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Identifying and quantifying *in vivo* methylation sites by heavy methyl SILAC

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Protein methylation is a stable post-translational modification (PTM) with important biological functions. It occurs predominantly on arginine and lysine residues with varying numbers of methyl groups, such as mono-, di- or trimethyl lysine. Existing methods for identifying methylation sites are laborious, require large amounts of sample and cannot be applied to complex mixtures. We have previously described stable isotope labeling by amino acids in cell culture (SILAC) for quantitative comparison of proteomes. In heavy methyl SILAC, cells metabolically convert [$^{13}CD_3$]methionine to the sole biological methyl donor, [$^{13}CD_3$]*S*-adenosyl methionine. Heavy methyl groups are fully incorporated into *in vivo* methylation sites, directly labeling the PTM. This provides markedly increased confidence in identification and relative quantitation of protein methylation by mass spectrometry. Using antibodies targeted to methylated residues and analysis by liquid chromatography–tandem mass spectrometry, we identified 59 methylation sites, including previously unknown sites, considerably extending the number of *in vivo* methylation sites described in the literature.

Methylation of DNA is known to be responsible for transcriptional repression, genomic imprinting and modulation of chromatin structure[1]. Protein methylation, which was discovered nearly half a century ago[2], has been implicated in protein-protein interactions[3], cellular localization[4] and maturation of heterogeneous ribonucleoproteins (hnRNPs)[5]; in addition, it is thought to have crucial roles in cellular signaling[6]. Methylation is an important part of the histone code that regulates cell type–specific gene expression programs[1]. Protein methylation modifies primarily arginine and lysine residues, but carboxymethylation of isoprenylated cysteine residues is also known to occur in membrane-associated proteins such as Ras, Rac, Rho and Cdc42 (see, for example, refs. 7–9). The modification does not substantially alter the positive charge of arginines or lysines, but it does increase their hydrophobicity. The increased steric hindrance and decreased hydrogen bonding of methylated arginines may affect interaction of proteins with other cellular substrates[10]. Methylation is a very stable protein modification, and no biological lysine or arginine demethylase activity has thus far been identified.

Cellular methyltransferases share well-conserved structural features[11], and bioinformatic analyses have predicted a large number of putative class I methyltransferases in yeast. Methylation may thus be a far more prevalent and important PTM than previously thought[12]. However, identification of the substrates of even well-described methyltransferases has been delayed, because methods used during the past few decades are laborious and require large amounts of sample. These methods involve incorporation of radioactive [^{3}H]methyl, enzymatic digest and HPLC separation of peptides, followed by thin-layer chromatography, gas chromatography–mass spectrometry, Edman sequencing or amino acid analysis[7]. Alternatively, a recombinant methyltransferase is used in *in vitro* assays for arginine methylation[13]. All these approaches can be used only with single proteins and require a priori knowledge of their identity.

Because PTMs have central roles in practically all aspects of cellular function, their study is one of the holy grails of proteomics[14]. Detection of dimethylated arginine residues[15] in a peptide mixture is possible with precursor-ion scanning in triple-quadrupole mass spectrometers. Characteristic fragment-ion losses upon fragmentation of methylated peptides in collision-induced dissociation can identify methylation sites[16,17]. However, not all types of methylation generate characteristic fragment ions, and this approach depends on high-quality tandem mass spectrometry (MS/MS) spectra.

Here we report a new method for the identification and quantification of *in vivo* protein methylation based on metabolic labeling using SILAC[18–20]. Cells are grown in medium containing either only 'light' or only 'heavy' methionine, which is metabolically converted to 'heavy' *S*-adenosyl methionine (AdoMet), resulting in complete incorporation of labeled methyl groups into all methylation sites. This provides increased confidence in identification of methylation sites and their relative quantification.

RESULTS

Incorporation of stable isotope–labeled amino acids

MS-based quantification typically relies on the incorporation of such stable isotopes as ^{13}C, ^2H, ^{15}N and ^{18}O, resulting in pairs of peptides identical in all respects except their separation in mass. In

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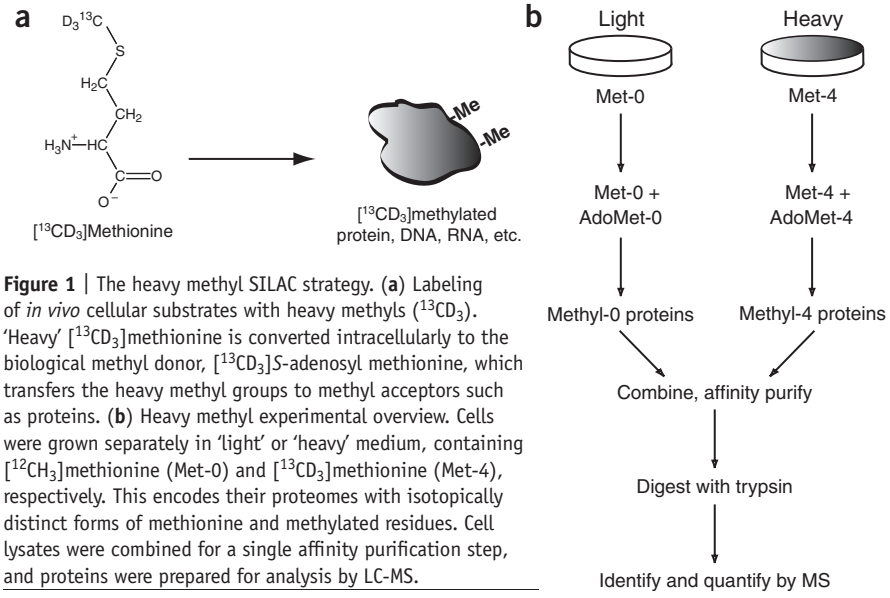

