Methylation of lysine residues on histone proteins is known to play an important role in chromatin structure and function. However, non-histone protein substrates of this modification remain largely unknown. An effective approach for system-wide analysis of protein lysine methylation, particularly lysine monomethylation, is lacking. Here we describe a chemical proteomics approach for global screening for monomethyllysine substrates, involving chemical propionylation of monomethylated lysine, affinity enrichment of the modified monomethylated peptides, and HPLC/MS/MS analysis. Using this approach, we identified with high confidence 446 lysine monomethylation sites in 398 proteins, including three previously unknown histone monomethylation marks, representing the largest data set of protein lysine monomethylation described to date. Our data not only confirms previously discovered lysine methylation substrates in the nucleus and spliceosome, but also reveals new substrates associated with diverse biological processes. This method hence offers a powerful approach for dynamic study of protein lysine monomethylation under diverse cellular conditions and in human diseases. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.044255, 329–339, 2015.

Within proteins, the lysine residue can be modified at its ε-amino side-chain by mono, di, and trimethyl groups (1, 2). Over the past few decades, study of lysine methylation (Kme) has mainly focused on core histones. Early studies demonstrated critical roles of this modification in chromatin structure and function (3–5). The methylation status of lysine is regulated by two groups of enzymes with opposing enzymatic activities: lysine methyltransferases and lysine demethylases (5, 6). To date, several dozens of protein lysine methyltransferases and lysine demethylases have been identified (7–9). Dysregulation of histone methylation can result in reprogramming of gene expression networks and has been associated with diverse disease states, including cancer (4, 5, 7). Accordingly, enzymes regulating lysine methylation have emerged as a group of promising drug targets (7, 10, 11).

All post-translational modifications (PTMs)1 that have been identified in histones are also present in non-histone proteins. Identification of a large family of Kme-regulatory enzymes, the non-nuclear localization of some of these enzymes, and the recent discovery of non-histone Kme substrates clearly suggest that Kme is likely abundantly present in non-histone proteins (12–14), as has been observed for lysine acetylation. Nevertheless, non-histone methylation substrates remain largely unknown. Identification of protein substrates is a critical step for functional characterization of a PTM pathway, a principle well illustrated by the history of lysine acetylation biology. Progress in elucidating the roles of lysine acetylation in chromatin function, transcriptional regulation, and DNA-independent pathways (e.g. metabolism) followed shortly after the discovery of lysine acetylation substrates among the three groups of cognate cellular pathways (2, 15–19). Likewise, proteomic identification and quantification of Kme substrates will reveal chromatin-independent downstream protein targets and pathways of this modification, thus laying a concrete foundation for studying its functions outside the nucleus.

Nevertheless, detection of Kme substrates is not simple. The relatively low-energy radiation emitted by $^3$H or $^{14}$C makes it difficult to detect Kme substrates by a radio labeling approach, as is done for protein phosphorylation using $^{32}$P (20). Kme, especially monomethylation (Kme1), induces only a small structural change to the substrate lysine residue. Be-
cause of this very limited difference in physiochemical properties between monomethyllysine and unmodified lysine residues, it is difficult to use chemical methods to isolate methylated peptides from unmodified ones as is done, for example, for phosphopeptides using immobilized metal affinity chromatography (21). In addition, it has been challenging to develop a highly specific antibody against monomethyllysine with workable affinity (22). Thus, it is still a daunting challenge to develop sequence-independent (or pan) anti-Kme antibodies with adequate affinity and specificity. As a consequence, progress in identifying Kme substrates has been slow, largely because of a lack of suitable affinity enrichment technology for isolation of Kme peptides that can be used for mass spectrometry-based proteomic screening (23–26).

To overcome this technical challenge, we developed a new chemical proteomic approach for efficient enrichment and global analysis of protein lysine mono-methylation. In our approach, we first chemically derivatized the monomethyl ε-amine group of the lysine residue, adding propionyl moiety. Then, the propionyl monomethylated peptides were enriched using a pan anti-propionyl monomethyllysine antibody, followed by HPLC/MS/MS analysis of the enriched peptides for peptide identification and propionyl mono-methylation site mapping. This method was clearly validated by identifying 446 mono-methylation sites on 398 proteins with high accuracy, the largest monomethyllysine data set ever reported.

