Published in final edited form as:

Nat Protoc. 2014 January ; 9(1): 37–50. doi:10.1038/nprot.2013.164.

Proteome-wide enrichment of proteins modified by lysine methylation

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Abstract

We present a protocol for using the triple malignant brain tumor domains of L3MBTL1 (3×MBT), which bind to mono- and di-methylated lysine with minimal sequence specificity, in order to enrich for such methylated lysine from cell lysates. Cells in culture are grown with amino acids containing light or heavy stable isotopic labels. Methylated proteins are enriched by incubating cell lysates with 3×MBT, or with the binding-null D355N mutant as a negative control. Quantitative liquid chromatography and tandem mass spectrometry (LC-MS/MS) are then used to identify proteins that are specifically enriched by 3×MBT pull-down. The addition of a third isotopic label allows the comparison of protein lysine methylation between different biological conditions. Unlike most approaches, our strategy does not require a prior hypothesis of candidate methylated proteins, and it recognizes a wider range of methylated proteins than any available method using antibodies. Cells are prepared by growing in isotopic labeling medium for about 7 d; the process of enriching methylated proteins takes 3 d and analysis by LC-MS/MS takes another 1–2 d.

INTRODUCTION

Recent work has shown that a wide range of nonhistone proteins are modified by the addition of up to three methyl groups to the ε-nitrogen of lysine side chains[1–4]. Proteome-wide enrichment of specific post-translational modifications (PTMs) has been used to map the extent of other PTMs, including phosphorylation, acetylation and ubiquitylation. In most cases, these procedures are based on pan-specific enrichment using antibodies or chemical

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AUTHOR CONTRIBUTIONS S.M.C. and K.E.M. developed the 3×MBT pull-down protocol. S.M.C. optimized the protocol for mass spectrometry. E.M.G. conducted the Rkm1 experiment. G.M.M. generated SILAC yeast strains and adapted the protocol to yeast. S.M.C. and K.E.M. wrote the protocol. O.G. supervised the work.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests: details are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

affinity[5–7]. However, applying similar approaches to methylated lysine has proven to be challenging. We recently demonstrated an approach that uses a naturally occurring methyl-lysine binding domain as a tool for enrichment and identification of proteins modified by lysine methylation[2]. The triple malignant brain tumor domains (3×MBT) of the protein L3MBTL1 bind to mono- and di-methylated lysine with minimal sequence specificity. Expressing 3×MBT as a fusion with glutathione S-transferase (GST) allows 3×MBT to be anchored to beads functionalized with reduced glutathione (GSH), thereby providing a generalized tool for enriching lysine methylation that is inexpensive and reproducible (Fig. 1). Proteins enriched by 3×MBT from cell lysate can be analyzed by western blotting or by separation on SDS-PAGE, followed by in-gel digestion with trypsin and LC-MS/MS.

Protein pull-downs from cell lysate are generally contaminated by residual abundant proteins and proteins bound through nonspecific interactions to the beads, to GST or to 3×MBT. A point mutation that specifically abrogates methyl-lysine recognition, D355N, provides a negative control for nonspecific binding ($3\times MBT_{D355N}$)[8,9]. To separate candidate methylated proteins from background signals, we incorporate stable isotopic labeling in cell culture (SILAC)[10]. This allows a direct quantitative comparison between proteins bound by 3×MBT and proteins bound by the $3\times MBT_{D355N}$ negative control (Fig. 1b). Strong enrichment (e.g., more than twofold) by 3×MBT indicates a candidate methylated protein. The SILAC approach can be extended to three quantitative channels, allowing simultaneous quantitative comparison among two biological conditions and the negative control (Fig. 1c).

Applications of methyl-lysine protein enrichment

The protocol enables the identification of methylated proteins and quantitative comparison of methylation between different biological conditions in cell culture. It is applicable to any biological system that can be prepared in culture with amino acids containing defined light and heavy stable isotopes. Alternative experimental designs such as ‘super SILAC’ may enable analysis of biological systems that are not amenable to labeling with heavy amino acids[11].

The use of SILAC allows small differences in protein methylation to be measured accurately and reproducibly between different conditions. Technical and biological variations depend on protein abundance, repeatability of the biological system and the number of peptides identified during LC-MS/MS analysis. We have used this approach to identify candidate substrates of the lysine methyltransferases G9a and GLP by examining changes after treatment of cells with the small-molecule inhibitor UNC0638 (refs. 2, 12). We have also applied this approach to yeast after knockout of the methyltransferase Rkm1, and we observed reduced methylation of its known substrate Rpl23 (ref. 13) (see ANTICIPATED RESULTS). The same methodology could be applied to examine methylation dynamics during temporal processes such as the cell cycle, or after treatment with biologically active molecules such as growth factors.

This protocol makes use of the 3×MBT domain of L3MBTL1, because it has pan-specific affinity for mono- and di-methylated lysine[2,8]. Many other protein domains bind specifically to methylated lysine with varying degrees of target specificity. A variation on this approach may be used to identify methylation-specific interactions for these domains by using them in

place of the 3×MBT domain[4]. Such a procedure could begin by identifying a point mutation that disrupts the methyl-lysine-binding pocket. Proteins bound by the wild-type and inactive domains can then be compared and candidate methyl-specific interactions can be selected for further validation.

Comparison with other methods

Pan-specific antibodies have been used for proteomic analysis of other PTMs[5–7]. Unfortunately, commercially available pan-methyl antibodies have shown high nonmethyl-specific binding and preference for particular sequences, and they are therefore not suitable for pan-methyl enrichment[14]. Coupling enrichment of methylated proteins with a pan-methyl antibody with heavy methyl SILAC has been used to identify some methylation events, but the pan-methyl antibodies were found to contribute little in terms of methyl-specific enrichment[15,16]. Other research groups have generated their own polyclonal antibodies to conduct methyl-peptide enrichment, and they have succeeded in enriching methylated peptides[3]. The variable nature of polyclonal antibodies makes it difficult to use this approach as a consistent tool. Another recent publication identified methylated proteins from the interactome of the chromodomain of HP1β, but this affinity enrichment is presumably limited to proteins bearing an appropriate recognition sequence[4].

Experimental design

This method is used for performing quantitative comparisons between pull-downs by 3×MBT and $3\times MBT_{355N}$. This is achieved by using SILAC—the differential labeling of cells in culture with amino acids, usually arginine and lysine, containing specific stable isotopes. There are many amino acids and isotopic labels that may be used depending on the needs of a particular experiment. We perform two-way experiments using normal amino acids for one culture condition (light) and $^{2}H_{4}$-L-lysine/$^{13}C_{6}$-L-arginine (medium) or $^{15}N_{2}{}^{13}C_{6}$-L-lysine/$^{15}N_{4}{}^{13}C_{6}$-L-arginine (heavy) for the other. This design is suitable for comparing 3×MBT with $3\times MBT_{D355N}$ under a single biological condition. For a three-way comparison, we culture cells in light, medium and heavy media. This design is suitable for comparing 3×MBT pull-down between two experimental conditions along with one pull-down using $3\times MBT_{D355N}$ as a negative control.

In a three-way experiment, the protein sample for the $3\times MBT_{D355N}$ pull-down should be prepared by using the condition that is expected to have the most lysine methylation (e.g., overexpression of a lysine methyltransferase (KMT), or the absence of a KMT inhibitor). If there is no *a priori* expectation about the amount of methylation, then we alternate the condition used for the negative control. We use the medium SILAC condition for the $3\times MBT_{D355N}$ pull-down because it is possible for strong signals in the medium channel to carry into the heavy channel. As the $3\times MBT_{D355N}$ pull-down has the lowest expected signal, this design minimizes carryover. Other SILAC conditions and amino acids may be used, depending on the needs of a particular experiment[10, 17]. As information regarding the source of a given peptide is encoded in the isotopic label, samples from different labeling conditions should be combined as early as possible to minimize variability during preparation (i.e., before cell lysis, for samples that will both be used for 3×MBT pull-down)

and after the pull-down (Step 20) for samples from pull-downs with different domains (e.g., 3×MBT and 3×MBT_{D355N}).

