Comprehensive identification of arginine methylation in primary T cells reveals regulatory roles in cell signalling

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The impact of protein arginine methylation on the regulation of immune functions is virtually unknown. Here, we apply a novel method—isomethionine methyl-SILAC—coupled with antibody-mediated arginine-methylated peptide enrichment to identify methylated peptides in human T cells by mass spectrometry. This approach allowed the identification of 2,502 arginine methylation sites from 1,257 tissue-specific and housekeeping proteins. We find that components of T cell antigen receptor signal machinery and several key transcription factors that regulate T cell fate determination are methylated on arginine. Moreover, we demonstrate changes in arginine methylation stoichiometry during cellular stimulation in a subset of proteins critical to T cell differentiation. Our data suggest that protein arginine methyltransferases exert key regulatory roles in T cell activation and differentiation, opening a new field of investigation in T cell biology.
Post-translational modifications (PTMs) govern cellular homeostasis and responses to changes of internal and external conditions. Thus, knowledge of the type and extent of PTMs in tissue proteomes should provide more exhaustive insights into physiological and pathophysiological mechanisms. Comprehensive mass spectrometry (MS)-based studies on highly reversible PTMs, such as protein phosphorylation and ubiquitination, have already revealed regulation of cellular signalling pathways correlating with physiological or pathological settings. However, other PTMs have been more difficult to tackle at a global scale, such as protein arginine methylation, thought to be rather permanent. In higher eukaryotes, protein arginine methylation can occur symmetrically or asymmetrically at the arginine side chain guanidino group and is mediated by at least nine different arginine methyltransferases (PRMTs). Methylation reduces the number (up to five) of arginine hydrogen bond donors weakening interactions in protein–protein and protein–nucleic acid complexes, potentially generating differential binding preferences. However, arginine-rectio-heavy isobaric may be favoured by methylation as suggested for Tudor domain binding to symmetrically methylated arginine sites. Mice deficient for PRMT1, PRMT4 or PRMT5 show embryonic or perinatal lethality, demonstrating the importance of this PTM. Arginine methylation is an epigenetic histone modification and impacts on transcription and DNA-repair but the extent and potential plasticity of this PTM in cellular functions remains unclear. Initial MS-based proteomics investigations have beenm by inefficient enrichment for arginine-methylated peptides. Moreover, confident identification of methylated sites in complex mixtures has been problematic due to the increased search space when matching fragmentation spectra, as several amino acid substitutions are isobaric to methylation. The elegant heavy methyl-SILAC labelling strategy by Ong et al. increased confidence in identification of methylated peptides, but it did not allow the discrimination between methylated and methionine-containing peptides.

Primary T cells from human blood are ideal tissues to investigate at proteomics scale the extent and impact of PTMs in normal and pathological cellular processes. Cell subsets can be readily purified in sufficient amounts with minimal manipulation and stimulated to differentiate in vitro. Thus, for example, resting naive or memory T cells can be induced by appropriate stimuli mimicking in vivo conditions, to turn into the effector cells that fend off microbial pathogens or tumours but also into T cells that initiate or control inflammatory responses. The central role played by T cells in autoimmune inflammation and make them an ideal target for monitoring alterations of PTM signatures in diseased individuals. T cells appear to be sensitive to perturbations of arginine methylation as T cell development is blocked in PRMT4-null embryos and earlier studies indicated that arginine methylation augments substantially during T cell activation.

Here, we use isomethionine methyl-SILAC (iMethyl-SILAC), an improved procedure to exclusively detect methylated peptides, different proteases and anti-mono-methylated arginine antibodies (Abs) recently described that effectively enrich for arginine-methylated peptides. When applied to Jurkat T cells and TCR/CD28-stimulated primary T cells, this comprehensive approach allowed us to identify the largest number of arginine methylation sites and proteins known to date implicating PRMT action in most, if not all cell functions, including TCR-proximal signalling and cell fate programs. Furthermore, we demonstrated that arginine methylation stoichiometry changes during cell differentiation and show this to occur in mRNA splicing factors critical in T cell differentiation.