### MATERIALS AND METHODS

**Isotopic Methionine Labeling and In Vitro Propionylation—**SILAC labeling medium Roswell Park Memorial Institute medium 1640 (RPMI1640) or Eagle’s minimal essential medium (DMEM) (Life Technologies Corp., Carlsbad, CA) was reconstituted with methionine of interest (either [3,3,3]H3-methionine or [3,3,3]C3-methionine; Sigma-Aldrich), 10% dialyzed PBS (Invitrogen Corp.), and 1× penicillin (Hyclone Laboratories Inc., South Logan, UT). The cell lines were grown in either RPMI or DMEM medium at 37 °C supplied with 5% CO2. The labeling efficiency of cells cultured in medium containing [3,3,3]C3-methionine was greater than 98% as determined by mass spectrometry prior to the proteomics experiment. Cells were washed three times with ice-cold Dulbecco’s PBS (Mediatech Inc., Manassas, VA) before harvesting, collected in chilled lysis buffer (8.0 ml urea in two parts 0.1 M NaH2CO3 and one part 0.1 M NaHCO3 (v/v) with 1× protease inhibitor) and incubated on ice for half an hour. The debris was removed by centrifugation, and the supernatant was collected.

The protein concentration of the supernatant was determined by a 20 mg protein lysate and vortexed. The solution was removed by centrifugation, and the supernatant was collected.

The reaction was terminated by adding 10 μl ethanolamine at RT for 30 min. The modified protein was precipitated with trichloroacetic acid. The protein pellet was resuspended in 2 ml digestion buffer (0.1 M NaH2CO3, pH 8.5), followed by in-solution trypsin digestion (28). The tryptic peptides were centrifuged at 20,000 × g for 10 min before subsequent HPLC separation.

Liver Cancer Sample Preparation—With the informed consent of the donor, liver tissue was collected from a liver cancer patient at Zhongshan Hospital (Shanghai, China). Ethical approval for the use of human subjects was obtained from the Research Ethics Committee of Zhong Shan Hospital. The fresh tissue was frozen in liquid nitrogen immediately after surgery. To extract the proteins, the liver tissue was quickly dissected using surgical scissors and washed with chilled Dulbecco’s PBS (Mediatech Inc., Manassas, VA) to remove residual blood. The dissected tissue was homogenized using a Dounce homogenizer in chilled Dulbecco’s PBS. Connective tissue was removed with cell strainer (70 μm size, Cell strainer, Falcon One Inc., Franklin Lakes, NJ). Hepatocytes were collected by centrifugation and lyzed as described above.

HPLC Fractionation of Tryptic Peptides and Affinity Purification—Before affinity purification, the tryptic peptides were separated by HPLC (29). Briefly, the separation was performed on a Varian SD1 LC system (Agilent Technologies Inc., Folsom, CA) with an Xbridge C18 column (19X150 cm, Waters Corp., Milford, MA) using a 70-min gradient from 2% to 40% of buffer B (10 mM ammonium formate/80%ACN, pH 8.5) at a flow rate of 10 ml/min. The sample was collected into 60 fractions based on equal time intervals (about 10 ml/tube), and combined into 20 fractions. Each fraction was dried in a SpeedVac. The peptides were redisolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 0.5% (w/v) Nonidet P-40) for affinity enrichment.

Affinity enrichment of the propionyl-methyl peptides was carried out using protein A-agarose beads conjugated with 20 μg of anti-propionyl-methyllysine antibody (Jingjie PTM BioLab Co. Ltd, Hangzhou, China) at 4 °C for 4 h. The beads were washed three times with 1 ml of NETN buffer and three times with ETN (20 mMTris-HCl, pH 8.0, 100 mMNaCl, and 1 mM EDTA). The bound peptides were eluted from the beads by washing three times with 100 μl of 0.1%TFA. The eluates were combined and dried in a SpeedVac.