We perform all experiments in duplicate with the biological conditions reversed between light and heavy SILAC labels. This label swap allows contaminant signals to be easily eliminated (e.g., ambient proteins such as keratins will be found in the light channel in both experiments). Additional replicates are necessary if the goal is the statistical analysis of differential protein pull-down.

Identification of methylated proteins depends on the depth of coverage achieved during LC-MS/MS. The number of proteins identified can often be increased by dividing each sample into several molecular-weight fractions after SDS-PAGE (Steps 27 and 28), or by adjusting the length of the HPLC gradient used during LC-MS/MS. We generally divide proteins from human cells into five molecular-weight fractions and analyze each with a 2-h gradient. Similarly, we divide protein from yeast cells into three fractions, also analyzed over 2-h gradients. Subcellular fractionation can also be used to further enrich for proteins targeted by a specific methyltransferase. For example, nuclear extracts can be used for pull-down experiments to identify targets of a nuclear methyltransferase, thereby limiting nonspecific signals from cytosolic proteins. It is also possible that some proteins enriched by 3×MBT are not methylated themselves but are enriched as part of a complex with at least one methylated subunit. We routinely validate candidate methylated proteins by immunoprecipitation from cells; this is followed by comprehensive analysis using LC-MS/MS to map modified residues. We also validate candidate methyltransferase substrates by *in vitro* methylation assays.

For the LC-MS/MS analysis, we recommend HPLC gradients between 2 and 4 h with reverse-phase chromatography on a C18 resin, the use of an electrospray tip flowing no faster than 200 nl min^{-1} and a mass spectrometer running in data-dependent acquisition mode with capabilities similar to or better than an LTQ Orbitrap XL. We have used Thermo Scientific Q-Exactive, LTQ Orbitrap Elite and Velos instruments. MS1 scans are performed at high resolution in the Orbitrap mass analyzer, followed by MS/MS scans on the top ten peptides with dynamic exclusion. We find similar results by performing MS/MS with collision-induced dissociation (CID) or higher-energy collisional dissociation (HCD) fragmentation, and with MS/MS spectra collected using either ion trap or Orbitrap mass analyzers.

Limitations of the method

The quantitative readout of this protocol represents a bulk measurement of the methylation status of a protein; it does not distinguish among methylation of individual lysine residues within a protein or determine stoichiometry of methylation at any particular site. For example, the methyltransferase G9a mono- and di-methylates histone H3 at lysine 9 *in vivo*[18], but inhibiting G9a only reduces the methyl-dependent pull-down of H3 by 3×MBT by ~10% (ref. 2), almost certainly because H3 is modified at multiple lysines by many methyltransferases[19]. In addition, the 3×MBT domain does not recognize tri-methylated lysine, nor can it distinguish between mono- and di-methylation. Therefore, methylation states that change between mono- and di-methyl, and changes in the amount of tri-

methylation that do not alter mono- or di-methylation, will not be observed using this protocol.

Although knowing that a protein is methylated is helpful, addressing specific biological questions often requires the determination of the particular site of methylation. One limitation of the 3×MBT approach is that specific methylated residue(s) are not identified from every enriched protein. This is because analysis by LC-MS/MS only identifies a subset of peptides from each protein. This is especially true for proteins that are large, expressed in low abundance or methylated with low stoichiometry. In other cases, the methylated residue may occur in a sequence context that is digested by trypsin into peptides that are either too short or too long for identification by LC-MS/MS. Protein sequence coverage may be improved by performing additional fractionation at the protein or peptide levels or by using alternative proteases such as chymotrypsin or Glu-C[20].

Proteomic strategies for detecting other PTMs, such as phosphorylation and acetylation, identify modified residues directly by conducting the affinity enrichment on peptides produced by tryptic digest, so that modified peptides are enriched directly without coenrichment of unmodified peptides from the same proteins[5]. We have been unable to efficiently enrich peptides from digested cellular extracts with 3×MBT[2]. This may be an area for future development of the 3×MBT domain and related approaches.

MATERIALS

REAGENTS

- Plasmids: 3×MBT-pGEX6P-1 and 3×MBT$_{D355N}$-pGEX6P-1 constructs[2] (AddGene plasmid nos. 46987 and 46988, respectively)
- Mammalian SILAC medium: DMEM (Thermo Scientific/Pierce, cat. no. 88420) and RPMI (Thermo Scientific/Pierce, cat. no. 88421)
- Dialyzed FBS for SILAC (Thermo Scientific/Pierce, cat. no. 88440)
- Yeast SILAC medium (based on Kubota *et al.*[21]): Dropout mix minus leucine, lysine, arginine and adenine (US Biological, cat. no. D9515F)
- Yeast nitrogen base without amino acids (Sigma, cat. no. Y0626)
- Leucine (Sigma, cat. no. L8912)
- Adenine (Sigma, cat. no. A8626)
- Glucose (Invitrogen-Gibco, cat. no. 15023021)
- SILAC amino acids: L-lysine-2HCl (Thermo Scientific/Pierce, cat. no. 88429), L-arginine-HCl (Thermo Scientific/Pierce, cat. no. 88427), $^{2}H_{4}$-L-lysine-2HCl (Thermo Scientific/Pierce, cat. no. 88438), $^{13}C_{6}$-L-arginine-HCl (Thermo Scientific/Pierce, cat. no. 88433), $^{15}N_{2}{}^{13}C_{6}$-L-lysine-2HCl (Thermo Scientific/Pierce, cat. no. 88209), $^{15}N_{4}{}^{13}C_{6}$-L-arginine-HCl (Thermo Scientific/Pierce, cat. no. 89990), L-proline (Thermo Scientific/Pierce, cat. no. 88430)

- Yeast strain from any background (for example, Fisher cat. no. NC0537738), but they must be auxotrophic for arginine and lysine (*arg4 lys2*) as described in ref. 21
- LB medium (Sigma, cat. no. L3522)
- BL21-competent *Escherichia coli* (New England BioLabs, cat. no. C2530 H)
- Ampicillin sodium salt (Sigma, cat. no. A0166)
- IPTG (Sigma, cat. no. I5502)
- Tris base (Fisher, cat. no. BP152)
- Hydrochloric acid (HCl; Fisher, cat. no. A144) **! CAUTION** Concentrated acid is corrosive.
- Sodium chloride (NaCl; Sigma, cat. no. S3014)
- Sodium hydroxide solution, 1 N (NaOH; Sigma, cat. no. 38215) **! CAUTION** Concentrated bases are corrosive.
- Nonidet P-40 (NP-40; American Bioanalytical, cat. no. AB01425)
- cOmplete, EDTA-free protease inhibitor cocktail tablets (Roche, cat. no. 04693132001)
- Protease inhibitor cocktail for use with fungal and yeast extracts (Sigma, cat. no. P8215)
- PMSF (Sigma, cat. no. 93482)
- Lysozyme (Affymetrix, cat. no. 18645)
- Glutathione (GSH) Sepharose 4B (GE Healthcare, cat. no. 17-0756)
- Acid-washed glass beads, 425–600 mm (Sigma, cat. no G8772)
- Laemmli sample buffer, 4× (Bio-Rad, cat. no. 161-0747)
- SDS-PAGE Tris/Glycine/SDS running buffer (Bio-Rad, cat. no. 161-0732)
- L-glutathione, reduced (Sigma, cat. no. G4251)
- SilverQuest silver staining kit (Invitrogen, cat. no. LC6070)
- Ethanol, 95% (vol/vol) (Gold Shield, cat. no. 412602)
- Glacial acetic acid (Fisher, cat. no. A490) **! CAUTION** Concentrated acid is corrosive.
- Deionized water (Milli-Q purified, Millipore)
- Ammonium bicarbonate (Sigma, cat. no. A6141)
- DTT (Bio-Rad, cat. no. 161-0611)
- Iodoacetamide (Sigma, cat. no. I1149)
- Acetonitrile (EMD Millipore, cat. no. AX0151-1)