Results
Discovery of arginine methylation sites using iMethyl-SILAC. In heavy methyl-SILAC, cells are labelled with \( ^{15} \text{N} \)-Methionine or \( ^{13} \text{C},^{15} \text{N} \)-Methionine. Presence of a 1:1 methyl-SILAC pair in the precursor scan corroborates the assignment of the fragmentation spectrum to a methylated peptide. However, because the light or heavy methionine is incorporated into proteins, peptides containing methionine will also generate 1:1 methyl-SILAC pairs in precursor scans. To eliminate this ambiguity, we designed an improved labelling strategy, replacing \( ^{15} \text{N} \)-Methionine with \( ^{13} \text{C},^{15} \text{N} \)-Methionine (Fig. 1a). The two stable isotope-labelled methionines are nearly isobaric but differ in the distribution of the additional mass; we, therefore, termed this labelling strategy isomethionine methyl-SILAC (iMethyl-SILAC). The methyl groups transferred during protein methylation are still either light or heavy, but methionines incorporated during protein synthesis are nearly isobaric. As a result, methyl-SILAC pairs only arise from methylated peptides. To demonstrate the specificity and efficacy of iMethyl-SILAC, Jurkat T cells were labelled according to the heavy methyl-SILAC and iMethyl-SILAC strategies. Arginine-methylated peptides were immuno-affinity purified using mAbs DSA12 or M6R4100 raised against arginine mono-methylated sites in the context of various sequence contexts followed by LC-MS/MS (liquid chromatography-tandem mass spectrometry) analysis. When used in HeLa cells or HCT116 (ref. 21) cell extracts, these Abs were recently shown to effectively enrich for methylated peptides, which resulted in the identification of 1,000–1,700 arginine methylation sites. As expected, heavy methyl-SILAC labelling produced methyl-SILAC pairs from methionine-containing peptides and methylated peptides that were indistinguishable (Fig. 1b, top). In contrast, iMethyl-SILAC labelling meant that only methylated peptides occurred as methyl-SILAC pairs, providing unambiguous corroboration (Fig. 1b, bottom) and contributing to reduced the false discovery rate (FDR, Fig. 1c).

We then performed a comprehensive analysis of arginine methylation using Jurkat T cells and peripheral blood lymphocyte-derived T cells from normal human donors that were activated with anti-CD3 and -CD28 Abs to allow iMethyl-SILAC labelling. Total cell extracts from Jurkat and primary T cells were digested with either one of three different proteases (Fig. 2a) and protein digests subject to immuno-affinity enrichment of methylated peptides using DSA12 or M6R4100 mAbs. LC-MS/MS detected a total of 82,338 methyl-SILAC pairs matched to arginine-methylated peptides, at an FDR of 0.93% (Fig. 2b). At the minimum accepted identification threshold (iProphet probability 0.25), iMethyl-SILAC led to a sixfold reduction in FDR when compared with label-free identification of methylation sites (Fig. 1c). When comparing iMethyl-SILAC and methyl-SILAC labelling, the FDR was further reduced, although to a lesser extent (Fig. 1c). Most of the methylated peptides were sequenced multiple times, which corresponded to 2,031 and 1,411 unique arginine methylation sites identified in Jurkat T cells and in primary T cells, respectively (Supplementary Fig. 1a and see below). Since an overlap of 940 methylation sites between Jurkat T cells and primary T cells was found (Supplementary Fig. 1a), together the two cellular sources gave a total number of 2,502 unique arginine methylation sites (Supplementary Data 1) derived from 1,257 unique proteins (Supplementary Data 2). This is the highest number of arginine methylation sites and proteins identified to date. Enrichment with the M6R4100 and DSA12 antibodies led to the identification of 1,939 and 1,421 unique sites, respectively (Supplementary Fig. 1b). On average, 11 or 6.7% of immuno-affinity purified peptides from primary T cells or Jurkat T cells, respectively, were arginine methylated (Supplementary Fig. 2), an approximate 560-fold enrichment as...
The RXR methylation motif was observed as a consensus outlier. Consensus outliers were previously noticed when methylated arginine. Proline at position (arginine methylation site predictions, although a sizable portion with another tyrosine at was significantly overrepresented (Supplementary Fig. 1c). Reflecting the Abs specificity, the detection of 355 additional sites not found using trypsin with trypsin, chymotrypsin and GluC led to the identification of with mix 1:1 digest mass spectrometry. Methionine-containing peptides and methylated peptides are observed as indistinguishable methyl-SILAC pairs when cells are labelled by heavy methyl-SILAC (top). When cells are labelled by isomethionine methyl-SILAC, only methylated peptides give rise to methyl-SILAC pairs (bottom). Without methyl-SILAC, the FDR is lowered rapidly to unacceptable levels as the minimum accepted iProphet probability is lowered (red line). By requiring a hit to be corroborated by a methyl-SILAC pair, the FDR is lowered in heavy methyl-SILAC-labelled samples (blue line) and further lowered in iMethyl-SILAC-labelled samples (green line).