HPLC/MS/MS Analysis and Protein Sequence Database Searching—The enriched propionyl-methyl peptide samples were dissolved in 3-μl HPLC buffer A (0.1% formic acid in water, v/v) and delivered onto the capillary RPLC trap column (2 cm length with 100 μm inner diameter (ID), packed with Luna C18 resin, 5 μm particle size, 100 Å pore size, Dikma Technologies Inc., Lake Forest, CA) through the auto-sampler at a maximum pressure of 250 bar in 100% buffer A. After loading and washing, the peptides were transferred to the analytical column (10 cm length with 75 μm ID, packed with C18 resin, 3 μm particle size, 90Å pore size, Dikma Technologies Inc.) connected to an EASY-nLC 1000 HPLC system (Thermo Fisher Scientific Inc.) connected to an Xbridge C18 analytical column (19X150 cm, Waters Corp.) through a syringe pump. The peptides were eluted with an acetonitrile gradient (2% to 90% HPLC buffer B (0.1% formic acid in acetonitrile, v/v) in buffer A at a flow rate of 300 nL/min. The eluted peptides were ionized and introduced into an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific Inc.) using a nanospray source. Survey full-scan MS spectra (from m/z 300 to 2000) were acquired in the Orbitrap with resolution r = 24,000 at m/z 400. The ten most intense ions were sequentially isolated in the linear ion trap and subjected to collisionally induced dissociation (CID) with a normalized energy of 35%. The exclusion duration for the data-dependent scan was 30 s, the repeat count was two, and the exclusion window was set at +2 Da and −1 Da. In addition, all HPLC/MS/MS data were analyzed by Mascot (v2.3, Matrix Science Ltd., London, UK). Peak lists were generated by Proteome Discoverer software (version 1.4) from Thermo Fisher, Precursor mass tolerance for Mascot analysis was set at ±10 ppm, and fragment mass tolerance was set at ±0.6 Da. The proteome was set to trypsin/P (which accounts for in-source fragmentation at lysine or arginine residues followed by proline), allowing for a maximum of three missed cleavage sites. All data were searched against the UniProt Human database (88,817 sequences) including fixed modifi-
cation of cysteine residues by carbamidomethylation as well as the following variable modifications: 13CD3-methionine, 13CD3-methionine oxidation, lysine propionylation (lysine +56.0262), lysine propionyl-13CD3-methylation (lysine +74.0640) for the heavy coded samples, and additional lysine propionyl-CH3-methylation (lysine +70.0418) for the SILAC labeled HeLa sample. While for the liver cancer, only methionine oxidation, lysine propionylation and lysine propionyl-CH3-methylation were considered as variable modifications. All spectra with a Mascot ion score of more than 20 were manually inspected using stringent criteria as previously described (30). All peptides identified with a C-terminal lysine were filtered out unless the lysine was followed by proline.

Isolation and Mass Spectrometric Analysis of CDC5L—Purification was performed as described previously (31). Briefly, heavy13CD3-methionine-labeled K562 cells were lysed in 1x NETN buffer. Homogenates were centrifuged at 6000 g at 4 °C for 10 min to remove cell debris. Cleared lysates were incubated overnight at 4 °C with anti-CDC5L antibody (C20, Santa Cruz Biotechnology Inc., Dallas, TX) followed by incubation with protein A/G beads (Santa Cruz Biotechnology Inc.) for another 4h at 4 °C. After extensive washing, immunoprecipitates were boiled in loading buffer, and then separated on a NuPAGE Novex 4–12% Bis-Tris Mini Gel (Invitrogen Corp). The gel bands of the target proteins were cut out and subjected to in-gel trypsin digestion. Tryptic peptides were resolubilized in 0.1% TFA buffer and analyzed by HPLC-MS/MS to map lysine methylation sites.

Subcellular Localization Analysis—Manually curated protein subcellular localization data were downloaded from the PENCE Proteome Analyst Specialized Subcellular Localization Server v2.5.

Functional Annotation Analysis—Enrichment analysis of Gene Ontology (GO) (32), KEGG pathway (33), and PFAM domain (34) databases were performed using DAVID 6.7 (35) (The Database for Annotation, Visualization, and Integrated Discovery) tools with the total Homo sapiens genome information as the background. GO biological process, molecular function, and cellular compartment categories were analyzed separately. All p values were adjusted with a Benjamin-Hochberg false discovery rate using a cutoff rate of 0.05 (36).

Motif Analysis—An iceLogo standalone version (version 1.2) was used for consensus flanking sequence analysis of methylation sites. Six neighboring amino acids residues on each side of the methylation site were selected as the positive set. All peptides identified with a C-terminal lysine were filtered out unless the lysine was followed by proline.

Protein–Protein Interaction Network Analysis—All interactions for Homo sapiens with high confidence (0.7) in the STRING 9.05 database were used for protein–protein interaction analysis. The network was visualized in Cytoscape (v.3.0.1 beta) (37) and highly connected clusters were identified by the MCODE plug-in (38).

Protein Complex Analysis—The manually curated CORUM protein complex database for Homo sapiens was used for protein complex analysis (39). Overrepresented complexes were identified using Fisher’s exact test with a cutoff of <0.05.