- Formic acid (Sigma, cat. no. 94318) **! CAUTION** Concentrated acid is corrosive. Use glass pipettes to handle concentrated formic acid and store diluted formic acid solutions in glass containers to avoid leaching material from plastic that could interfere with LC-MS/MS.
- Sequencing-grade modified trypsin (Promega, cat. no. V5113)
- Urea (Sigma, cat. no. U5378)
- Liquid nitrogen **! CAUTION** Liquid nitrogen is cryogenic and should be used in a well-ventilated space.
- Polyacrylamide gel, 10% or 12%, (wt/vol) (Bio-Rad, cat. no. 456-1031, 456-1041)

EQUIPMENT

- Wide-bore P200 micropipette tips (VWR, cat. no. 10011-326)
- Gel-loading micropipette tips (USA Scientific, cat. no. 1022-0600)
- Graduated cylinder, 1 liter (Fisher Scientific, cat. no. and S63461)
- Glass graduated cylinder, 10 ml (VWR, cat. no. 70188-950)
- Microcentrifuge tubes, 1.5 ml (Fisher Scientific, cat. no. 21-403-196)
- Conical tubes, 15 ml (Denville Scientific, cat. no. C1017-P)
- Conical tubes, 50 ml (Denville Scientific, cat. no. C1062-P)
- High-speed 14-ml centrifuge tubes (Fisher Scientific, cat. nos. NC9164146 and NC9164111)
- Heat block for microcentrifuge tubes at 42 °C and 100 °C (VWR, cat. no. 89133-528)
- Ice and insulated container
- Culture shaker spinning at 220 r.p.m. and maintaining temperatures of 20–37 °C
- Sterile polystyrene tubes (Fisher Scientific, cat. no. 352057)
- Erlenmeyer flasks, 250 ml to 1 liter (Fisher Scientific, cat. no. FB-500-250)
- Beaker, 4 liter (Fisher Scientific, cat. no. 02-555-25 K)
- Centrifuge with rotors for 15-ml tubes and high-*g* tubes (Beckman Coulter Avanti J-20 XP)
- Centrifuge bottles suitable for 6,000*g* (such as Beckman Coulter, cat. no. 361691)
- Refrigerated microcentrifuge (such as Fisher Scientific, cat. no. 05401203)
- Rotisserie for microcentrifuge tubes (Fisher Scientific, cat. no. 4002110Q)
- Magnetic stirrer (Fisher Scientific, cat. no. 11-675-910Q)
- Multitube vortexer: Scientific Industries Vortex Genie 2 (cat. no. SI-0236) with Turbomix attachment (cat. no. SI-0564)

- Indicator strips, pH 5–10 (EMD Millipore, cat. no. 9588)
- pH meter (Fisher Scientific, cat. no. 13-636-AB15BC)
- Tip sonicator (such as Branson Digital Sonifier S-250D; output indicated in Step 8 is specific to this sonicator)
- Slide-A-Lyzer MINI dialysis devices, 3.5 kDa molecular weight cutoff (Thermo Scientific/Pierce, cat. no. 69552)
- Floating tube holder for Slide-A-Lyzer dialysis devices (Thermo Scientific/Pierce, cat. no. 69588)
- Apparatus for SDS-PAGE (Bio-Rad, cat. no. 165-8001)
- Razor blades (Fisher Scientific, cat. no. 12-640)
- Vacuum concentrator system (SpeedVac) (such as Thermo Scientific, cat. no. SPD111VP1)
- LC-MS/MS system (see Experimental design for system configuration recommendations)
- Stage tips, C18 material, 200-μl tip (Thermo Scientific, cat. no. SP301)
- LTQ Orbitrap Velos (Thermo Scientific)
- Computer with software for MS/MS data analysis (see PROCEDURE Step 54)

REAGENT SETUP

SILAC medium—Follow the manufacturer's instructions for the preparation of mammalian cell culture medium or the instructions given in Kubota *et al.*[21] for the preparation of yeast growth medium. We recommend verifying complete incorporation of the SILAC label in your particular cellular system[10].

Protein sample from mammalian cell culture—Split the cell type of interest into each SILAC medium and grow it in culture for at least seven population doublings before performing treatments or cell lysis[22]. We recommend preparing the entire experiment in duplicate with light and medium labels swapped (in a two-way SILAC), or light and heavy labels swapped and the medium label duplicated (in a three-way SILAC). Any procedure may be used for protein extraction, but the pH should be ~7.4 with 50–100 mM buffer (Tris in this protocol), salt concentration should be ~150 mM (NaCl in this protocol) and the sample should contain 0.05–0.1% (vol/vol) nonionic detergent such as NP-40 or TritonX-100 (SDS is not recommended)[2]. If you are using three-way SILAC, then the light and heavy cells should be combined immediately before lysis (referred to as light + heavy, and heavy + light samples to represent the label swap), whereas the medium lysate is processed separately until the high-salt dialysis (Step 20). Each sample volume should be 0.5–1 ml and should contain between 600 μg and 5 mg of protein. To account for small differences in protein levels, a small aliquot of each protein sample is removed immediately before starting the 3×MBT pull-down and used to calculate a correction factor for protein loading (Step 13).

Protein sample from yeast extract—Yeast strains must be auxotrophic for arginine and lysine to prevent biosynthesis of unlabeled amino acids (*arg4 lys2*)[21]. Grow yeast in 5 ml of each SILAC medium overnight at 30 °C. Dilute each culture to OD_{600} = 0.2 in 50 ml of SILAC medium and grow it to OD_{600} = 0.6–1.0 at 30 °C. Pellet the cells, by combining equivalent cell numbers of light and heavy cultures if you are using three-way SILAC. Wash the cells once in 5 ml of lysis/binding buffer and resuspend them in 1 ml of lysis/binding buffer supplemented with 0.5 mM PMSF and 2 μl of yeast protease inhibitor cocktail. Bead-beat the yeast in a 1.5-ml microcentrifuge tube by using glass beads, and then vortex the tube for 15 min at 4 °C. Transfer the lysates to a fresh tube and pellet the debris at 20,000*g* for 15 min at 4 °C.

NaCl, 5 M—Add 292.2 g of NaCl to a graduated cylinder and add deionized water to a final volume of 1 liter. Place the solution in a large beaker and mix it by using a magnetic stir rod until the NaCl is fully dissolved. Store it at room temperature (20 °C) for 6 months.

Tris, 1 M, pH 7.4—Add 121.1 g of Tris base to a graduated cylinder and add deionized water to 900 ml. Place the solution in a large beaker and mix it with a magnetic stir rod. Add HCl while monitoring the pH with a pH meter until the pH reaches 7.4 (this will probably require 60–65 ml of HCl). Once the pH is reached and Tris is dissolved, return the solution to the graduated cylinder and adjust the final volume to 1 liter by using deionized water. Store it at room temperature for 6 months.

Tris, 1 M, pH 8.0—Prepare this the same way as 1 M Tris (pH 7.4), but instead adjust the pH to 8.0 (this will probably require 40–45 ml of HCl). Store it at room temperature for 6 months.

Lysis/binding buffer—Lysis/binding buffer contains 150 mM NaCl, 50 mM Tris HCl (pH 7.4), and 0.05% (vol/vol) NP-40. Combine 920 ml of deionized water, 50 ml of 1 M Tris (pH 7.4), 30 ml of 5 M NaCl and 0.5 ml of NP-40. Mix thoroughly at room temperature until the detergent is fully dissolved. Store the buffer at 4 °C for 6 months.

IPTG, 1 M—Place 715 mg of IPTG in a graduated cylinder and add deionized water to 3 ml. Vortex the mixture until IPTG is fully dissolved. Store IPTG at −20 °C for 6 months.

Ampicillin, 50 mg ml^{-1}—Dissolve 50 mg of ampicillin in 1 ml of deionized water. Store it at −20 °C for 6 months.