Figure 1 | The heavy methyl SILAC strategy. (**a**) Labeling of *in vivo* cellular substrates with heavy methyls ($^{13}CD_3$). 'Heavy' [$^{13}CD_3$]methionine is converted intracellularly to the biological methyl donor, [$^{13}CD_3$]*S*-adenosyl methionine, which transfers the heavy methyl groups to methyl acceptors such as proteins. (**b**) Heavy methyl experimental overview. Cells were grown separately in 'light' or 'heavy' medium, containing [$^{12}CH_3$]methionine (Met-0) and [$^{13}CD_3$]methionine (Met-4), respectively. This encodes their proteomes with isotopically distinct forms of methionine and methylated residues. Cell lysates were combined for a single affinity purification step, and proteins were prepared for analysis by LC-MS.

'heavy methyl SILAC', we grow cells in medium with methionine containing a 'heavy' methyl group bearing one carbon-13 and three deuterium atoms. Cells metabolically convert the [$^{13}CD_3$]methionine to the cell's sole methyl donor, [$^{13}CD_3$]AdoMet, in a process requiring ATP. This activated intermediate is used to methylate cellular substrates such as protein, DNA, RNA and phospholipids[11], thereby transferring the 'heavy' methyl group to the methyl acceptor and incorporating the mass tag into all methylated proteins (**Fig. 1**).

Cells grown in heavy SILAC medium were indistinguishable from cells grown in 'light' medium in terms of cell growth and overall morphology. We analyzed proteins from HeLa cell lysates grown in medium with [$^{13}CD_3$]methionine using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Methylated peptides and methionine-containing peptides were detectable only in their heavy forms, indicating full incorporation (**Fig. 2**). Therefore, direct *in vivo* labeling of this PTM can be achieved.

After transfer of the methyl group from AdoMet to a methylated substrate, *S*-adenosyl homocysteine is formed and converted to homocysteine, which can subsequently be re-methylated to methionine in a transmethylation reaction involving tetrahydrofolate and cobalamin. LC-MS/MS analysis of cells encoded with heavy methionine did not identify any peptides with methionine in its light form, indicating that the large excess of [$^{13}CD_3$]methionine from the culture medium reduces any potential contribution from re-methylated methionines to a negligible amount.

Quantification of protein methylation by heavy methyl SILAC

In heavy methyl SILAC, relative quantification of methylation in two cellular states is achieved by measuring the relative signal heights of methylated peptide pairs as in any approach that uses stable-isotope labeling for mass spectrometric quantification. To differentiate changes in methylation from changes in protein abundance, we additionally labeled cells with lysine-D4, resulting in labeling of all peptides containing lysine, methionine and/or methylated residues. Pairs of lysine- or methionine-containing peptides indicate the protein ratio, whereas the ratios of methylated peptides are the product of protein abundance and methylation change.

We mixed lysates from 'light' and 'heavy' labeled cells in known ratios and analyzed these proteins by MS (see Methods). The ratio of signal intensities from the 'heavy' methylated peptide form to the 'light' unmethylated peptide yields the quantitative ratio of protein methylation directly (**Fig. 3**). Methylated peptide pairs from hnRNP A2/B1, Ewing sarcoma breakpoint isoform (EWS) protein and polyadenylate-binding protein 4 (PABP-4) matched the 1:1 and 1:3 ratios of mixing as expected. From three separate immunoprecipitation experiments with 1:1 mixed lysates, we quantified 58 methylated peptides and found the average ratio to be 1.03 $\pm$ 0.17 (see **Supplementary Table 1** online). As expected, the quantitative accuracy of heavy methyl SILAC was equivalent to other SILAC experiments. These accuracies depend on the experiment and operating parameters, including instrument type. For instance, peptide pairs with high signal/noise ratios yield quantitative ratios with lower standard deviations. The variables that affect the quantitative precision achievable in SILAC have been described in greater detail elsewhere[21].

Increased confidence in identification of methylation sites

Proteomic analysis of protein methylation or methylated peptides is challenging from the perspective of database searching. The sheer number of mass modifications (monomethyl, dimethyl arginine, monomethylated, dimethylated, trimethylated lysine, among others) results in a larger number of false positives arising from the combinatorial increase in the number of possible peptide matches in the expanded search space. For example, a peptide

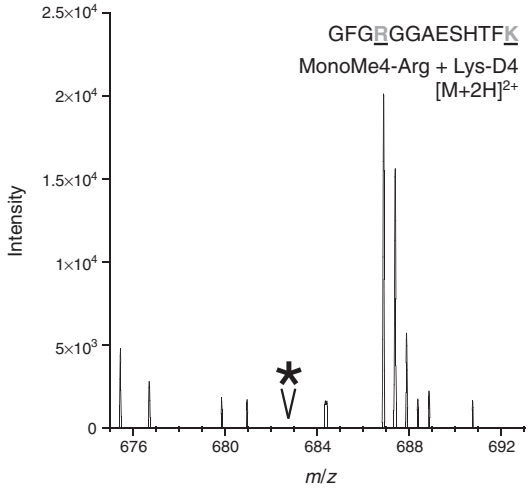

Figure 2 | Full incorporation of heavy methyls. Mass spectrum contains only the distinct peptide cluster from the peptide containing 'heavy' ($^{13}CD_3$) monomethyl arginine. The asterisk marks the position where the 'light' form of the peptide should occur if present.