RESULTS

Affinity Enrichment of Lysine Monomethylated Peptides—Affinity enrichment is a key step toward efficient global analysis of a PTM, as has been well demonstrated in large-scale studies of protein phosphorylation and lysine acetylation (40–42). To this end, we developed a novel strategy to purify peptides containing monomethyllysine, involving three main steps (Fig. 1A): 1) chemical derivatization of the monomethylatedlysine-containing peptides by chemical propionylation and immuno-affinity enrichment followed by MS analysis. B. The propionylation reaction occurs at monomethylated lysine but not di or trimethylated lysine. C. Specificity of the anti-propionyl monomethyllysine antibody by dot-blot assay. Kpr+me, Kpr, Kbu, Kme1, Kme2, Kme3, and Kcr denote peptide libraries of sequence xxxxxxxxxx, where K represents a fixed lysine residue modified by propionyl-methyl, propionyl, butyryl, monomethyl, dimethyl, trimethyl, and crotonyl groups, respectively. D. A typical annotated ion trap CID spectrum of a propionyl-13CD3-methyllysine-bearing peptide from protein HSP90.
Global Lysine Mono-Methylation Analysis

Hydride to form propionyl-methyl ε-amine; 2) affinity enrichment of the propionyl-methyllysine-containing peptides using a pan antibody against propionyl-methyllysine; and 3) HPLC/MS/MS analysis of the resulting peptides to identify the modified peptides and map the modification sites. For the proposed procedure for proteomic screening to be efficient, the propionylation reaction should be as complete as possible. The chemical propionylation of lysine residues was first introduced by Garcia et al. to enhance the hydrophobicity of tryptic peptides and increase sequence coverage during mass spectrometric analysis of histone modifications (27). However, this chemistry has not yet been applied to proteomics analysis of lysine methylation. To determine whether this chemistry works efficiently in the more complex environment of a protein lysate, we subjected HeLa whole-cell lysate to the chemical reaction using propionyl anhydride in NH4CO3-NaHCO3 buffer (pH 8.5). After the reaction, we digested the proteins with trypsin and then analyzed the proteolytic peptides by HPLC/MS/MS. The propionylation reaction occurs at unmodified lysine and monomethyllysine residues and the N-terminal amine, but not at di or trimethylated lysine residues (Fig. 1B) (27). If the propionylation reaction goes to near completion, the percentage of tryptic peptides with unmodified lysine should be low. Thus, the intensity ratio of modified to unmodified lysine-containing peaks provides a relevant measure of the efficiency of the propionylation reaction. Our analysis showed that after treatment with propionyl anhydride, less than 2% of the detected peptides contained unmodified lysine, suggesting that our procedure for lysine propionylation is efficient (supplemental Fig. S1).

Antibody Generation—The pan antibody against propionyl monomethyllysine was developed in Jingjie PTM BioLab (Hangzhou, China) Co. Ltd. Briefly, propionyl-methyllysine-conjugated keyhole limpet hemocyanin (KLH) was used to immunize rabbits. The anti-propionyl monomethyllysine antibody was isolated from the resulting serum. The specificity of the pan antibody was then tested by dot-blot assay with peptide libraries containing propionyllysine (Kpr), butyryllysine (Kbu), crotonyllysine (Kcr), or mono, di, or trimethyllysine residues (Kme1, Kme2, and Kme3) in a fixed position (Fig. 1C). The antibody showed more than 100-fold specificity for propionyl monomethyllysine relative to the other modified lysine residues. Because propionyl monomethyllysine is structurally similar to propionyllysine and butyryllysine, this result clearly suggests that the anti-propionyl monomethyllysine antibody has high affinity and specificity. We therefore used the antibody for enriching propionyl monomethyllysine peptides in our subsequent proteomic screenings.

Confident Detection of Lysine Propionyl Monomethylated Peptides—When identifying monomethylated peptides, false positives can arise for two reasons. Lysine monomethylation induces a mass shift of 14.0157 Da, which is the same as the shift observed for several amino acid substitutions or mutations: Ser to Thr, Asp to Glu, Val to Leu or Ile, and Asn to Glu. (22). In addition, propionyl monomethylation has the same mass shift as lysine butyrylation, a protein modification that occurs in vivo (43). Therefore, our strategy should include a proper control to prevent false identification of propionyl monomethylated peptides. To address these concerns, we used the well-known method of isotopic labeling, using 13CD3-methionine. The isotopic label results in a mass shift of 18.037 at the lysine residue. Therefore, propionyl-13CD3-methyllysine induces a unique mass shift of 74.0640 localized at the lysine residue, which can be distinguished by MS from the mass shift caused by any known PTM (including butyrylation), or any amino acid mutation (44). As an example, we identified a propionyl monomethylated peptide from HSP90 (Fig. 1D), where the propionyl monomethylation can be unambiguously localized by the mass shift of 74.0640 Da at residue K3 within the peptide.