Lysozyme, 50 mg ml^{-1}—Dissolve 50 mg of lysozyme in 1 ml of deionized water. Store it at −20 °C for 6 months.

Urea, 8 M—Place 0.48 g of urea in a graduated cylinder and add deionized water to 1 ml. Vortex the mixture until urea is fully dissolved. Store it at room temperature for 1 week.

DTT, 1 M—Place 154 mg of DTT in a graduated cylinder and add deionized water to 1 ml. Vortex the mixture until DTT is fully dissolved. Store it at −20 °C for 6 months.

Iodoacetamide, 10 mg ml^{-1}—Dissolve 10 mg of iodoacetamide in 1 ml of deionized water. Prepare the solution immediately before use.

Formic acid, 10% (vol/vol)—Measure 10 ml of concentrated formic acid by using a glass graduated cylinder and glass pipettes, and then add this to 90 ml of deionized water. Store it at room temperature in a glass bottle for 3 months.

Elution buffer—Elution buffer contains 100 mM Tris HCl (pH 8.0) and 10 mg ml^{-1} reduced glutathione. Combine 885 μl of deionized water, 100 μl of 1 M Tris (pH 8.0), 10 mg of glutathione and 15 μl of 1N NaOH. Prepare the buffer on the day of use.

High-salt dialysis buffer—High-salt dialysis buffer contains 750 mM NaCl and 50 mM Tris (pH 7.4). Combine 1.6. liters of deionized water with 300 ml of 5 M NaCl and 100 ml of 1 M Tris (pH 7.4). Store the buffer at 4 °C for 6 months.

Digest buffer—Digest buffer is 50 mM ammonium bicarbonate. Dissolve 395 mg of ammonium bicarbonate in 100 ml of deionized water. Store the buffer in a glass container for up to 6 months.

Acidifying buffer—Combine 450 μl of deionized water, 50 μl of formic acid and 500 μl of acetonitrile. Mix the buffer in a plastic tube and use it immediately or store it in a glass container for up to 1 month.

PROCEDURE

Prepare 3×MBT beads ● TIMING 4 d

1| Add 1 ng each of 3×MBT-pGEX6p-1 and 3×MBT$_{D355N}$-pGEX6p-1 plasmid to separate 10-μl aliquots of competent BL21 *E. coli* cells, incubate them on ice for 5 min, transfer them to a heat block at 42 °C for 45 s and transfer them back onto ice for 2 min. These two cultures are processed in parallel to (and including) Step 12.

2| Add the transformed bacteria to 3 ml of LB medium with 50 μg ml^{-1} ampicillin. Grow this starter culture at 37 °C with shaking at 220 r.p.m. overnight.

3| Dilute 1 ml of the starter culture into 100 ml of LB medium at room temperature with 50 μg ml^{-1} ampicillin (this culture can be scaled up to 0.5 liters as needed, maintaining the 1–100 ratio of starter culture to final culture volume). Grow the culture at 37 °C with shaking at 220 r.p.m. for 2.5 h.

4| Add 10 μl of 1 M IPTG per 100 ml of culture (final concentration 100 μM) to induce protein expression. Grow the culture at 20 °C overnight with shaking at 220 r.p.m.

5| Pellet the bacteria by centrifugation at 6,000*g* for 10 min at 4 °C. Pour off the medium.

! CAUTION Liquid nitrogen is cryogenic and should be used in a well-ventilated space.

■ **PAUSE POINT** The bacterial pellet may be flash-frozen by immersing the tube in liquid nitrogen, and it can be stored at −80 °C for 3 months.

6| On ice, resuspend the pellets in lysis/binding buffer supplemented with 1 mM PMSF and protease inhibitor cocktail (one tablet per 50 ml of buffer). Use 10 ml of lysis/binding buffer for every 100 ml of bacterial culture volume pelleted.

7| Add 50 µl of lysozyme per 10 ml of lysis/binding buffer (final concentration 0.25 mg ml^{-1}). Incubate the mixture on ice for 30 min.

8| Disrupt the bacterial cells by sonication. Use the Branson Digital Sonifier to sonicate three times for 10 s on (1 s on, 1 s off) at 18% output.

9| Clear the lysate by spinning at 20,000*g* for 25 min at 4 °C.

10| While you are waiting for the spin in Step 9, prewash the GSH-Sepharose beads three times by adding 10 ml of lysis/binding buffer, pelleting the beads by spinning at 1,000*g* for 2 min at 4 °C and removing the buffer. Use 100 µl of GSH-Sepharose per 50 ml of original bacterial culture volume.

11| Pour the cleared lysate from Step 9 onto the washed GSH-Sepharose beads and rotate the tube on the rotisserie at 4 °C overnight.

12| Pellet the beads by spinning them at 500*g* for 2 min at 4 °C. Carefully aspirate and discard the supernatant. Wash the beads three times by adding 10 ml of lysis/binding buffer on ice, rotating them on the rotisserie at 4 °C for 5 min and pelleting them as before. After aspirating the third wash, add 820 µl of lysis/binding buffer per 100 µl of original GSH-Sepharose volume (Step 10) and keep it on ice or at 4 °C until Step 14. We recommend preparing beads to be used the same day.

? TROUBLESHOOTING

3×MBT pull-down ● TIMING 2 d

13| Remove an aliquot of each protein sample (Reagent Setup) to use for normalization of protein loading (input samples, Step 41). For protein samples from a two-way SILAC, follow option A (Fig. 1b); for samples from a three-way SILAC, follow option B (Fig. 1c).

A. Two-way SILAC

i. Combine 1 µl of each protein sample in a 1.5-ml tube.

ii. Add 18 µl of 8 M urea and store the samples at 4 °C until Step 41 (up to 1 week).

B. Three-way SILAC

i. Combine 2 µl of the light + heavy protein sample (1 µl of light material and 1 µl of heavy material, combined before cell lysis) with 1 µl of the medium protein sample. In another tube, combine 2 µl of the label-swap protein sample (heavy + light) with 1 µl of the medium protein sample.

ii. Add 17 µl of 8 M urea to each input sample and store it at 4 °C until Step 41 (up to 1 week).

14| Set up 3×MBT and 3×MBT_{D355N} pull-downs, depending on whether the experiment uses two- or three-way SILAC. For a two-way SILAC, follow option A; for a three-way SILAC follow option B.

A. Two-way SILAC

i. Gently suspend the 3×MBT and 3×MBT_{D355N} beads from Step 12 by inverting or flicking the tube until the beads are well mixed, and then immediately use a wide-bore micropipette tip to transfer 450 µl of each bead type to two 1.5-ml tubes (four tubes total).

ii. Spin the beads at 450*g* for 15 s at 4 °C and carefully remove the supernatant. This leaves ~50 µl of packed beads.

iii. Combine half of the light SILAC protein sample with 3×MBT beads and half of the medium sample with 3×MBT_{D355N} beads. Combine the other half of each sample with the other type of beads (the label swap).

B. Three-way SILAC

i. Gently suspend 3×MBT beads from Step 12 and immediately use a wide-bore micropipette tip to transfer 450 µl to two 1.5-ml tubes. Similarly transfer 450 µl of 3×MBT_{D355N} beads to a separate 1.5-ml tube.

ii. Spin the beads at 450*g* for 15 s at 4 °C and carefully remove the supernatant.

iii. Add the combined (light + heavy) SILAC protein sample to one tube of 3×MBT beads and the label-swap (heavy + light) protein sample to the other. Combine the medium SILAC protein sample with the aliquot of 3×MBT_{D355N} beads.

15| Incubate the pull-down samples rotating on the rotisserie overnight at 4 °C.

16| Pellet the beads at 1,500*g* for 2 min at 4 °C and carefully remove the supernatant. Wash the beads four times by adding 750 µl of binding buffer, rotating the beads on a rotisserie at 4 °C for 5 min, pelleting at 1,500*g* for 2 min at 4 °C and removing the supernatant.

