications of PRMT5 or its binding partner MEP50 could affect the activity of PRMT5. For instance, JAK2-V617F-mediated phosphorylation of PRMT5 occurs on its N-terminus in a highly conserved region (42, 43) resulting in decreased affinity of PRMT5 with its histone substrate, as well as a significant decrease in H4R3 methylation. However, modifications of PRMT5 other than phosphorylation and acetylation have not been identified. In the present work, we provide experimental evidence that glutathionylation regulates PRMT5 activity. Glutathionylation is a post-translational modification caused by the interplay of ROS and cellular antioxidant systems. We discovered that PRMT5 is glutathionylated at C42 in conditions of oxidative stress or in the kidneys of aged mice. Furthermore, we demonstrate that Grx1 catalyzes deglutathionylation of PRMT5 both in vitro and in vivo.

It has been shown that PRMT5 interacts with MEP50 to form an octamer which functions as a methyltransferase in vivo. Glutathionylation of C42 of PRMT5 affects its interaction with MEP50, possibly due to the large steric hindrance caused by GSH, resulting in reduced methyltransferase activity and consequent decreased histone methylation. Proteomic analysis of C42D mutant that mimics PRMT5 glutathionylation revealed that PRMT5 glutathionylation affects cellular proteostasis. We also found that overexpression of the PRMT5-C42D mutant induced cell cycle arrest in the G2/M phase and inhibited cell growth. Previous studies have shown that the tumor suppressor p53 is involved in cell cycle regulation and cell death, and is methylated at R333, R335, and R337 by PRMT5 in response to DNA damage (44). Furthermore, E2F1 has also been identified as a substrate of PRMT5, whose methylation can promote cell growth by reducing its inhibitory
PRMT5 glutathionylation affects methyltransferase activity (45). Our results suggest that PRMT5 glutathionylation may partially contribute to a decrease in methylation of p53 and E2F1 and inhibit cell growth (Fig. S6).

In summary, our results indicate that glutathionylation of PRMT5 is a new mechanism to regulate PRMT5 activity. The data presented here suggest that glutathionylation makes an important contribution to the ROS-mediated cell-growth inhibition observed in aged animals.
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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Data Availability: The proteomic data have been deposited to the ProteomeXchange Consortium via the PRoteomics IDEntifications (PRIDE) partner repository with the dataset identifier PXD020428.
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Figure 1. Characterization of PRMT5 glutathionylation in kidney tissues from old mice or formed under oxidative stress. (a) the glutathionylation level on C42 of PRMT5 was significantly increased in kidney tissues from old mice, represented by the ratio of glutathionylated vs unmodified peptide; (b) LC-traces of un-glutathionylated and glutathionylated PRMT5-C42 peptides with or without H2O2 treatment; (c) Fragment ions of the recorded in MS/MS spectrum of the doubly-charged ion at m/z 847.41 corresponding to the unmodified PRMT5 peptide QGFDFLCMPVFHPR; (d) Fragment ions of the recorded in MS/MS spectrum of the doubly-charged ion at m/z 1007.93 corresponding to the glutathionylated PRMT5 peptide QGFDFLC(+GSH)(+O)PVFHPFR. the little panel shows an MS spectrum of an HPLC run. Glutathionylation product is indicated by +305.07 Da; (e) 1 mM H2O2 significantly increased glutathionylation on C42 of PRMT5. “-SSG” means the location of glutathionylation.
Figure 2. Grx1 catalyzes deglutathionylation of PRMT5. (a) Immunoprecipitation of PRMT5 by anti-flag (Grx1) antibody with or without prior cross-linking; (b) MALDI-TOF analysis of a glutathionylated PRMT5 peptide (QGFDFLC(+GSH)MPVFHPR) formed from reaction with oxidized glutathione (GSSG), in which the mass of unmodified peptide is 1,692.55, while the mass for the glutathionylated peptide is 1,997.57. Deglutathionylation of the modified peptide was catalyzed by Grx1 in the presence of glutathione reductase (GR), reduced nicotinamide adenine dinucleotide (NADH), and glutathione (GSH); (c) The content of Grx1 affects the glutathionylation on C42 of PRMT5. “-SSG” means the location of glutathionylation.
**Figure 3.** The effects of PRMT5 glutathionylation on histone methylation *in vitro* and *in vivo*. (a) Methyltransferase activity of PRMT5 under different experimental conditions, in which H4 peptide was used as a substrate; (b) Western blotting and qPCR of PRMT5 in different cell lines, in which either PRMT5-WT or its mutant (PRMT5-C42A, PRMT5-C42D) was overexpressed; (c)-(d) The level of symmetric dimethylation of H4R3, H3R2, and H3R8 in PRMT5-WT and its mutant (PRMT5-C42A, PRMT5-C42D) overexpression cells; (e)-(g) Quantitative analysis of histone methylation levels in PRMT5-WT, PRMT5-C42A and PRMT5-C42D overexpression cells by LC-MS/MS.
**Figure 4.** The glutathionylation on C42 of PRMT5 may decrease its binding affinity with MEP50 or RIOK1. (a) The structural organization of PRMT5/MEP50 complex in which the glutathionylation on Cys42 was illustrated by red color; (b)-(c) Quantitative analysis of MEP50 and RIOK1 immunoprecipitation by flag antibody (PRMT5) in PRMT5-WT and its mutant (PRMT5-C42A, PRMT5-C42D) overexpression cells.
**Figure 5.** Quantitative proteomics analysis of differentially expressed proteins between PRMT5-WT and PRMT5-C42D mutant overexpression cells. (a) Venn diagram of proteins identified in three biological replicates; (b) The correlation of three biological replicates from proteomics; (c) A volcano plot of differentially expressed proteins in PRMT5-WT and PRMT5-C42D overexpression cells (p < 0.05); (d) Ingenuity Pathway Analysis (IPA) of the differentially expressed proteins in PRMT5-WT and PRMT5-C42D overexpression cells (p < 0.05); (e)-(f) The activation (e) and inhibition (f) pathways associated with differentially expressed proteins in PRMT5-WT and PRMT5-C42D overexpression cells. Data were analyzed using Student’s t-test (N = 3). *p < 0.05, **p < 0.01, and ***p < 0.001. p < 0.05 is considered statistically significant. Error bars represent ± SEM.
Figure 6. Effects of PRMT5-C42D overexpression on cell proliferation. (a) Inactivation of G2/M DNA damage checkpoint pathway in PRMT5-C42D overexpression cells as compared to PRMT5-WT cells by IPA. The upregulated proteins were red color coded while downregulated proteins were green color coded; (b)-(c) Growth curves of cells in which either PRMT5-WT or PRMT5-C42D was overexpressed in 293T cells (b) and A549 cells (c); (d)-(e) Cell cycle analysis for cells in which either PRMT5-WT or PRMT5-C42D was overexpressed in 293T cells (d) and A549 cells (e).