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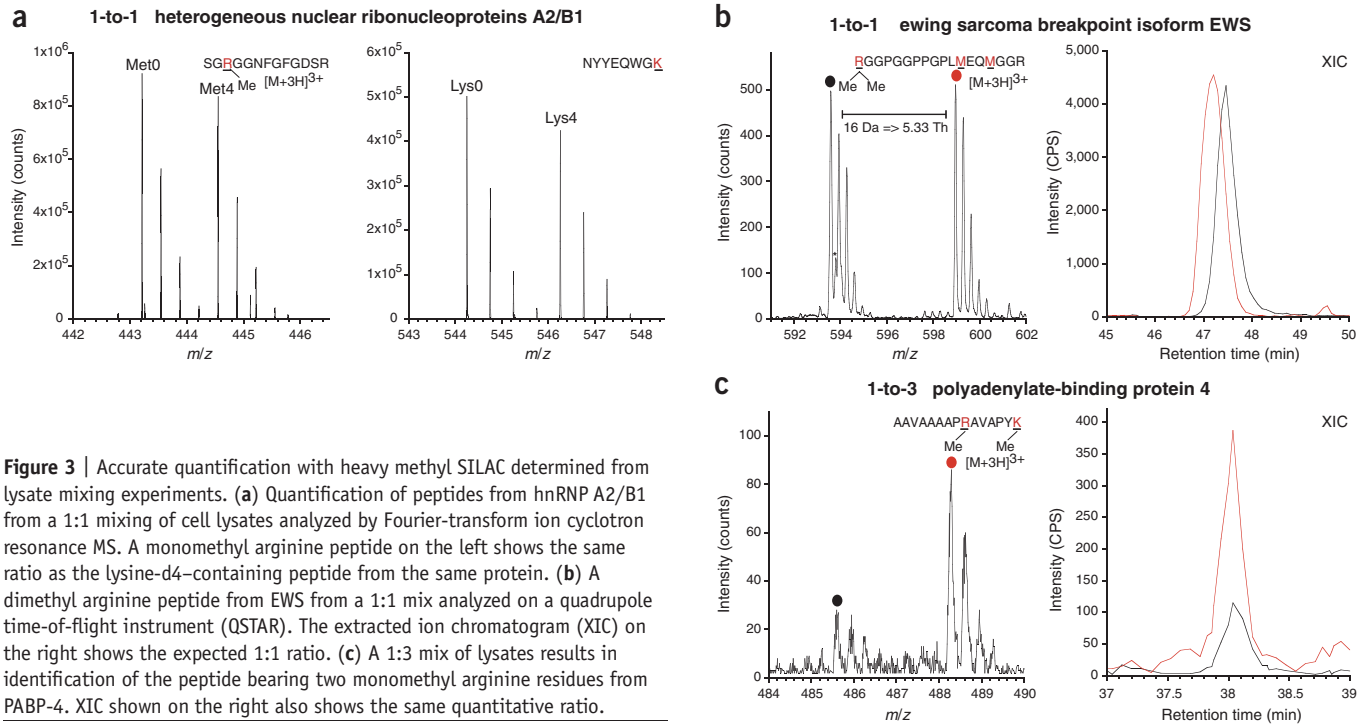

Figure 3 | Accurate quantification with heavy methyl SILAC determined from lysate mixing experiments. (**a**) Quantification of peptides from hnRNP A2/B1 from a 1:1 mixing of cell lysates analyzed by Fourier-transform ion cyclotron resonance MS. A monomethyl arginine peptide on the left shows the same ratio as the lysine-d4–containing peptide from the same protein. (**b**) A dimethyl arginine peptide from EWS from a 1:1 mix analyzed on a quadrupole time-of-flight instrument (QSTAR). The extracted ion chromatogram (XIC) on the right shows the expected 1:1 ratio. (**c**) A 1:3 mix of lysates results in identification of the peptide bearing two monomethyl arginine residues from PABP-4. XIC shown on the right also shows the same quantitative ratio.

containing methionine, arginine and/or lysine could exist in 24 different combinations (two methionine × three arginine × four lysine modifications). Additionally, trypsin does not cleave as efficiently C-terminally to methylated lysines or arginines, producing an even larger peptide search space. Consequently, fragmentation spectra have to be matched to a much larger list of theoretical sequences generated from these putative peptide matches, and identification confidence suffers.

Several amino acids have chemical structures that differ by a CH_2 group. This difference in mass of 14 Da would be indistinguishable from monomethylation. For instance, a glycine and methylated arginine on the same peptide would be identical in elemental composition to the same peptide with a single G→A substitution without methylation. We have found that database search software can mistakenly assign fragmentation spectra to methylated peptides, particularly if the amino acid substitution occurs close to the putative methylation site, and consequently, most of the fragments do not distinguish between the two possibilities. A substitution matrix (**Fig. 4**) indicates pairs of amino acids differing by masses close to multiples of $(CH_2)_n$. Because this only takes into account single-amino-acid substitutions, the actual number of potential amino acid substitutions leading to an increased false positive rate is much larger.

Heavy methyl SILAC circumvents all these problems by differentiating methylated peptides from unmodified ones. After mixing proteins from unlabeled and labeled samples, methylated peptides exist in pairs separated by the mass difference introduced by the methyl groups; thus, a dimethyl arginine pair would be separated by 2 × $^{13}CD_3$, or 8 Da (**Fig. 5**).