17| After the final wash, remove all excess liquid, being careful not to remove any beads. For proteomic analysis, continue immediately to Step 18. Samples may be analyzed by western blotting by adding Laemmli sample buffer directly to the beads and heating the beads between 95 and 100 °C for 5 min. Samples for western blotting may be stored at 4 °C for 1 week or at −20 °C indefinitely.

▲ **CRITICAL STEP** Residual buffer from the GSH-Sepharose beads will increase the volume of the sample and make it difficult to load for SDS-PAGE.

? TROUBLESHOOTING

Release of methylated proteins and 3×MBT removal ● TIMING 2 d

18| Add 50 µl of elution buffer to each pull-down and incubate it for 4 h on a rotisserie at 4 °C. During this time, chill the dialysis buffer for Step 21.

19| Pellet the beads by centrifugation at 1,500*g* for 2 min at 4 °C. Eluted proteins may be analyzed by western blotting as in Step 17.

? TROUBLESHOOTING

20| Use a gel-loading micropipette tip to collect all of the eluate (supernatant) from the beads. For a two-way SILAC experiment, follow option A; for a three-way SILAC experiment, follow option B.

A. Two-way SILAC

i. Combine the eluates from the light 3×MBT and medium 3×MBT_{D355N} pull-downs in a new 1.5-ml tube, and combine the eluates from the label swap (medium 3×MBT and light 3×MBT_{D355N} pull-downs) in another 1.5-ml tube.

B. Three-way SILAC

i. Combine the eluate of one (light + heavy) 3×MBT pull-down with half of the eluate from the medium 3×MBT_{D355N} pull-down. This results in a sample with equal amounts of light, medium and heavy material. Combine the eluate of the label-swapped (heavy + light) 3×MBT pull-down with the other half of the eluate from the 3×MBT_{D355N} pull-down.

21| Transfer each combined eluate into a Slide-A-Lyzer mini dialysis tube. Place the tube in a floating tube holder and dialyze the contents overnight at 4 °C against 2 liters of high-salt dialysis buffer, stirring the buffer on a magnetic stirrer.

▲ **CRITICAL STEP** Position the dialysis tube so that the liquid level inside the tube is higher than that of the buffer outside the tube. This prevents the sample volume from increasing as the two levels equalize.

▲ **CRITICAL STEP** Overnight dialysis is necessary to remove enough free glutathione so that 3×MBT can be depleted by rebinding to fresh GSH-Sepharose beads.

22| Prewash 150 µl of GSH-Sepharose beads for each pull-down by adding 1 ml of lysis/binding buffer, pelleting the beads by spinning at 1,000*g* for 2 min at 4 °C and removing the buffer (wash three times total). Carefully remove all extra liquid after the last wash to minimize the volume added to the sample in Step 23.

▲ **CRITICAL STEP** The amount of GSH-Sepharose beads must be at least 1.5 times the amount of beads used in the pull-down in order to ensure sufficient depletion of the 3×MBT domain. This amount takes into consideration the total volume of beads represented by the eluates combined in Step 20.

▲ **CRITICAL STEP** Residual buffer from the GSH-Sepharose beads will increase the volume of the sample and make it difficult to load for SDS-PAGE.

23| Add the dialyzed eluate from Step 21 to the prewashed GSH-Sepharose to rebind the 3×MBT. Rotate the mixture on the rotisserie at 4 °C for 4 h.

24| Pellet the GSH-Sepharose, which has bound most of the 3×MBT domain, by spinning it at 1,500*g* for 2 min at 4 °C. The domain should be depleted by >90%. Excess 3×MBT can distort the SDS-PAGE gel in later steps (Fig. 2a,b).

25| Transfer each supernatant to a fresh 1.5-ml tube. Spin the supernatant once more at maximum speed for 5 min at 4 °C to ensure that all beads are removed.

▲ **CRITICAL STEP** Use a gel-loading micropipette tip to remove as much liquid as possible from the packed beads; leaving behind any sample will reduce the protein yield.

26| Transfer the supernatant containing methylated proteins to a new tube and add 4× Laemmli buffer. Incubate the samples at 95–100 °C for 5 min.

■ **PAUSE POINT** After heating, the samples may be stored at −20 °C for up to 2 weeks. Heat the frozen samples as in Step 26 before continuing.

In-gel digest ● TIMING 2 d

▲ **CRITICAL** Perform Steps 33–40 in parallel with Steps 41–45.

27| Run the samples on an SDS-PAGE gel. Load the samples into the fewest possible lanes to minimize the gel volume used and separate the samples from label-swap experiments by at least one lane containing only Laemmli buffer. To ensure that even low-molecular-weight proteins are retained, the dye front should not be run off the gel.

? TROUBLESHOOTING

28| Stain the gel according to the directions in the silver staining kit.

▲ **CRITICAL STEP** From this point forward, keep the gel covered during all incubation steps to minimize contamination from proteins in the air.

▲ **CRITICAL STEP** A dedicated container should be used for silver staining; it should never have been exposed to any type of protein or blocking buffer, such as milk or BSA.

? TROUBLESHOOTING

29| Use a clean razor blade to remove and discard the band corresponding to the remaining 3×MBT in the sample (Fig. 2c). Use a fresh razor blade for each label-swap experiment to prevent cross-contamination. Cut each sample into the desired number of molecular-weight fractions (Fig. 2c). The use of more fractions increases proteomic coverage but also increases the mass spectrometry time required.

30| Dice each fraction into small pieces (~1 mm^3). All lanes containing the same label can be cut together.

31| Transfer the gel pieces from each fraction into a 1.5-ml tube and destain the gel pieces according to the instructions for the silver staining kit.

32| Wash the gel pieces for 5 min with 1 ml of digest buffer.

▲ **CRITICAL STEP** When you are changing solutions, remove as much liquid as possible.

33| Replace the buffer with 1 ml of digest buffer supplemented with 10 mM DTT. Incubate the gel pieces at 55–65 °C for 1 h.

34| Wash the gel pieces for 5 min in 1 ml of digest buffer.

35| Replace the buffer on the gel pieces with digest buffer supplemented with 10 mg ml^{-1} iodoacetamide. Incubate the gel pieces at room temperature in darkness for 1 h.

▲ **CRITICAL STEP** Prepare iodoacetamide solution immediately before use.

36| Remove the iodoacetamide from the gel pieces and wash them in 1 ml of acetonitrile for 5 min with shaking or rotation. The gel pieces will dehydrate, becoming off-white or yellow. Remove the acetonitrile and wash the pieces again for 5 min with fresh acetonitrile.

▲ **CRITICAL STEP** Gel pieces must dehydrate completely for trypsin to be efficiently absorbed in Step 38. Repeat Step 36 if the gel pieces are not uniformly opaque.

37| Use the SpeedVac to dry the gel pieces for 15 min.

38| Remove the dehydrated gel pieces from the SpeedVac and place them on ice. Cover the gel pieces in digest buffer with 12.5 ng μl^{-1} trypsin (31 µl of trypsin per ml of digest buffer) and incubate them on ice for 30 min.

39| Remove any remaining trypsin buffer from the gel pieces and cover them in a minimal volume of digest buffer.

40| Leave the gel pieces overnight at room temperature in darkness to complete digestion.

Digest the input samples ● TIMING 2 d

▲ **CRITICAL** Perform Steps 41–45 in parallel with Steps 33–40.

41| Add 2 µl of digest buffer supplemented with 100 mM DTT to each input sample from Step 13. Incubate the input samples at 55–65 °C for 1 h.

42| Add 2 µl of digest buffer supplemented with 100 mg ml^{-1} iodoacetamide to each input sample. Incubate the samples at room temperature in darkness for 1 h.

43| Add 80 µl of digest buffer to each input sample.

44| Add 3 µl of trypsin to each input sample (to reach about 12.5 ng μl^{-1}) and incubate the samples overnight in darkness at room temperature.

45| Add 10 µl of 10% (vol/vol) formic acid to each input sample to stop the digestion.

■ **PAUSE POINT** Digested input samples may be stored at 4 °C for 1 week or at −20 °C for 1 month.