To test reliability of this approach, we cultured two groups of HeLa cells in parallel in DMEM media containing either 13CH3-methionine or 12CH3-methionine. After more than six doublings, we mixed the two pools of cells and lysed them. The resulting protein lysate was propionylated, digested with trypsin and resolved into fractions by preparative HPLC. In each fraction, the propionyl monomethylated peptides were enriched using the pan anti-propionyl monomethyllysine antibody and then subjected to nano-HPLC/MS/MS analysis. The MS/MS spectra of the enriched peptides were searched against the human Uniprot proteins database with the Mascot algorithm, and positive identifications were manually inspected using stringent criteria to verify each identification as previously described (30). Using this method, we identified 180 propionyl monomethylated peptides from HeLa cells, with the enrichment specificity in the fractions ranging from 3 to 41% (Fig. 2A). A truly propionyl monomethylated peptide will have two parent ions with a mass difference of 4.02 Da derived from 13CD3-SAM and 12CH3-SAM, and the fragment ions will have an identical pattern if both light and heavy peptides are selected for MS/MS (see Fig. 2B and 2C for characteristic spectra). When manually checking the raw data, 179 of the 180 putative propionyl monomethylated peptides were found with paired precursor ions, corresponding to a total of 185 methylation sites on 163 proteins. Only one “heavy” peptide was identified without a counterpart ion (Fig. 2D), suggesting that our procedure is reliable for mapping monomethylation sites, and filtering out false positives. In the proteomic screening experiments reported below, we used only 13CD3-methionine-labeled cells.

Landscape of Protein Lysine Monomethylome—We next extended the method of using 13CD3-methionine labeling to profile Kme1 in four other human cancer cell lines: K562 (chronic myelogenous leukemia), SW620 (colon cancer), A549 (lung cancer), and SMM7721 cells (liver cancer). Using the strategy described above, we identified 434 nonredundant monomethylated peptides corresponding to 446 sites on 398 proteins. Most of the monomethylation sites have not been
Fig. 2. **Proteome-wide analysis of lysine monomethylation using an isotopic labeling approach.** A, Affinity enrichment efficiency and the number of methylated peptides identified in each fraction. B, MS spectrum of a “Light” and “Heavy” methyl-coded monomethylated peptide of protein EZH2 from HeLa cells, showing twin peaks with a mass difference of 4.02 Da induced by isotopic labeling using $^{13}$CD$_3$. C, Ion trap CID spectra from the “Light” and “Heavy” methylated peptide YSQADALK$_{me1}$YVGIER of protein EZH2 from HeLa cells. The 4-Da mass shift of the b- and y-ions of the “Heavy” peptide relative to its “Light” counterpart was induced by the isotopic methyl group. D, The overlap of the methylated peptides from “Light” and “Heavy” methionine-labeled HeLa cells.
reported previously (24, 25, 45, 46). A complete list of the methyllysine peptides and proteins identified in this study is provided in supplemental Table S1 and supplemental Fig. S2.

The core histone proteins have been extensively analyzed for lysine methylation, thus providing a good positive control. As expected, we identified 12 histone Kme1 sites. In addition to the well-studied methylation sites at H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20 (47), we identified the little-studied H3K18 monomethylation (48). Most of these sites were identified in all five of the cell lines, indicating that our method is robust for detecting Kme1 peptides. Intriguingly, we identified three previously unknown monomethylation sites: H2AK5, H1K33, and H1K145 (Fig. 3A).

In addition to the histone sites, we identified new Kme1 sites on proteins previously known to be monomethylated. For instance, we found two new monomethylated sites (K1321 and K1463) on the protein WIZ, in addition to the previously reported site at K967 (26).

Next, we applied our method to analyze the scope of Kme1 substrates in human liver cancer tissue taken directly from a patient. With our stringent criteria, we identified 59 monomethylation sites located on 49 proteins. We compared the Kme1 data sets among the five types of cells. Only 29 monomethylation sites among 27 monomethylated peptides were found in all five cell types, including 20 novel methylation sites and five histone H3 Kme1 sites (Fig. 3B and 3C).

**Validation of the Novel Kme1 Sites**—Because site-specific anti-Kme1 antibodies for non-histone proteins are not available, we validated the identified Kme1-containing peptides by analyzing corresponding synthetic peptides. Because synthetic peptides and their cell-derived counterparts will generate identical MS/MS patterns under the same mass spectrometric conditions, this approach is the gold standard for peptide validation. To this end, we randomly selected and synthesized 10 propionyl-monomethylated peptides identified with Mascot ion scores ranging from 20 to 60. The MS/MS spectra of these synthetic propionyl-methyllysine peptides matched perfectly with those of their cell-derived counterparts (Fig. 4A and supplemental Fig. S3), confirming the identification of all of these propionyl-methyllysine peptides. These results suggest that our approach for identifying Kme1 peptides is reliable and highly accurate.

