Proteomic Analysis of Arginine Methylation Sites in Human Cells Reveals Dynamic Regulation During Transcriptional Arrest*

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The covalent attachment of methyl groups to the side-chain of arginine residues is known to play essential roles in regulation of transcription, protein function, and RNA metabolism. The specific N-methylation of arginine residues is catalyzed by a small family of gene products known as protein arginine methyltransferases; however, very little is known about which arginine residues become methylated on target substrates. Here we describe a proteomics methodology that combines single-step immunoenrichment of methylated peptides with high-resolution mass spectrometry to identify endogenous arginine mono-methylation (MMA) sites. We thereby identify 1027 site-specific MMA sites on 494 human proteins, discovering numerous novel mono-methylation targets and confirming the majority of currently known MMA substrates. Nuclear RNA-binding proteins involved in RNA processing, RNA localization, transcription, and chromatin remodelling are predominantly found modified with MMA. Despite this, MMA sites prominently are located outside RNA-binding domains as compared with the proteome-wide distribution of arginine residues. Quantification of arginine methylation in cells treated with Actinomycin D uncovers strong site-specific regulation of MMA sites during transcriptional arrest. Interestingly, several MMA sites are down-regulated after a few hours of transcriptional arrest. In contrast, the corresponding dimethylation or protein expression levels are not altered, confirming that MMA sites contain regulated functions on their own. Collectively, we present a site-specific MMA data set in human cells and demonstrate for the first time that MMA is a dynamic post-translational modification regulated during transcriptional arrest by a hitherto uncharacterized arginine demethylase. Molecular & Cellular Proteomics 13: 10.1074/mcp.O113.032748, 2072–2088, 2014.

Post-translational modifications (PTMs) greatly increase the complexity of proteins far beyond the combinatorial possibilities of the 20 amino acids. As a result, the ability to characterize and identify PTM patterns in cells, tissues, and organisms on a proteome-wide scale has become important to better understand the molecular details of the individual PTMs (1, 2). Often PTMs are underrepresented in proteomic studies because of their low abundance and temporal lifespan unless specific enrichments are utilized. Such PTM-specific enrichment methods combined with advances in liquid chromatography mass spectrometry (LC-MS) allow for proteome-wide characterization of PTMs by pinpointing the exact amino acid localization within the investigated protein. This has greatly expanded current knowledge of modified proteins and the amino acid localization for a wide range of PTMs, such as phosphorylation (3, 4), acetylation (5), ubiquitylation (6, 7), and glycosylation (8). These modifications indisputably play important roles in many biological processes, and have been extensively studied on a proteome-wide scale. In comparison, large-scale analysis of other types of PTMs, such as protein methylation, has only recently started to emerge (9, 10).

In mammalian cells, protein methylation most commonly occurs as N-methylation of several amino acid residues. These include the ε-amine of lysine, the imidazole ring of histidine, the guanidine moiety of arginine, and the side chain amid nitrogen of glutamine and asparagine (11). Although

1 The abbreviations used are: PTMs, post-translational modifications; ActD, actinomycin D; ADMA, asymmetric di-methylated arginines; DMEM, Dulbecco’s modified Eagle medium; DOX, doxycycline; ER, estrogen receptor; FCS, fetal calf serum; FDR, false discovery rate; GAR, glycine and arginine-rich regions; GO, Gene Ontology; GPProX, The Graphical Proteomics Data Explorer suite; HCD, higher-energy collisional activation dissociation; HEK293T, human embryonic kidney 293 cells; hnRNPs, heterogeneous nuclear ribonucleoproteins; IRS, insulin receptor substrate; LC-MS, liquid chromatography mass spectrometry; MMA, mono-methyl arginine; NR3C1, glucocorticoid receptor; PADI, peptidyl arginine deiminase; PRMTs, protein arginine methyltransferases; Rme, methylated arginine-specific antibody; RmeGG, methylated arginine-specific antibody for methylated arginines in RGG regions; SDMA, symmetric di-methylated arginines; SILAC, stable isotope labeling of amino acids in cell culture; U2OS, human osteosarcoma cells; UHPLC, ultra-high pressure liquid chromatography.
discovered 50 years ago, protein methylation has predominantly been studied as a mechanism of epigenetic regulation of histones, with the catalyzed transfer of methyl groups from S-adenosyl methionine to histones by enzymes known as histone methyltransferases (12). Recently, strategies were described for large-scale analysis of lysine methylation (9), yet methods for extensive characterization of arginine methylation still awaits to be fully established.