Extract peptides from the in-gel digest ● TIMING 6 h

46| Prepare a 1.5- or 15-ml tube to collect peptides from each of the digested fractions.

47| Transfer the digest buffer from each gel fraction into its respective collection tube. From this point, pool all subsequent washes and extracts into the same respective tubes.

48| Wash the gel pieces with a minimal volume (to cover the gel pieces) of acidifying buffer for 5 min. Transfer the buffer to the collection tubes.

49| Dehydrate the gel pieces with 600 µl of acetonitrile for 5 min with rotation or shaking. Transfer the acetonitrile to the appropriate collection tubes.

▲ **CRITICAL STEP** Gel pieces must be completely dehydrated to ensure maximum peptide recovery. Repeat Step 49 if the gel pieces are not uniformly opaque.

50| Rehydrate the gel pieces with a minimal volume of digest buffer.

51| Pool residual digest buffer into the collection tubes and repeat the dehydration in Step 49.

52| Dry down the pooled fractions in the collection tubes with a SpeedVac. It may be helpful to divide the sample among several tubes to increase the drying rate.

■ **PAUSE POINT** Dried peptides may be stored at 4 °C for 1 week or at −20 °C for 1 month.

LC-MS/MS ● TIMING 2–24 h

53| Analyze each fraction collected in Step 52 separately, as well as 10% of the digested input material (collected in Step 45) to be used in normalizing for protein loading. See the Experimental design section for more information. Analysis of proteins from in-gel digestion is routine for most mass spectrometry facilities, but the sample preparation and procedure for LC-MS/MS analysis depends on the configuration of the mass spectrometer and liquid chromatography system. We remove salts using C18 Stage Tips and analyze each fraction by using a 2-h reverse-phase HPLC gradient with 100 nl min^{-1} flow into an LTQ Orbitrap Velos. Each MS1 survey scan in the Orbitrap is followed by ten data-dependent MS/MS scans in the ion trap using CID fragmentation.

? TROUBLESHOOTING

LC-MS/MS data analysis ● TIMING 2–8 h

54| Analyze LC-MS/MS data with one of the various software tools developed to identify and quantify peptides from LC-MS/MS data (for example, MaxQuant, MASCOT, Sequest)[23–25]. Search parameters should include variable modification of lysine with mono-, di- and tri-methylation (addition of 14.01565, 28.03130 and 42.04695 Da, respectively). We recommend consulting with a mass spectrometry core facility or laboratory regarding the analysis of your specific data set. MaxQuant[23] is a publically available tool for peptide identification that includes built-in support for protein quantification by SILAC. Any analysis should produce a spreadsheet of proteins identified and their light/medium and light/heavy SILAC ratios. Contaminant proteins

such as keratin should be identified and excluded at this step (many software tools can flag these proteins automatically). Identification of methylated peptides should be carefully validated because they are subject to more frequent false identification than unmodified peptides or peptides carrying other common modifications such as phosphorylation (Box 1).

? TROUBLESHOOTING

55| To normalize for differences in protein loading, calculate the median value of light/medium and light/heavy SILAC ratios for proteins identified in the input material (Step 45). Divide SILAC ratios for proteins identified in the pull-down by the median values from the input material.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● TIMING

Steps 1–12, preparing 3×MBT beads: 4 d

Steps 1 and 2, day 1: 20 min

Steps 3 and 4, day 2: 3 h

Steps 5–11, extract peptides, day 3: 2 h

Step 12, day 4: 1 h

Steps 13–17, 3×MBT pull-down: 2 d, the first of which overlaps with day 4 above

Steps 13–15, day 4: 20 min

Steps 16 and 17, day 5: 40 min

Steps 18–26, release of methylated proteins and 3×MBT removal: 2 d, beginning on day 5

Steps 18–21, day 5: 5 h

Steps 22–26, day 6: 5 h

Steps 27–52, in-gel digest: 2 d (note that Step 27 may begin immediately after Step 26, resulting in a 13-h procedure)

Steps 27–47, day 7: 8 h

Steps 46–52, extract peptides, day 8: 6 h

Step 53, LC-MS/MS: 2–24 h

Steps 54 and 55, LC-MS/MS data analysis: 2–8 h

ANTICIPATED RESULTS

A single LC-MS/MS analysis from human cells in culture will identify several hundred to almost a thousand proteins depending on the amount of starting material, the cell line, the

number of separate fractions produced before LC-MS/MS and the length of the HPLC gradients. Analysis of proteins from yeast will identify up to several hundred enriched proteins. We often eliminate proteins from the data set that are represented by only a single peptide. We also exclude from the data set any proteins that were observed in only one of the two label-swap experiments and any known contaminants (i.e., keratins, L3MBTL1 and trypsin).

Identification of candidate methylated proteins from quantitative pull-down data

In experiments with human material, at least 60% of proteins should be at least twice as abundant in 3×MBT pull-down compared with 3×MBT_{D355N} pull-down. This threshold for candidate methylated proteins is set to exclude almost the entire range of natural variability among biological replicates, but some proteins with lower enrichment are also methylated[2]. A pair of label-swap experiments can be easily visualized by placing proteins on a scatterplot according to their 3×MBT over 3×MBT_{D355N} ratios in each experiment (Fig. 3a). It is helpful to log-transform the ratios such that proteins that do not change between conditions cluster at the origin. Before plotting pull-down ratios, we normalize for protein loading by dividing each SILAC ratio from the 3×MBT pull-down by the median value of the same SILAC ratio in the input samples (Step 55); this accounts for differences in the amount of protein originating from each cell lysate. In an experiment analyzing proteins captured by 3×MBT from yeast lysate, the most strongly enriched proteins include well-known methylated species such as histones H3 and H4, elongation factors EF1a and EF2 and ribosomal proteins including Rpl23 (Fig. 3a).

Determination of differential methylation from quantitative data

To identify proteins that may be differentially methylated between two biological conditions, we first consider only proteins that are enriched by 3×MBT relative to 3×MBT_{D355N}. Proteins that change between biological conditions but are not enriched by 3×MBT are likely to be differentially expressed, not methylated, and bound nonspecifically in the pull-down. Second, we consider whether the amount of protein bound by 3×MBT changes reproducibly in both experiments with labels swapped. The size of effect depends on several factors, especially the number of methylated residues on a protein. For example, human histone H3 can be methylated at a number of residues (major sites include K4, K9, K27, K36 and K79), and thus treatment with an inhibitor that blocks methylation of a specific residue results in only a small change in the amount of H3 captured by 3×MBT. Our experience indicates that knockdown, inhibition or overexpression of a methyltransferase often results in a 50% change in capture of known substrates of that enzyme[2].

Example: analysis of Rkm1 knockout in yeast

As an example of how to identify candidate KMT substrates by 3×MBT pull-down and quantitative LC-MS/MS, we conducted a three-way SILAC experiment comparing lysine-methylated yeast proteins from wild-type and *rkm1*Δ strains[21]. Rkm1 is a KMT known to methylate the ribosomal protein Rpl23 (ref. 13). In the experiment, we used wild-type and *rkm1*Δ strains grown in light or heavy SILAC medium, and the experiment was conducted as two replicates with light and heavy labels swapped between experiments. In each

experiment, the medium SILAC condition contains 3×MBT$_{D355N}$ pull-down from wild-type cells. A complete list of proteins identified in both labeling directions and their quantitative data is shown in Supplementary Table 1.

To identify candidate substrates of Rkm1, we first remove proteins that are not strongly enriched by 3×MBT over 3×MBT$_{D355N}$, in this case requiring a ratio greater than 2 averaged between the two replicates. We also calculate the wild-type over knockout ratio from the two experiments with label-swap and visualize the results as a scatter plot (Fig. 3b). In this case, signal for Rpl23, the best characterized substrate for Rkm1, is noticeably stronger from wild-type cells than *rkm1*Δ (Fig. 3b, c). The effect size of about 50% is consistent with the fact that Rpl23 is methylated by both Rkm1 and Rkm2 (ref. 26). Pull-down of other ribosomal proteins is not similarly disrupted in the *rkm1*Δ cell sample, probably because ribosomal proteins are not an intact complex after high-salt extraction in the sample preparation and overnight pull-down.