To test experimentally whether heavy methyl SILAC increases the ability to identify methylated peptides, we analyzed in depth the LC-MS/MS data from a single gel band. Among 2,743 MS/MS fragmentation events, 30 were assigned to methylated peptides with a Mascot score of ≥17 at a mass accuracy of <25 p.p.m. In this experiment, the statistical significance threshold was 28 when not considering methylation but 39 when including this modification, as a result of the vastly expanded search space described earlier. We then determined whether the peptides were indeed methylated by the presence or absence of an accompanying methylated peptide in the heavy form with the correct mass offset. Only 13 of the peptides were methylated and, notably, some of the wrongly assigned peptides had scores at or above the significance threshold of 29 (see **Supplementary Table 2** online). Conversely, methylated peptides with scores as low as 18 (below the significance threshold in the 'nonmodified' search), could be 'rescued' because of the presence of the confirming companion peak and independent identification of the same peptide sequence through fragmentation of that peak.

In these experiments, high mass accuracy helps not only by limiting the search space considered but also by identifying genuine peptide pairs. Additionally, high mass accuracy distinguishes between acetylation (monoisotopic mass 42.01056 Da) and trimethylation (monoisotopic mass 42.04695 Da) of lysines. For example, in the experiment just described, a modified peptide had a mass difference of 0.8 p.p.m. to the acetylated and 22 p.p.m. to the trimethylated form. However, in this case, the absence of a companion peak already revealed it to be acetylated, even without high-mass-accuracy data (see **Supplementary Table 2** online).

MS/MS spectra for both light and heavy peptides are acquired in most cases, depending on the complexity of the sample analyzed. This increases overall confidence in peptide identification, because the same peptide is identified from two independent fragmentation spectra. 'Overlaying' the two fragmentation spectra helps in unambiguously locating the actual sites of methylation, through the presence of 'doublet' ions arising from the tag[21]. Characteristic

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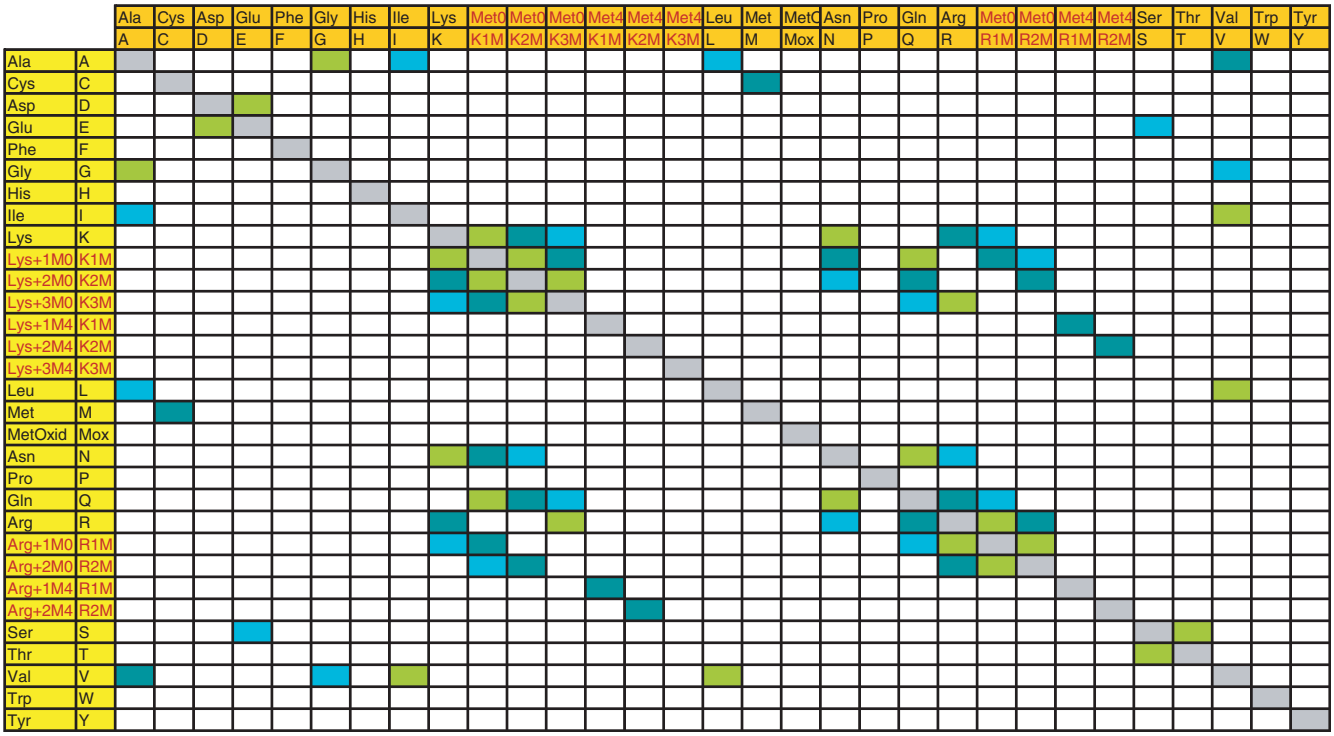

Figure 4 | $(CH_2)_n$ substitution matrix for amino acids. Mass differentials between single amino acids (including methylated residues) are calculated in a matrix. Filled boxes indicate a mass difference corresponding to a multiple of $(CH_2)_n$ within 0.05 Da. Olive boxes are ±14 Da, dark green boxes are ±28 Da, cyan boxes are ±42 Da and gray boxes are ±0 Da.

losses[16,17] that eliminate the methyl groups from the precursors (such as methylguanidine from monomethyl arginine) will lead to collapse of the fragments from the two precursors into a single peak (**Fig. 6**), providing information about the nature of the methylation. Characteristic fragment losses for light and heavy methyl groups are listed in **Table 1**.