In addition to testing our method using synthetic peptides, we also validated the monomethylation sites in vivo for CDC5L, a protein involved in cell cycle control (49). To this end, we first metabolically labeled the methyl group using...
L-ANTQGK\textsubscript{me1} from cultured K562 cells by immunoprecipitation for MS analysis. We successfully identified the K164 \textsuperscript{13}CD\textsubscript{3}-methylated peptide, LANTQGK\textsubscript{me1} from the\textit{ in vivo}\textsuperscript{-}derived protein CDC5L (Fig. 4B). To further confirm this peptide, we analyzed the MS/MS spectrum of its corresponding synthetic counterpart, which showed an almost identical MS/MS spectra, except for the 4-Da difference caused by isotopic labeling (Fig. 4B). Taken together, our data clearly confirmed monomethylation at the K165 residue of CDC5L.

\textbf{Bioinformatics Analysis of the Methylated Proteins—} We next performed bioinformatics analysis to investigate the possible roles of protein lysine monomethylation in cellular regulation. We first analyzed the subcellular localizations of the Kme1 substrates. Earlier studies suggest that most arginine-methylated proteins are associated with RNA processing and are mainly located in the nucleus or ribosome (12). In our study, nuclear proteins accounted for the largest subgroup (40.37%) of the identified lysine monomethylated proteins. Thirty-four percent of the Kme1 proteins were predicted to localize to the cytoplasm, with an additional 8% of proteins being found in the ribosome or spliceosome. This result suggests that lysine monomethylation may have diverse previously unknown functions in the cytosolic compartment. Intriguingly, we also identified 11 mitochondrial Kme1 proteins. Most of these proteins play roles in regulating various aspects of mitochondrial function (\textit{supplemental Table S2}). For example, SLC25A4, SLC25A5, and SLC25A6, three members of the SLC25 protein family, catalyzes the exchange of cytoplasmic ADP for mitochondrial ATP across the mitochondrial inner membrane (50). Intriguingly, the monomethylation site of these three proteins is located at lys147, an evolutionary conserved domain. The data suggest that mitochondrial functions may be regulated by protein methylation.

We then carried out analysis of the Kme1-containing proteins using the Gene Ontology (GO) categories. Within the molecular function (MF) domain, the largest group of proteins belong to the “Binding” category, and the majority of these carry RNA-binding activity. We additionally found that the second-largest category of Kme1 proteins has the function of catalytic activity (Fig. 5B). Analysis of the Biological Process (BP) domain suggested that the methylated proteins have roles in diverse biological processes, of which metabolic...
process, cellular process, cell cycle, and cell communication comprised the largest part (supplemental Fig. S4).

Further assessment revealed considerable enrichment for RNA-related GO terms and Pfam database categories, such as chromosome organization and RNA processing (BP), RNA binding (MF), nucleolus (cellular component (CC) domain of GO), and RNA recognition motif (i.e. RRM, RBD, or RNP domain, in Pfam). Additional KEGG pathway analysis underscored the significant overrepresentation of the methylated proteins in RNA-related spliceosome and ribosome pathways (supplemental Fig. S5 and supplemental Table S3).

We further used the STRING protein interaction database (51) and Comprehensive Resource of Mammalian protein complex (CORUM) database (39) to visualize the protein–protein interaction networks and identify overrepresented protein complexes. The interaction network showed that the methylated proteins were highly connected to each other and formed a number of overrepresented subclusters, the largest of which were proteins involved in the cellular functional categories of spliceosomes, RNA splicing and cell cycle (supplemental Fig. S6 and supplemental Table S4). By querying the CORUM database, we found 30 enriched protein complexes among the lysine monomethylated proteins (p < 0.05). Among these are the spliceosome, the Nop56p-associated pre-rRNA complex, Large Drosha complex, and CDC5L complex (Fig. 5C–5F and supplemental Table S5), consistent with the study reported previously (24).