Figure 1 | Isomethionine methyl-SILAC (iMethyl-SILAC) strategy. (a) Methionine is incorporated into proteins and also converted to the methyl group donor S-adenosyl methionine. Isotope-labelled methionines are nearly isobaric, but the distribution of label differs, therefore only methylated peptides will be observed as methyl-SILAC pairs. (b) Isomethionine methyl-SILAC labelling removes ambiguity arising from methionine-containing peptides. Methionine-containing peptides and methylated peptides are observed as indistinguishable methyl-SILAC pairs when cells are labelled by heavy methyl-SILAC (top). When cells are labelled by isomethionine methyl-SILAC, only methylated peptides give rise to methyl-SILAC pairs (bottom). (c) False discovery rate (FDR) among identified arginine-methylated peptides as a function of peptide identification confidence (iProphet probability). Primary T cells were labelled by heavy methyl-SILAC or iMethyl-SILAC. Without corroboration by methyl-SILAC pairs, the FDR rises rapidly to unacceptable levels as the minimum accepted iProphet probability is lowered (red line). By requiring a hit to be corroborated by a methyl-SILAC pair, the FDR is lowered in heavy methyl-SILAC-labelled samples (blue line) and further lowered in iMethyl-SILAC-labelled samples (green line).

Arginine methylation is implicated in T cell fate decisions. In line with previous work, arginine-methylated proteins found in this study were implicated in regulating transcription (> 200), post-transcription and translation (> 230 in mRNA processing proteins, including eight proteins involved in RNA-mediated silencing) and chromatin re-modelling. Indeed, several components of histone modification machineries (for example, HAC, HDAC, lysine methyltransferases, JMJ) were found arginine methylated. As previously noted, actin- and tubulin-mediated cytoskeleton re-modelling and intracellular protein transport, including nuclear pore components were also significantly enriched (170 proteins) among the identified methylated proteins (Fig. 2c). A novelty was the presence of 60 housekeeping enzymes (including several from mitochondria) involved in various metabolic pathways and ion channels. However, most surprising was to find arginine methylation sites in a relatively surprising was to find arginine methylation sites in a relatively