Identifying *in vivo* methylation sites after antibody enrichment

The analysis of PTMs of proteins is not straightforward, because the overwhelming majority of peptides derived from a biological sample are unmodified and because modified peptides are present in substoichiometric amounts. Some degree of enrichment for modified peptides or proteins is therefore important. For example, phosphopeptides can be enriched by immobilized metal-affinity chromatography. However, because methylation does not alter the physicochemical properties of arginine or lysine residues to any great extent, we found that antibody-based enrichment of methylated proteins was necessary to observe methylated peptides. Antibodies recognizing proteins methylated on residues such as monomethyl and dimethyl arginine (ab412) and all three forms of methyl lysine (ab7315) were first evaluated in immunoprecipitation–western blotting experiments. This resulted in many distinct bands for the ab412 and an undefined staining for the ab7315 antibody (data not shown).

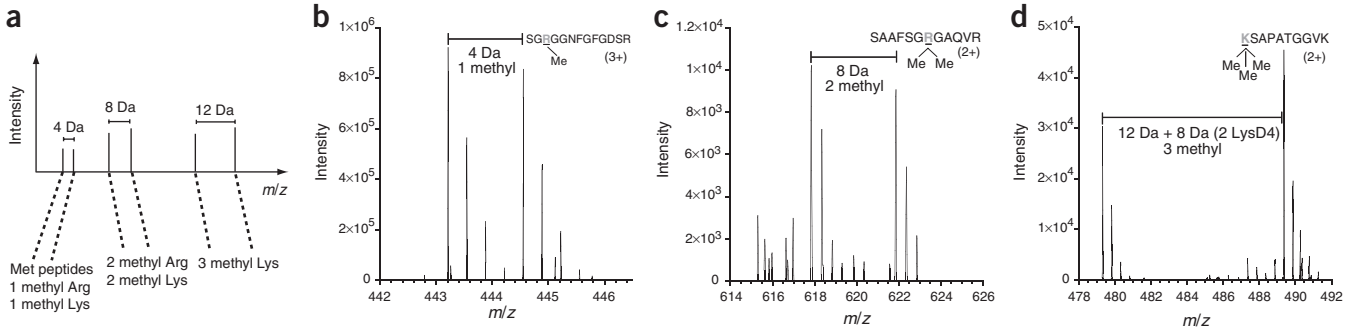

Figure 5 | Heavy methyl SILAC encodes methylated peptide pairs with a modification-specific mass tag. (**a**) The type of methylated residue can be identified on the basis of mass separation of the peptide pair. (**b**) A peptide with a single arginine methylation with a mass separation of 4 Da. (**c**) Dimethyl arginine peptide shows the corresponding mass separation of 8 Da. (**d**) Trimethyl lysine–containing peptide with two lysine-D4 (from SILAC-Lys-D4 labeling) is separated by a sequence-specified mass difference of 20 Da.

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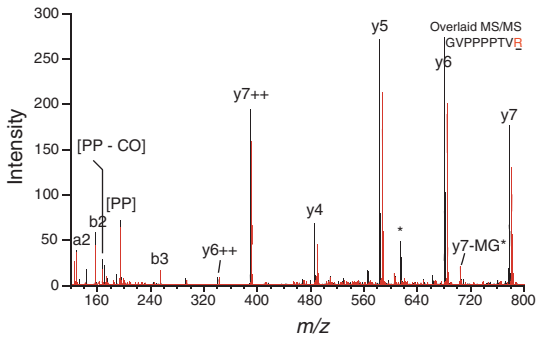

Figure 6 | Informative MS/MS spectra and characteristic fragment losses in heavy methyl SILAC. MS/MS spectra from 'light' and 'heavy' methylated peptide are overlaid, clearly specifying the position of the labeled methylated residue. The y-ion series is displaced by 4 Da, corresponding to the C-terminal position of the methylated arginine. The y_7-MG ion is a singlet resulting from the fragment loss of methylguanidine from y_7 (see **Table 1**). The abundant singly charged ion at *m/z* 195.10 in the low mass range originates from the internal fragment PP. A contaminating doubly charged ion peak with a larger mass than the precursor ion (GVPPPPTVR) at *m/z* 615.33 only present in the MS/MS spectrum of the unlabeled peptide is marked with an asterisk.

MS analysis resulted in a large number of identified proteins, suggesting only modest enrichment of methylated proteins. In a background of 1,952 proteins from HeLa S3 cellular lysates, we identified 58 methylated peptides, corresponding to 59 unique methylation sites in 33 different proteins (**Table 2**). We also made use of an antibody against the RNA-binding protein Sam68 to enrich this protein for a more targeted analysis of protein methylation. Sam68 methylation has previously been analyzed by [^{3}H]methionine labeling and matrix-assisted laser desorption/ionization analysis[4] or with precursor ion scanning[15]. We identified five methylated peptides from Sam68 from our pull-down experiment; two of these are already known, two fall within the region previously suggested to be methylated and the last one was new.

Table 2 lists methylation sites identified in these experiments. We found 14 arginine methylation sites on 10 different hnRNPs, a class of RNA-binding proteins known to be methylated in Arg-Gly-Gly motifs (RGG box[5]). These were primarily dimethylated arginine residues, although we found evidence for monomethylated arginines in hnRNP A2/B1 and U. Monomethylation within the RGG box is also known to occur but in much lower proportions. We also observed a previously undescribed arginine dimethylation site in the RGG box of fragile X mental retardation syndrome–related protein 1 (expected by sequence homology[22]). We further identified specific sites on methylated proteins, such as PABP-1 (ref. 23), the p90 subunit of NFAT[24] and Sm proteins[25], and three previously unknown methylation sites for EWS. Immunoprecipitation with pan-methyl lysine did yield known lysine methylation sites from histones but, notably, no other lysine methylation sites. It did, however, yield a considerable number of arginine methylation sites, suggesting that this polyclonal antibody cross-reacts to a considerable extent with arginine-methylated residues.