We also find that the heat-shock protein Ssa4 is strongly enriched by 3×MBT from wild-type cells relative to knockout cells, making it a candidate target for Rkm1. It remains possible that Ssa4 is simply more abundant in wild-type cells or that it is captured through association with Rpl23. This result could be validated by using targeted mass spectrometry to verify that Ssa4 is methylated, and then by performing an *in vitro* methylation reaction with recombinant Rkm1 and Ssa4.

Data availability

Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the Proteomics Identifications Database (PRIDE) partner repository[27] with data set identifier PXD000360.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by a grant from the US National Institutes of Health (NIH) to O.G. (R01 GM079641). S.M.C. was supported by an American Cancer Society Illinois Division Postdoctoral Fellowship award 123711-PF-13-093-01-TBE. K.E.M. was supported by a Hubert Shaw and Sandra Lui Stanford Graduate Fellowship. This material is based on work supported by the National Science Foundation Graduate Research Fellowship under grant no. DGE-1147470 to K.E.M. G.M.M. was supported by the Generalitat de Catalunya Beatriu de Pinos award BP-A 2010. O.G. is a recipient of an Ellison Senior Scholar in Aging Award. We thank the PRIDE team for assistance in making mass spectrometry data available.

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Box 1

Identification and validation of methylated peptides by LC-MS/MS

Methylation is a challenging modification to identify by mass spectrometry, because the mass shift of 14 Da mimics the mass difference between many amino acids (e.g., glycine and alanine, serine and threonine). We routinely observe much higher incidence of false positives than is predicted by searching against a reverse sequence database. In particular, automated analysis is likely to misidentify related peptide sequences that differ by a single methyl group. These hits often have excellent scores for both peptide identification and position of the modification. It is therefore crucial that someone experienced with interpretation of mass spectra manually inspects any candidate methylated peptides. We apply two validation criteria that are specific for methylated peptides when confirming modification sites identified by high-throughput proteomics[2]:

Diagnostic fragment ions constrain the position of the methyl group to a single amino acid. It is standard practice to require that a modification be constrained to a specific residue, but methylation differs from other modifications because any amino acid should be considered a potential site of methylation if it could be exchanged for another amino acid by the addition of a methyl group (e.g., glycine, serine, etc.).

There are no nonmethylated peptides in the relevant proteome with the same mass and a similar peptide sequence. For example, tryptic peptides from closely related proteins such as human TOP2A and TOP2B differ by a single methyl group adjacent to the terminal lysine. In this example, a nonmethylated peptide from TOP2B is frequently misidentified as a methylated peptide from TOP2A:

GFQQISFVNSIATSK*(TOP2A amino acids 307–319, * indicates methylation)

GFQQISFVNSIATTK (TOP2B amino acids 328–342)

A strategy to simplify the identification of methylated residues uses cells grown with heavy methionine to change the mass shift of the PTM[15]. In this approach, cells are grown in medium containing $^{2}H_{3}{}^{13}C$-methionine. Methionine is the precursor for biosynthesis of S-adenosyl methionine, the methyl-donating cofactor for protein methylation, and thus methylated residues gain 18 Da instead of 14 Da. This gives methylated peptides a unique mass shift that is unlikely to be mistaken for similar nonmethylated sequences.

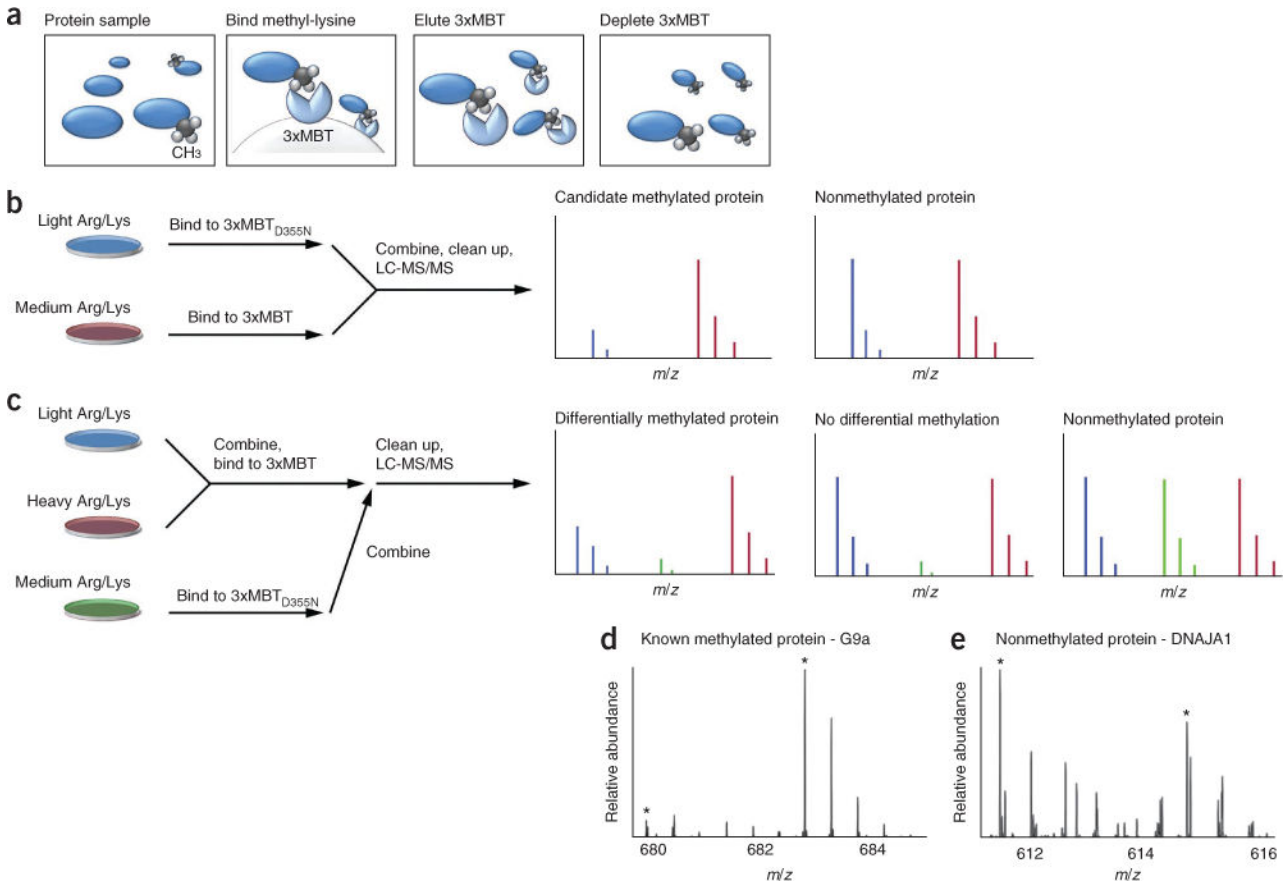

Figure 1.

Overview and experimental design. (**a**) Overview of the procedure. Proteins modified by lysine mono- or di-methylation are selectively bound by the GST-3×MBT fusion protein immobilized on beads. The fusion protein and any bound proteins are eluted with reduced glutathione, the glutathione is removed and GST-3×MBT is depleted by rebinding to beads while using high salt to disrupt 3×MBT binding to methylated proteins. (**b**) SILAC for identifying candidate methylated proteins. Protein extracts are prepared from cells grown in medium containing amino acids with either light or medium stable isotopes. The two samples are incubated with 3×MBT or the $3\times MBT_{D355N}$ negative control, bound proteins are combined and proteins enriched by 3×MBT are identified by quantitative LC-MS/MS. (**c**) Experimental design to compare methylation states between two biological conditions. Cells are prepared in light or heavy amino acids under the treatment conditions being compared. The two sets of cells are combined before lysis. The medium amino acids are again used to distinguish the $3\times MBT_{D355N}$ negative control. (**d, e**) Mass spectra from an experiment using human cell lysate and the experimental design from panel **b**. SILAC data for a peptide from a known methylated protein, the lysine methyltransferase G9a, is shown in **d**, whereas **e** shows a protein that appears to be nonspecifically bound by 3×MBT and $3\times MBT_{D355N}$. The asterisk (*) indicates light and heavy forms of the peptide, with the low *m/z* peak representing $3\times MBT_{D355N}$ pull-down and the high *m/z* peak the 3×MBT pull-down.