Calculated from the 0.012% of arginine-methylated peptides present in Jurkat T cell lysate (Supplementary Fig. 2). Digestions with trypsin, chymotrypsin and GluC led to the identification of 2,147, 497 and 274 sites, respectively, with the latter two allowing detection of 355 additional sites not found using trypsin (Supplementary Fig. 1c). Reflecting the Abs specificity, the majority of methylation sites were identified as MMA (Supplementary Fig. 1d), which is considered to be an intermediate methylation state towards di-methylation4,15. Using pLOGO23, we confirmed that arginine-methylated sites commonly occur in glycine-rich sequences (Supplementary Fig. 3). However, prolines were also overrepresented in close proximity to methylation sites (Proline Rich Arginine Methylation—PRAM—motifs)15, particularly at positions –1 and +4 relative to the methylated arginine. Proline at position +4 (n = 380) was associated with a high occurrence of methionine and phenylalanine at position +1. The RXR methylation motif was observed as a significant overrepresentation of arginine at +2. Tyrosine at –4 was significantly overrepresented (n = 88) and was associated with another tyrosine at +3. These findings should improve arginine methylation site predictions, although a sizable portion (n = 675, 27%) of methylated sites substantially deviated from those motifs. Consensus outliers were previously noticed when methylated peptides were selected on the basis of hydrophilicity or negative charge content15. Moreover, some histone arginine methylation sites were not glycine-rich or PRAM motifs (Supplementary Data 1). These findings suggest that PRMT catalytic sites may be quite adaptable or that some PRMTs have propensity to methylate non-glycine-rich and non-PRAM sites and shows that the anti-monomethyl-arginine Abs used here are efficient at detecting a wide range of methylation sites.
methylated peptides are identified by mass spectrometry and matched to methyl-SILAC pairs. (GluC. Peptides are desalted and methylated peptides are enriched by immuno-affinity purification using D5A12 or MeR4100 antibodies. Putative Isomethionine methyl-SILAC-labelled Jurkat or primary T lymphocytes are combined 1:1 and lysed. Proteins are digested with trypsin, chymotrypsin or of interferon-γ, the hallmark Th1 inflammatory cytokine. Eomesodermin, a paralogue of TBX21, is induced upon stimulation of naive CD8 T cells, and together with RUNX3 and TBX21 lead to expression of IFN-γ, perforin and granzyme B. RUNX1 is expressed in Th2 cells and helps promote Th17 cell differentiation by up-regulating expression of RORγt, the Th17-signature transcription factor. FoxP3, the master transcription factor required for regulatory T cell (Tregs) development so that these cells can limit autoimmunity and inflammation, cooperates with NFAF1, cRel and RUNX1 (ref. 18). Moreover, NOTCH1, Ikaros and Pax5 determine T and B cell fate at an early developmental stage but also during antigen stimulation. Interestingly, we observed that none of the arginine methylation site of those factors fell within their respective DNA binding domains, suggesting that they may rather regulate domains that interact with various protein partners, which may be the case for RUNX1, RUNX3 and FOXP3 whose methylation sites were found at C-and N-regions moieties (Supplementary Data 3), respectively, previously mapped to interact with transcription partners.

**Differential arginine methylation during T cell activation.** We then set out to ask whether T cell activation induces changes in arginine methylation in general, as previously suggested, and whether methylation sites undergo stoichiometry changes, as such sites would likely be important to T cell functions and differentiation (Fig. 3a). Primary CD4+ T cells from human blood were stimulated and labelled by conventional SILAC so that methylated and non-methylated peptides could be quantified.
Proteins showing changes in arginine methylation occupancy during T-cell stimulation. Proteins are coloured according to the change in arginine methylation occupancy during primary T cell differentiation. Thirty-seven per cent (134/365) of arginine-methylated peptides significantly increase (red) or decrease (green) in occupancy (right). (a) Differential arginine methylation during T-cell differentiation. Thirty-seven per cent (134/365) of arginine-methylated peptides significantly increase (red) or decrease (green) in Heavy/Light (H/L) ratio during stimulation of T cells (left). After normalizing for changes in corresponding protein levels, >10% (27/282) of arginine-methylated peptides significantly increase (red) or decrease (green) in occupancy (right). (c) Interaction network of proteins showing changes in arginine methylation occupancy during T-cell stimulation. Proteins are coloured according to the change in arginine methylation occupancy, where a protein contains multiple sites, each is coloured individually.

Figure 3 | Quantifying changes in arginine methylation occupancy during primary T cell differentiation.

Discussion

Arginine methylation is still commonly regarded as a rare protein modification that has attracted limited attention in cell biology. The moderate interest for this PTM may be primarily due to the scarcity of PRMT substrates and sites known (SWISSProl reports so far less than a 100 arginine-methylated proteins); to its intrinsic stability, which is seen as incompatible with a role in regulating cellular pathways, as compared with intrinsically dynamic protein modifications (for example, phosphorylation, ubiquitination, acetylation); the belief that mostly generic cellular processes are regulated by PRMTs, as only a few pioneering functional studies exist so far on the subject; and that detection of arginine methylation is technically very demanding and may carry unacceptable uncertainty for MS-based PTM identification. Our work demonstrates the widespread and dynamic occurrence of arginine methylation in primary cells and dispels earlier impressions, it can now be considered a PTM of major importance. Pan-Abs with specificity for arginine-methylated