Table 1 | Characteristic neutral losses arising from heavy methyl SILAC–labeled methylated residues

Modification	Me0 (Da)	Me4 (Da)
Monomethyl arginine or symmetric dimethyl arginine (methylamine)	−31.0422	−35.0644
Monomethyl arginine (methylguanidine) or MG*	−73.0640	−77.0862
Asymmetric dimethyl arginine (dimethylamine)	−45.0578	−53.1022
Trimethyl lysine (trimethylamine)	−59.0735	−71.1401

Several types of protein methylation give rise to informative fragment losses in MS/MS spectra that can help confirm the form of methylation within the peptide. The y_7 doublet collapses to a single peak after the loss of methylguanidine (as seen in **Fig. 6**).

DISCUSSION

In traditional tritium methyl labeling, protein synthesis has to be inhibited with drugs to avoid background signals arising from incorporation of radioactive methionine. Analysis of protein methylation by MS has obvious advantages over cumbersome classical methods. Recently, antibodies to methyl arginine were used to enrich arginine-methylated proteins and associated proteins[26]. This identified >200 proteins by MS, but no actual methylation sites. Instead, these proteins were deemed to be methylated if they contained Arg-Gly repeats and were absent in a control pull-down experiment. Such an approach, though useful as a screen, would still require considerable effort for validation. Other MS-based investigations of methylation have focused on limited numbers of proteins and also did not provide quantification.

Heavy methyl SILAC allows unbiased identification and simultaneous quantification of protein methylation sites. Because quantification in SILAC is based on individual peptides, site-specific quantification of protein methylation is achieved by simply determining intensity ratios of methylated peptide pairs. These ratios can then be normalized against the protein ratio determined from unmethylated SILAC-labeled peptides (for example, Lys-D4 labeling). Heavy methyl SILAC is, to our knowledge, the first quantitative labeling approach that metabolically labels the PTM directly at the substituting group. This is particularly important in methylation analysis, because many different forms of methylation are known in proteins (for example, MMK, DMK, TMK, MMA, sDMA, aDMA), several of which do not give rise to methyl-specific fragment ions. We have shown that we can confidently identify and quantify methylated peptide forms based on the presence of the methyl-peptide pair, which flags methylated peptides.

The direct labeling of the PTM is distinct from previous strategies for labeling proteins or peptides, such as ^{15}N metabolic labeling to compare changes in the phosphoproteome[27], chemical approaches to differentially esterify phosphopeptides[28] or labeling the nonmodified residues of purified histones with deuterated acetic anhydride[23]. Although our discussion of this labeling strategy refers to analysis of proteolytic digests of methylated proteins, whole-protein methylation analysis with a 'top-down' approach (as is common with histone proteins[29]) would also benefit. Like other stable isotope–based strategies, heavy methyl SILAC allows relative quantification between two states. If absolute quantification is desired, peptide internal standards can be spiked into the sample.

The method is simple to use in standard cell culture, because it requires only cell cultivation in medium containing stable isotope–substituted amino acids. Although we have made no attempt to

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Table 2 | Methylation sites determined by heavy methyl SILAC

Accession no.	Protein name	Methylated peptide	Novel?	Site
Q13151	hnRNP A0	SNSGPYRGGYGGGGGYGGSSF + dimethylation (R)	Yes	R291
Q99729	hnRNP A/B	RGGHQNNYKPY + dimethylation (R)	Yes	R322
P09651	hnRNP core protein A1	QEMASASSSQRGR + dimethylation (R)	Known (by similarity)	R194
		KALSKQEMASASSSQRGR + dimethylation (R)	Known (by similarity)	R194
		ALSKQEMASASSSQRGR + dimethylation (R)	Known (by similarity)	R194
		SGSGNFGGGRGGGFGGNDNFGR + dimethylation (R)	Yes	R206
		GGNFSGRGGFGGSR + dimethylation (R)	Yes	R225
P22626	hnRNP A2 / hnRNP B1	SGRGGNFGFGDSR + monomethylation (R)	Yes	R261
Q14103	hnRNP D0	RGGHQNSYKPY + dimethylation (R)	Yes	R345
P38159	hnRNP G	APVSRGR + dimethylation (R)	Yes	R185
O14979	hnRNP JKTBP	ASRGGGNHQNNYQPY + dimethylation (R)	Yes	R408
Q07244	hnRNP K	RGPPPPPPGRGGRGGSR + 2 dimethylation (R)	Yes	R296,R299
		GPPPPPPGRGGRGGSR + 2 dimethylation (R)	Yes	R296,R299
Q8N174	hnRNP U	RGNMPQRGGGGGGSGGIGYPYPR + 2 dimethylation (R)	Yes	R714, R720
		LQAALDDEEAGGRPAMEPGNGSLDLGGDSAGR + monomethylation (R)	Yes	R50
Q8N3B3	Similar to hypothetical protein (49% similarity hnRNP U)	SRGQGYVGGQR + dimethylation (R)	Yes	R538
P43331	Small nuclear ribonucleoprotein Sm D3	SMKNKNQGSGAGRGK + dimethylation (R)	Yes	R97
P14678	Small nuclear ribonucleoprotein associated proteins B/B′	VPLAGAAGGPGIGRAAGRGIPAGVPMPQAPAGLAGPVR + 2 dimethylation (R)	Known	R108,R112
P23246	Splicing factor, proline- and glutamine-rich	SRGGGGGGFHR + dimethylation (R)	Yes	R9
		FRSRGGGGGGFHR + 2 dimethylation (R)	Yes	R7,R9
		RGGGGGRGGLHDFR + 2 dimethylation (R)	Yes	R19, R25
P35637	RNA-binding protein FUS/TLS	GGRGRGGSGGGGGGGGGGYNR + 2 dimethylation (R)	Yes	R215,217
		GGRGRGGSGGGGGGGGGGYNR + monomethylation (R); dimethylation (R)	Yes	R215,217
Q01844	RNA-binding protein EWS	GMPPPLRGGPGGPGGPGGPMGR + dimethylation (R)	Known	R471
		GSRGNPSGGGNVQHR + dimethylation (R)	Known	R506
		GPRGSRGNPSGGGNVQHR + 2 dimethylation (R)	Known	R503, R506
		RGGPGGPPGPLMEQMGGR + dimethylation (R)	Yes	R615
		RGGRGGPGKMDKGEHR + 2 dimethylation (R)	Yes	R633, R636
		RGGRGGPGKMDKGEHRQER + oxidation (M); 2 dimethylation (R)	Yes	R633, R636