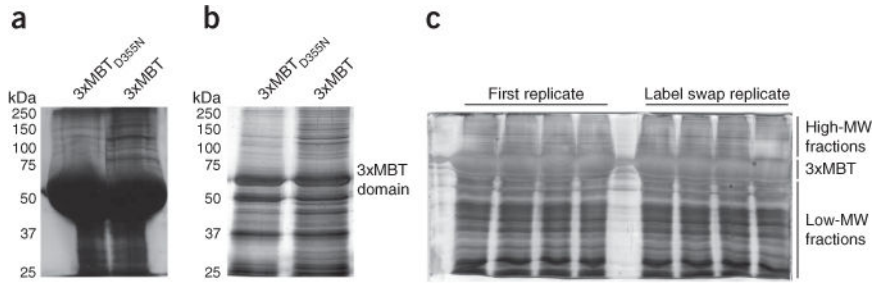

Figure 2.

Example of silver-stained gels showing protein pull-down by 3×MBT from protein extract of human cells. (**a**) SDS-PAGE and silver stain of the material from 3×MBT and 3×MBT$_{D355N}$ pull-downs before depleting excess 3×MBT domain (Step 23). (**b**) A similar pair of pull-downs after depleting the 3×MBT domain. There is still considerable residual domain, but it no longer distorts the gel lanes (panel **b** is reproduced with permission from Moore *et al.*[2], images in panels **a** and **b** are scaled to be directly comparable). (**c**) An example of the silver-stained SDS-PAGE gel for a complete 3×MBT proteomics experiment. The experiment includes a replicate with the light and heavy SILAC conditions reversed, analyzed in parallel and run on the same gel. Residual 3×MBT protein stains as a strong band, but it should not markedly distort the gel. The 3×MBT band should be excised and discarded. The high- and low-molecular-weight fractions may be divided into two or more molecular-weight regions to improve protein identification by LC-MS/MS. MW, molecular weight.

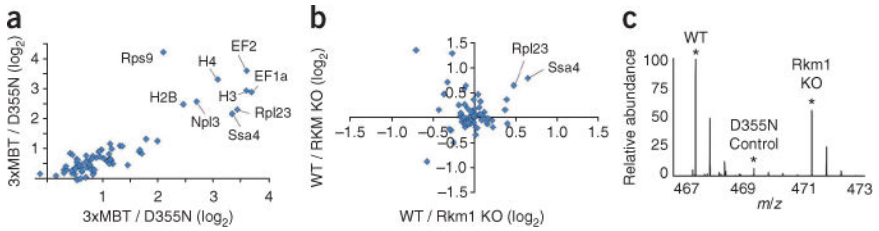

Figure 3.

Application of methyl-lysine enrichment to Rkm1 substrate identification. (**a**) Many well-known methylated proteins are enriched from yeast cell extract by 3×MBT relative to $3\times MBT_{D355N}$. The two axes show SILAC protein ratios from independent experiments. (**b**) Knockout (KO) of Rkm1 reduces the amount of Rpl23 and Ssa4 bound by 3×MBT by about 50%. Axes represent independent experiments with light and heavy SILAC labeling conditions reversed (wild-type (WT) heavy/*rkm1*Δ light on the *x* axis, WT light/*rkm1*Δ heavy on the *y* axis). Proteins in the upper-right quadrant show decreased 3×MBT pull-down upon loss of Rkm1 (potential Rkm1 substrates); proteins in the lower left quadrant show increased 3×MBT pull-down upon loss of Rkm1 (probably noisy outliers or proteins with increased expression in *rkm1*Δ cells); and proteins in the upper left quadrant represent contaminant ions. (**c**) The MS1 spectrum showing abundance of a representative peptide from Rpl23 in this experiment. The spectrum shows isotopically labeled peptides from three conditions, from left to right: 3×MBT capture of the peptide from WT cells, $3\times MBT_{D355N}$ capture of the peptide from WT cells, and 3×MBT capture of the peptide from *rkm1*Δ cells (each indicated by *). The amount of bound Rpl23 is clearly reduced after KO of Rkm1, and Rpl23 is almost absent from the negative control pull-down.

TABLE 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
12	GSH-Sepharose beads are difficult to pellet after binding to 3×MBT	Beads behave differently when saturated with protein	After pelleting the beads, reverse the direction of the tube relative to the centrifuge rotor and spin for 3–4 s
17, 19	Gel lanes or protein bands are warped in the western blot	Too much 3×MBT is loaded on the gel	Dilute the sample and load only a fraction on the gel
			Elute using GSH-Sepharose beads (as in Step 18) instead of boiling the beads in Laemmli buffer. Deplete the domain as in Steps 19–25
27	Sample volume is too large to load on a gel for SDS-PAGE	Buffers are not removed after wash steps	Use a gel-loading tip to remove all excess liquid from the packed beads in Steps 17 and 22
		Liquid is drawn into the dialysis tube while depleting GSH-Sepharose beads	During dialysis (Step 21), position the sample so that the bottom of the meniscus sits slightly above the buffer level
28	Lanes are warped or the 3×MBT band overwhelms the gel after silver stain	3×MBT was not efficiently depleted	Increase the volume of GSH-Sepharose beads in Step 22 to 200 µl
			Ensure complete removal of free glutathione by dialyzing overnight or against a larger volume of high-salt buffer in Step 21
53	PEG in the mass spectrometry runs	Plastic leaching into concentrated acids or organic buffers	Replace all buffers used during in-gel digest (Steps 27–52). Store the new buffers in glass containers
			Replace the concentrated formic acid stock. Pipette concentrated acids with glass
54	Poor protein coverage by LC-MS/MS	Low protein yield after 3×MBT pull-down	Increase the amount of protein going into the pull-down. We have used up to 5 mg of starting material
			Use 90 µl of 3×MBT beads per pull-down. Adjust the elution and rebinding volumes accordingly in Steps 18 and 22
		Chromatography gradients are too short or are not of sufficient molecular weight fractions	Use chromatography gradients up to 4 h during LC-MS/MS to maximize the number of peptides identified
			Divide the sample into more molecular weight fractions at Step 29
	Contamination by ambient proteins such as keratins	Protein gel or cut gel pieces are collecting protein from the environment	Keep the gel and gel pieces covered during the entire procedure
			Do not handle BSA, milk or other blocking reagents near the gel or gel pieces
	Known targets of a methyltransferase do not change after targeted perturbation	Targets are methylated by additional enzymes	Use methyl-specific antibodies or immunoprecipitation and LC-MS/MS to verify that known methylation sites change under the specific treatment condition
		The methylation site is stable over the duration of the treatment	Use western blotting or immunoprecipitation and LC-MS/MS to identify the treatment condition and time point that provides the largest change in known methylation site(s)
	Uneven protein levels between SILAC conditions	Cell cultures are not handled uniformly	Grow the cells for each SILAC condition in parallel from a single parent population
			Combine cells or cell lysates at the earliest possible step
	High background pull-down in 3×MBT_{D355N} samples	Insufficient washing	Wash the pull-down four times for at least 5 min with 1 ml of lysis/binding buffer in Step 16
		Precipitated protein is pelleting along with the beads	Spin the protein sample at 15,000*g* for 5 min at 4 °C before combining it with 3×MBT beads (Step 14)

Step	Problem	Possible reason	Solution
		Beads are not fully saturated with 3×MBT	Increase the bacterial culture volume to 200 ml per 100 µl GSH-Sepharose when preparing the domain