To examine whether any of the monomethylated lysine residues could be alternatively modified by acetylation or ubiquitination, we compared our data set with the public database of known PTMs from UniProt. In total, 37 Kme1 sites, including the known H3K9 and H3K27 sites (52) and 30 non-histone proteins, could be alternatively modified with either methyl or acetyl groups (supplemental Table S6). In ad-
in a window of
We identified 39 mutations (amino acid substitutions) located
inherited amino acid substitutions in humans from UniProt.
mutations are located around the Kme1 sites. We compared the
of modifications.
H3, could be alternatively modified by all three different types
found
Eight
merulosclerosis 5 (FSGS5) (supplemental Table S8).
cirrhosis (CIRRH), argininosuccinicaciduria (ARGINSA), Dia-
human diseases, including Rett syndrome, colorectal cancer,
molecular & cellular proteomics 14.2
337
other (supplemental Fig. S8)(24, 25).
rate of detection of Kme1 peptides (22). Although this method
residues cannot react with propionyl anhydride. We also had
to use heavy isotope-coded methionine to improve the accu-
methylated lysine because di and trimethylated lysine
residues cannot react with propionyl anhydride. We also had
to use heavy isotope-coded methionine to improve the accu-
Identification of the substrates is a critical step for func-
tional characterization of protein lysine methylation and re-
mains the most challenging for large-scale study. Despite the
biological significance of lysine di and trimethylation, our
method was designed to focus on the proteomic screening of
monomethylated lysine because di and trimethylated lysine
residues cannot react with propionyl anhydride. We also had
to use heavy isotope-coded methionine to improve the accu-
acy of detection of Kme1 peptides (22). Although this method
not directly applicable to clinical tissue samples, there
liability of peptide identification can be enhanced by alterna-
tive methods, such as stringent manual verification and
MS/MS analysis of synthetic peptides.
Among the Kme1substrates we identified, a noteworthy
subgroup of protein methyltransferases. In this study, we
identified lysine monomethylation among eight protein lysine
methyltransferases (SETDB1, EZH2, CAMKMT, EHMT1, EHMT2, SETD2, MLL4, and NSD1), and one methyltrans-
ferase interaction partner (ASH2L). Previous research showed
that automethylation of G9a methyltransferase at K239 led to
its interaction with HP1, consequently exerting its biological
functions (58). The automethylation motif of G9a flanking
K239 (ARKT) resembles the sequence around histone H3
lysine 9 (ARKS), which is the specific substrate of G9a (58).
Similarly, we found a novel methylation site (K490) on methyl-
transferase SETDB1 with a flanking motif of AKKST, which
resembles the ARKST motif of its specific substrate histone
H3K9 (59). Hence, it is likely that modification of K490 of
SETDB1 resulted from auto-methylation, which could conse-
quently lead to the recruitment of HP1 to form an HP1a-CAF1-
SETDB1 complex (58, 60). One question worthy of future
study is whether lysine automethylation among lysine methyl-
transferases has regulatory roles similar to those of autophos-
phorylation of receptor tyrosine kinases (61) and autoacety-
lation of acetyltransferases (e.g. p300 and CBP) (62).
Previous studies have identified arginine methylated protein
substrates and revealed the likely role of this modification in
RNA-related biological events such as gene transcription,
mRNA splicing and regulation of small RNAs (63). Our study
showed that proteins in these pathways are also lysine meth-
ylated. Given the high abundance of both arginine methylation
and lysine methylation, it is very likely that protein methylation
has an important role in the regulation of these processes.
Cross-talk among lysine modifications, such as between
methylation and acetylation, has been reported (64). For ex-
ample, H3K9 acetylation (H3K9ac) is generally associated
with active gene transcription, whereas methylation of H3K9
(H3K9me) correlates with inactivation of gene expression and
could compete with H3K9ac (65). In this study, we found
overlap of methylation sites with sites of acetylation and suc-
cinylation, including two sites which could be alternately modi-
fied with all three types of modification (supplemental Table
S8). Given that methylation retains the positive charge on
lysine, acetylation neutralizes the charge, and succinylation
converts the positive charge to a negative charge, these three
different types of modification provide a mechanism to pro-
duce distinct physicochemical states in the substrate
proteins.
In recent years, the biological importance of protein meth-
ylation has attracted extensive attention in the research com-
munity. Enzymes regulating lysine methylation have been pro-
posed as drug targets for diverse diseases (11). In addition,
some inhibitors of lysine methylation regulatory enzymes,
such as inhibitors of EZH2 and DOTL1, are currently under
clinical evaluation (66). However, current knowledge of lysine
methylation is restricted to a limited number of proteins, such
as histones and p53 (4, 67). As a consequence, non-histone
substrates of the enzymes and methylation substrate-based
biomarkers for compounds under clinical investigation remain
unknown, hindering our understanding of the mode of action
for these enzymes and their inhibitors. The proteomic tech-
nology described here offers a new approach to addressing
these issues by identifying methylation substrates in response
to either the enzymes or their inhibitors.
The study presented here not only provides a robust meth-
odology for global screening of lysine monomethylation sub-