Table 2 continued on following page

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exhaustively catalog the methyl proteome, our proof-of-concept experiment in HeLa cells resulted in a large number of new *in vivo* arginine methylation sites. The majority of sites detected with pan-methyl-specific antibodies were from proteins with roles in RNA binding. It is known that dimethyl arginines occur most frequently in Arg-Gly–rich regions and that these are common in RNA-binding regions. We also found that the currently available antibodies directed against all forms of methylation are not very specific. Note, however, that we did not test antibodies specific to particular forms of methylation—for example, asymmetric dimethyl arginine. The availability of better antibodies, similar to those targeted against phosphotyrosine, would allow more comprehensive study of protein methylation. Although our approach labels methylation throughout and is thus attractive for whole-proteome investigations, small subsets of proteins can be purified to greater homogeneity to provide a more directed analysis of specific methylation sites. In these focused studies, the emphasis must be on sequence coverage to allow the analysis of as many methylation sites as possible. Any improvements in these technological and practical problems, which are similar in all experiments with PTMs[14], would further enhance the usefulness of heavy methyl SILAC. Because the method is inherently simple, accurate and quantitative, it will be an important tool in unveiling the varied functions of protein methylation in living systems.

METHODS

Cell culture. SILAC labeling medium (RPMI 1640, Sigma-Aldrich) lacking lysine, leucine and methionine was reconstituted according to the manufacturer's instructions and supplemented with amino acid stocks prepared in PBS. To heavy medium, we added lysine-4,4,5,5-D4 and [$^{13}CD_3$]methionine (Sigma Isotec), and to light medium we added normal lysine and methionine (Sigma-Aldrich). We added 10% dialyzed FBS, glutamine, leucine and antibiotics to both light and heavy media. HeLa S3 cells (American Type Culture Collection) were cultured in suspension in heavy labeling medium for at least six cell doublings to allow adaptation and full incorporation of the stable isotope–containing amino acids. Cells were grown at 37 °C in a humidified atmosphere and 5% carbon dioxide in air.

Ratio mixing and immunoprecipitation experiments. Cells were harvested by centrifugation, washed twice with PBS and lysed in

Table 2 | Continued

Accession no.	Protein name	Methylated peptide	Novel?	Site
Q07666	Sam68	GAPAPRAR + dimethylation (R)	Yes	R346
		GVPPPPTVR + monomethylation (R)	Yes	R340
		GATVTRGVPPPPTVR + dimethylation (R)	Known	R331
		GRGAAPPPPPVPR + dimethylation (R)	Suggested (*mono*)	R291
		GAITRGATVTR + monomethylation (R)	Known	R325
Q86V81	Transcriptional coactivator Aly/REF	VNRGGGPIR + dimethylation (R)	Yes	R50
		NRGAGGFGGGGGTR + dimethylation (R)	Yes	R204
Q07955	Pre-mRNA splicing factor SF2, P33 subunit	GTGRGGGGGGGGGAPR + dimethylation (R)	Yes	R97
		SGRGTGRGGGGGGGGGAPR + monomethylation (R); dimethylation (R)	Yes	R93, R97
		GTGRGGGGGGGGGAPRGR + monomethylation (R); dimethylation (R)	Yes	R97, R109
Q12906	Nuclear factor of activated T cells-90, NF-AT-90	APVPVRGGPK + dimethylation (R)	Yes	R609
		RAPVPVRGGPK + dimethylation (R)	Yes	R609
Q13283	Ras-GTPase-activating protein–binding protein 1	GPGGPRGGLGGGMR + dimethylation (R)	Yes	R436
		GPPRGGMVQKPGFGVGRGLAPR + 2 monomethylation (R); dimethylation (R)	Yes	R447, R460, R465
Q9UN86	Ras-GTPase-activating protein–binding protein 2	GPPPRGGMAQK + dimethylation (R)	Yes	R457
		LGSGRGTGQMEGR + dimethylation (R)	Yes	R468
P51114	Fragile X mental retardation syndrome–related protein 1	RRPGGRGR + dimethylation (R)	Known (by similarity)	R445
P98179	Putative RNA-binding protein 3	SYSRGGGDQGYGSGR + dimethylation (R)	Yes	R105
P08729	Keratin, type II cytoskeletal 7	SAAFSGRGAQVR + dimethylation (R)	Yes	R19
P11940	Polyadenylate-binding protein 1	VANTSTQTMGPRPAAAAAAATPAVR + dimethylation (R)	Yes	R493
Q13310	Polyadenylate-binding protein 4	AAVAAAAPRAVAPYK + dimethylation (R)	Yes	R518
P17844	DEAD-box protein p68	RGGFNTFR + dimethylation (R)	Yes	R502
Q15815	Arginine/serine-rich splicing factor 10	SYRGGGGGGGGWR + dimethylation (R)	Yes	R141
Q8WWM7	Ataxin-2–related domain protein	VREGPRGGVR + dimethylation (R)	Yes	R361
Q92804	TATA-binding protein–associated factor 2N	GPMTGSSGGDRGGFK + dimethylation (R)	Yes	R203
Q9NZB2	Protein C9orf10	GPFPLQVVSVGGPARGRPR + dimethylation (R)	Yes	R982
Q16695	H3 histone	KSAPATGGVK + monomethylation (K)	Known	K27
		KSAPATGGVK + dimethylation (K)	Known	K27
		KSAPATGGVK + trimethylation (K)	Known	K27
P02304	Histone H4	KVLRDNIQGITKPAIR + dimethylation (K)	Known	K20