To maintain high confidence, methylated peptides were only accepted if they had also been identified in previous experiments by iMethyl-SILAC. We quantified 365 unique arginine-methylated peptides before and after stimulation of T cells, corresponding to 319 distinct arginine methylation sites in 202 proteins (Fig. 3b, left and Supplementary Data 4). A total of 134/365 (37%) peptides were altered in abundance as a result of stimulation. Because stimulation of T cells induces widespread changes in protein expression, non-methylated peptides remaining after IAP were fractionated by HILIC and analysed by MS to quantify changes in protein expression during stimulation. Changes in levels of methylated peptides were normalized to changes in protein expression to derive fold changes in occupancy of arginine methylation sites. This revealed that 27/282 (9.6%) arginine-methylated peptides did present altered methylation stoichiometry as a result of cellular stimulation (Fig. 3b, right and Supplementary Data 5), strongly suggesting arginine methylation-mediated dynamic regulation of a protein function, a notion that has not been convincingly explored before. Although the number of site-specific changes in stoichiometry is likely to be an underestimate, the data indicate that a select group of proteins undergo changes in methylation occupancy during T cell differentiation. Among the methylation sites showing altered occupancy, a large proportion were found in proteins involved in mRNA splicing, some of them known to interact with each other in large complexes (Fig. 3c), and are candidates for regulating important T cell functions during differentiation. In particular, SFPQ, which mediates TCR-signal induced alternative splicing of CD45 pre-mRNA, contains two carboxy (C)-terminal arginine methylated sites, which decrease in occupancy during T cell stimulation. These sites occur in the region known to interact with THRAP3 and are close to a phosphorylation site (Thr687), which also reduces in occupancy during T cell stimulation, allowing release of THRAP3 and association with CD45 pre-mRNA. Changes in arginine methylation in the same region may modulate the splicing activity of SFPQ in response to TCR signals. PRMT4 was up-regulated during T cell stimulation and is known to methylate numerous splicing factors and influence patterns of alternative splicing (Supplementary Fig. 4).
proteins/peptides largely independent of sequence context were recently generated\(^\text{20}\). They allowed considerable selection of methylated over non-methylated peptides (>500-fold purification, in this work), that together with iMethyl-SILAC labelling, development of software for methyl-SILAC pair matching (see Methods), use of multiple search algorithms and the use of different proteases made possible in our study a significant increase in the number of known PRMT substrates. Our work extends and improves upon very recent investigations significant increase in the number of known PRMT substrates.

The use of primary immune cells extended not only the PRMT substrates implicated in housekeeping functions and diverse cellular pathways but revealed that PRMTs control several T-cell-specific functions. Thus, our and other recent studies\(^{21,22}\) open a new area of investigation to test in mouse models (for example, arginine mutations) and humans (searching for mutations-causing disease at methylation sites) the in vivo relevance of arginine methylation for cell fate decisions and hematopoietic lineage diseases. Master transcription factors govern differentiation often by associating in the same or different cell subsets with different sequence-specific transcription factors, co-activators and co-repressors building up transcriptional landscapes that favour a particular lineage while repressing expression of an alternative one. Such a behaviour requires structural flexibility likely conferred by a code composed of combinations of PTM\(^5\), similar to histone modifications\(^7\), but also progressive stabilization towards more differentiated phenotypes that can be more permanently conferred by stable PTM\(^5\). Progressive changes or de novo methylation in proteins during differentiation programs may represent a relatively stable PTMs\(^5\). Progressive changes or de novo methylation in proteins during differentiation processes previously observed\(^{20,38}\) is not only due to de novo protein expression of methylated proteins, but also to changes in stoichiometry. Thus arginine methylation can be dynamic, at least at the time scale of differentiation processes taking several hours or days as shown here. It will be interesting to investigate by our comprehensive approach alterations of these pathways that may lead to immune and inflammation pathologies. Together with recent similar investigations\(^{21,22}\), the present work raises arginine methylation to the rank of other more well-known PTMs, such as acetylation, phosphorylation and ubiquitination\(^{2,3,39}\).