Discussion
Although affinity-based proteomic strategy has been de-
scribed for protein lysine methylation (22, 25, 26, 54–56), the
proteome-wide survey of protein lysine monomethylation re-
mains challenging, mainly because of the negligible physico-
chemical difference induced by the methyl group (57). The
446 methylation sites found on 398 proteins identified in this
study represent the largest data set of lysine monomethylated
protein ever reported. The overlap between the three inde-
pendent studies with different strategies was around 10%
suggested that there approaches were complement with each
other (supplemental Fig. S8) (24, 25).
Identification of the substrates is a critical step for func-
tional characterization of protein lysine methylation and re-
mains the most challenging for large-scale study. Despite the
biological significance of lysine di and trimethylation, our
method was designed to focus on the proteomic screening of
monomethylated lysine because di and trimethylated lysine
residues cannot react with propionyl anhydride. We also had
to use heavy isotope-coded methionine to improve the accu-
cy of detection of Kme1 peptides (22). Although this method
not directly applicable to clinical tissue samples, there
liability of peptide identification can be enhanced by alterna-
tive methods, such as stringent manual verification and
MS/MS analysis of synthetic peptides.
Among the Kme1substrates we identified, a noteworthy
subgroup of protein methyltransferases. In this study, we
identified lysine monomethylation among eight protein lysine
methyltransferases (SETDB1, EZH2, CAMKMT, EHMT1, EHMT2, SETD2, MLL4, and NSD1), and one methyltrans-
ferase interaction partner (ASH2L). Previous research showed
that automethylation of G9a methyltransferase at K239 led to
its interaction with HP1, consequently exerting its biological
functions (58). The automethylation motif of G9a flanking
K239 (ARKT) resembles the sequence around histone H3
lysine 9 (ARKS), which is the specific substrate of G9a (58).
Similarly, we found a novel methylation site (K490) on methyl-
transferase SETDB1 with a flanking motif of AKKST, which
resembles the ARKST motif of its specific substrate histone
H3K9 (59). Hence, it is likely that modification of K490 of
SETDB1 resulted from auto-methylation, which could conse-
quently lead to the recruitment of HP1 to form an HP1a-CAF1-
SETDB1 complex (58, 60). One question worthy of future
study is whether lysine automethylation among lysine methyl-
transferases has regulatory roles similar to those of autophos-
phorylation of receptor tyrosine kinases (61) and autoacety-
lation of acetyltransferases (e.g. p300 and CBP) (62).
Previous studies have identified arginine methylated protein
substrates and revealed the likely role of this modification in
RNA-related biological events such as gene transcription,
mRNA splicing and regulation of small RNAs (63). Our study
showed that proteins in these pathways are also lysine meth-
ylated. Given the high abundance of both arginine methylation
and lysine methylation, it is very likely that protein methylation
has an important role in the regulation of these processes.
Cross-talk among lysine modifications, such as between
methylation and acetylation, has been reported (64). For ex-
ample, H3K9 acetylation (H3K9ac) is generally associated
with active gene transcription, whereas methylation of H3K9
(H3K9me) correlates with inactivation of gene expression and
could compete with H3K9ac (65). In this study, we found
overlap of methylation sites with sites of acetylation and suc-
cinylation, including two sites which could be alternately modi-
fied with all three types of modification (supplemental Table
S8). Given that methylation retains the positive charge on
lysine, acetylation neutralizes the charge, and succinylation
converts the positive charge to a negative charge, these three
different types of modification provide a mechanism to pro-
duce distinct physicochemical states in the substrate
proteins.
In recent years, the biological importance of protein meth-
ylation has attracted extensive attention in the research com-
munity. Enzymes regulating lysine methylation have been pro-
posed as drug targets for diverse diseases (11). In addition,
some inhibitors of lysine methylation regulatory enzymes,
such as inhibitors of EZH2 and DOTL1, are currently under
clinical evaluation (66). However, current knowledge of lysine
methylation is restricted to a limited number of proteins, such
as histones and p53 (4, 67). As a consequence, non-histone
substrates of the enzymes and methylation substrate-based
biomarkers for compounds under clinical investigation remain
unknown, hindering our understanding of the mode of action
for these enzymes and their inhibitors. The proteomic tech-
nology described here offers a new approach to addressing
these issues by identifying methylation substrates in response
to either the enzymes or their inhibitors.
The study presented here not only provides a robust meth-
odology for global screening of lysine monomethylation sub-

Global Lysine Mono-Methylation Analysis

Molecular & Cellular Proteomics 14.2

MCP
strates, but also dramatically extends the inventory of lysine monomethylated proteins. A combination of this proteomic technology with genetic manipulation of expression of methylation regulatory enzymes can be used to identify substrates for methytransferases (for Kme1) and demethylases (for Kme1 and Kme2), and for dynamic analysis of Kme substrates under disease conditions. In addition, our data set is a valuable starting point for further biological research to study the biological functions of lysine-methylated proteins and the anatomy of lysine methylation pathways specific to human diseases.

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