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modified RIPA buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris (pH 7.5), 1% Nonidet P-40 (NP-40), 0.25% sodium deoxycholate and protease inhibitors (Complete Tablet; Roche A/S). Lysates were kept on ice, vortexed intermittently for 15 min and centrifuged at 10,000*g*.

Lysates were mixed 1:1 and 1:3 (Bradford assay; Bio-Rad). Protein from $\sim 2 \times 10^7$ cells, or 2 mg protein was immunoprecipitated with either 100 µl of monoclonal antibody for monomethylated and dimethylated arginines (clone 7E6, ab412; Abcam) or rabbit polyclonal pan-methyl lysine antibody (ab7315; Abcam). Antibody-protein complexes were recovered by incubation with 100 µl protein G (with ab412)–agarose beads or protein A–agarose (with ab7315) (Sigma-Aldrich) on an end-over-end rotator at 4 °C for 6 h. The immunoprecipitates were washed twice with lysis buffer and separated on a Novex 4–12% Bis-Tris gel with the MOPS buffer system and stained with colloidal Coomassie blue (Novex; Invitrogen A/S).

For immunoprecipitation of Sam68, cell lysates from the same preparation were mixed 1:1. Polyclonal antiserum specific for Sam68 (rabbit; 250 µl; Upstate) was conjugated to 0.5 ml protein A–agarose beads and cross-linked according to standard procedures[30]. Approximately 5×10^7 cells were subjected to immunoprecipitation with the antibody for 8 h at 4 °C in a PolyPrep column (Bio-Rad). Beads were washed four times with 20 column volumes of lysis buffer, equilibrated in buffer containing 50 mM Tris-HCl (pH 6.8), 50 mM NaCl, 0.2 mM EDTA and 0.5 mM DTT, and eluted with 2.5 ml buffer containing 50 mM Tris-HCl (pH 6.8), 6 M urea, 5% glycerol and 0.05% NP-40 for 1 h at room temperature. Eluted material was concentrated 25-fold (Centriprep Y3K centrifugal filter; Millipore), resolved by 10% (30:1) SDS-PAGE and stained with colloidal Coomassie blue.

MS analyses. The gel lane was divided into ten slices for 'GeLCMS'. For Sam68, the band corresponding to its approximate molecular weight was excised. Processing of the gel bands was essentially as described[21,31,32]. Nanoscale LC (Agilent 1100; Agilent Technologies Denmark A/S) was coupled to a quadrupole time-of-flight instrument (QSTAR-Pulsar; ABI-MDS-SCIEX) or a hybrid 7-Tesla linear ion-trap Fourier-transform ion cyclotron resonance mass spectrometer (LTQ-FT; Thermo Electron), both equipped with nanoelectrospray ion sources (Proxeon Biosystems) as described[21,32]. Linear gradient elution was from 95% buffer A (H_2O–acetic acid, 100:0.5, v/v) to 50% buffer B (H_2O–acetonitrile–acetic acid, 20:80:0.5, v/v) with a flow rate of 250 nl/min in 60 min.

MS/MS peak lists were converted into Mascot-compatible peak lists and searched with the Mascot software package (Matrix

Science) against the human International Protein Index protein sequence database (IPI, version 2.29, EBI, http://www.ebi.ac.uk/IPI/), to which we added frequently observed contaminants. Tryptic enzyme specificity with up to five missed cleavages was applied to all searches. Because Mascot allows a maximum of nine variable modifications to be searched concurrently, we set carbamidomethyl cysteine as a fixed modification. Normal oxidized methionine (MetOx-0), heavy methionine (Met-4), heavy oxidized methionine (MetOx-4), lysine-D4, monomethyl arginine, monomethyl(Met-4) arginine, dimethyl arginine, dimethyl(Met-4) arginine were searched as variable modifications for searches for arginine methylation. In methyl lysine searches, normal oxidized methionine (MetOx-0), heavy methionine (Met-4), Lys-D4, monomethyl-Lys, dimethyl-Lys, dimethyl(Met-4)-Lys-D4, trimethyl-Lys and trimethyl-LysD4 were set as variable modifications. Initial mass tolerances for protein identification of MS and MS/MS peaks with QSTAR-Pulsar data were both 0.2 Da, and 5 p.p.m. and 0.8 Da for LTQ-FT data, respectively. Peptide identifications were verified using software developed in house (http://msquant.sourceforge.net).

Our criteria for assignment of a methylation site were as follows: (i) presence of two peptide peaks of equal intensity (1:1 mixing) with the expected mass difference introduced by methyl groups, methionine and/or lysine residues; (ii) MS/MS spectra acquired from both peptide forms supporting the identification with major fragment ions assigned to the sequence. Precise masses and retention times were then used to obtain extracted ion chromatograms for quantification of peptide pairs.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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