**Methods**

**Labelling of cells by SILAC.** For SILAC labelling of cells, RPMI 1640 lacking t-methionine, t-arginine and t-lysine (Dundee cell products) was supplemented with 10% dialysed fetal bovine serum (GIBCO) and for methyl-SILAC labelling either: (a) t-methionine, (b) t-arginine and t-lysine (Sigma Isotec), (c) t-methionine-

methyl-\(^{13}\)C\(_3\) (Sigma) all at 0.1 mM together with 0.29 mM t-arginine and 0.219 mM t-lysine. For conventional SILAC, 1 mM methionine, 0.29 mM t-arginine-\(^{13}\)C\(_6\)-N\(_2\) and 0.219 mM t-lysine-\(^{13}\)C\(_6\)-N\(_2\) (Cambridge Isotope Laboratories) were used. Jurkat cells were cultured at 37°C for five to seven cell doublings in a humidified 5% CO\(_2\) atmosphere. Human primary CD4 + T lymphocytes were isolated from the blood of healthy donors by negative selection using a Dynal isolation kit (Life Technologies) according to the manufacturer’s protocol. For methyl-SILAC labelling, primary T lymphocytes were cultured with 2 μg ml\(^{-1}\) phorbol 12-myristate 13-acetate (Sigma) and 50 ng ml\(^{-1}\) IL-2 (AbD Serotec). For methyl-SILAC labelling, primary T lymphocytes were cultured for 7-9 days at 37°C in a humidified 5% CO\(_2\) atmosphere, medium was supplemented with 100 U penicillin and 100 μg ml\(^{-1}\) streptomycin (PAA). Methyl-SILAC-labelled cells were mixed 1:1 before collecting. Cells were collected, washed with phosphate-buffered saline and snap-frozen.

**Enrichment of arginine-methylated peptides.** 100 × 10\(^6\) Jurkat cells or 200 × 10\(^6\) primary T lymphocytes were lysed in 500 μl 8 M urea in 20 mM Tris pH 8 at room temperature (RT) for 15 min. Lysates were sonicated four times with a micro-tip sonicator set at 10 W and centrifuged at 20,000 g for 15 min at RT. The supernatant was collected and proteins reduced with 5 mM DTT for 30 min, then alkylated with 15 mM chloroacetamide for 30 min at RT. Protein was quantified using the Bradford dye binding assay. In the unstimulated-stimulated comparison, for each donor T cells were pooled and lysed for each biological and technical replicate, giving a total of 400 × 10\(^6\) unstimulated cells per peptide. An equal number of stimulated cells from the same donors were lysed and protein from unstimulated cells was mixed with stimulated cells 1:1. A total of nine donors were used, divided into three biological replicates. Urca was dialysed to 2 M (trispyridylsulphonato) ceric (II) (MTPS) (Cerlane) or 1 M (trispyridylsulphonato) ceric (II) (TCPS) (Cerlane). One micromolar of the tryp tic digests. Peptide/protein ratios were in the range 1:100–1:150. Proteins were digested overnight at 37°C. Digests were acidified to 0.1% TFA and centrifuged at 2,000 g for 5 min. Each 10 mg of peptides was desalted using 500 μg Sep-Pak C18 cartridges (Waters), eluting with 40% acetonitrile 0.1% TFA. Peptides were lyophilized and each 10 mg was resuspended in 1.3 ml imidazole-acetate buffer (IAB) (50 mM MOES, 10 mM Na\(_2\)PO\(_4\), 50 mM NaCl, pH 7.2). For each IAP, 200 μg DS15A antibody (CST # 8015, Cell Signalling Technology, Danvers, MA) or 270 μg MxR410 antibody (CST # 8711, Cell Signalling Technology, Danvers, MA) was bound to 80 μl protein A agarose beads (Roche) for 3 h at RT. For unstimulated-stimulated IAPs and IAPs before strong cation exchange (SCX), 100 μg DS15A and 135 μg MxR4100 were used in combination. Resuspended peptide were centrifuged at 20,000 g for 5 min, added to the beads and rotated overnight at 4°C. Beads were washed three times with 1 ml IAP buffer and once with water. Peptides were eluted with 2 × 0.15% TFA and passed through a 1.6 μm pore-size glass microfiltration plug. For SCX fractionation, peptides were dried and resuspended in 10 mM KH\(_2\)PO\(_4\), 20% acetonitrile, pH 2.7. SCX was performed in tips packed with SCX phase (Empore) using elutions of 30 mM, 50 mM, 70 mM, 100 mM, 160 mM, 350 mM, 500 mM KCl in 10 mM KH\(_2\)PO\(_4\), 20% acetonitrile pH 2.7. Peptides were desalted with C18 (Empore)-packed tips and dried.

**HILIC fractionation.** Supernatant remaining after immuno-affinity purification of arginine-methylated peptides from unstimulated-stimulated cells was desalted using 500 μg Sep-Pak C18 cartridges (Waters). Peptides were dried, resuspended in 85% acetonitrile, 0.1% formic acid and applied to a HILIC column (ZIC HILIC 150 × 4.6 mm, 3.5 μm, 200 Å, Merck). Fractionation was performed at 0.5 ml min\(^{-1}\) with buffer A (0.1% formic acid) and buffer B (95% acetonitrile, 0.1% formic acid) as follows: 0–4.5 column volumes gradient to 40% buffer B, 4.5–14.5 column volumes gradient to 40% buffer B, 14.5–17 column volumes 40% buffer B, 17.1–20 column volumes 90% buffer B.

**Mass spectrometry data acquisition.** Peptides were resuspended in 0.1% TFA and analysed on an Ultimate RSLC nano (Dionex) system run in line with a Q Exactive mass spectrometer (Thermo Scientific). Peptides were resolved on a C18 reverse phase 50 cm × 75 μm Easy-Column (Thermo Scientific) using a linear gradient of 5–44% Buffer B (80% acetonitrile, 0.1% formic acid) at 300 μl min\(^{-1}\) over 130 min. The mass spectrometer was operated in a ‘Top 10’ data-dependent acquisition mode with dynamic exclusion enabled (40 s). Survey scans (mass range 300–1,650 Th) were acquired at a resolution of 70,000 at 200 Th with the 10 most abundant multiply charged (±2) ions selected with a 3-Th isolation window for HCD fragmentation. MS/MS scans were acquired at a resolution of 17,500 at 200 Th.

**Mass spectrometry data analysis.** MS/MS spectra were de-isotoped and charge deconvoluted with Progenesis ClearSpc (Nonlinear Dynamics). The .mgf files were uploaded to the UTSouthwestern Central Proteomics Facility Pipeline\(^{40}\) and searched against a concatenated and reversed decoy UniProt human database with OMSSA, X! Tandem native scoring and X! Tandem k-score. Cysteine carbamidomethylation and arginine and t-lysine N-methylations were set as fixed modifications. Acetylation of protein amino (N)-termini, methionine oxidation, light monomethylarginine, light dimethylarginine, heavy
monomethylarginine, heavy dimethylarginine were set as variable modifications. Precursor mass tolerance was 20 p.p.m., fragment mass tolerance was 0.1 Da. Localization of arginine methylation was performed using ModLS. A maximum of three mis-cleavages were allowed for tryptic specificity, two mis-cleavages were allowed for chymotrypsin and GluC. FDR was set to 5% at the peptide level and results filtered for arginine-methylated peptides. Raw data files were searched for matching methyl SILAC pairs using a programme developed in-house. MethylQuant (programme available on request). Peptides were retained if they satisfied the following criteria: a matching methyl SILAC pair at a 1:1 intensity ratio, mass error <9.3 p.p.m., IP probability score > 0.25 and ModLS score > 8. When the ModLS score was <8, the peptide was retained if all arginines were methylated and all methyl-arginines were mis-cleaved (trypsic digest). All methylated arginines had a PTM score ≥ 0.95. After these filters, the FDR was calculated by determining the proportion of decoy methylated peptide spectrum matches that matched to a methyl-SILAC pair. FDR for arginine-methylated peptides was 0.93%. To generate Fig. 1c, identified methylated peptides, including decoy hits for calculation of FDR, generated from experiments on heavy methyl-SILAC-SLAC-labelled primary T cells and I766–773 (2010).

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Author contributions
V.G. performed all the experiments and conceived the isomethionine labelling strategy. V.G. and O.A. designed the experiments. V.G., D.T. and O.A. analysed the data. V.G. wrote MethylQuant. A.G. provided the antibodies and expertise with immuno-affinity purifications. B.T. provided expertise with sample preparation and data analysis. V.G. and O.A. wrote the manuscript.

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