Arginine methylation is a PTM that increases the structural diversity of proteins and modulates their function in living cells, and proteins modified by arginine methylation are involved in a number of cellular processes, including transcriptional regulation, RNA metabolism and DNA damage repair (13). However, for many of the target proteins it remains to be determined how methylation of arginines affect their molecular activity, which is often because of the lack of methods to pinpoint the specific methylation site within the protein.

Methylation of the arginine side-chain is catalyzed by protein arginine methyltransferases (PRMTs), which mainly target arginine residues of substrates located in glycine- and arginine-rich regions, the so-called GAR motifs. The PRMTs can be divided into two major classes (type I and II) depending on the type of methylarginine they catalyze (14), and both type I and type II enzymes are able to generate omega-N-methylarginine (MMA; arginine mono-methylation) in proteins. In contrast, generation of asymmetric N,N-dimethylarginine (ADMA) is catalyzed by type I enzymes only (PRMT1, PRMT4/CARM1, PRMT6, and PRMT8), whereas type II enzymes (PRMT5 and PRMT9) catalyze the formation of symmetric N,N-dimethylarginine (SDMA). Recently, a type III methylase (PRMT7) was demonstrated to form only MMA, and thus currently constitutes the only enzyme with this activity (15). Although PRMTs are fairly well characterized and known to target certain sequence specific motifs, very little is known about the site-specific localization of arginine methylation in human proteins.

Because all PRMTs are able to catalyze mono-methylation, MMA may only represent a transient methylation form used as a substrate for further methylation into ADMA or SDMA. However, MMA sites may still contain physiologically relevant functions on their own, as supported by the restricted MMA-sequences for RG sites, whereas surrounding residues reveal a strong enrichment for glycine residues only. Although arginine methylation has been reported to primarily locate within RNA-binding regions, such as the RGG-box, we find that MMA sites locate to these regions merely because of the proteome-wide distribution of arginine residues and the occurrence of surrounding glycine residues. Still, the majority of MMA sites belong to proteins harboring RNA-binding domains, and are involved in RNA metabolism, transcription, and chromatin remodeling. To further investigate the biological implications of MMA sites in RNA metabolism, we performed a temporal proteomics experiment aiming at mapping regulated methylation sites upon transcriptional arrest by Actinomycin D (ActD). ActD is a widely used transcriptional inhibitor that intercalates into G-C rich DNA regions and prevents the progression of RNA polymerase (17). Interestingly, our analysis identifies several MMA sites regulated upon inhibition of transcription, whereas no regulation is observed for the corresponding di-methylation or protein turnover. In summary, the presented methodology allows for rapid and quantitative analysis of in vivo arginine mono-methylation sites in response to cellular perturbations.

PROTEOMIC ANALYSIS OF ARGinine METHYlation SITES

EXPERIMENTAL PROCEDURES

Cell Culture—HEK 293T (Human Embryonic Kidney) cells were grown in DMEM media (Invitrogen, Carlsbad, CA) supplemented with 10% FCS and penicillin/streptomycin (100 U/ml) (Invitrogen, Carlsbad, CA). Stable HeLa-Kyoto cells expressing THRAP3 tagged with C-terminal GFP under the control of an endogenous promoter were generated by transfecting BAC transgenes and were kindly provided by Anthony Hyman (Max Planck Institute, Dresden, Germany). Selection was maintained by adding 400 μg/ml G418 to the culture medium. Stable HEK 293T expressing inducible PADI4, were a kind gift from Maria Christophorou (Kouzarides lab, Gurdon Institute, University of Cambridge) and were maintained in DMEM supplemented with 10% FBS, penicillin/streptomycin, glutamine, and Blasticidin (3 μg/ml) and hygromycin (100 μg/ml). For doxycycline induction, 2 μg/ml doxycycline was added for the given time points.

For SILAC labeling, U20S (human osteosarcoma) cells and PADI4 expressing cells were grown in SILAC-DMEM (PAA Laboratories, Pittsburg, PA) supplemented with 10% dialyzed FBS (PAA Laboratories), sodium-pyruvate, l-glutamine, penicillin/streptomycin, and either l-lysine (Lys0) and l-arginine (Arg0) or l-lysine-U-13C6-15N2 (Lys8) and l-arginine-U-13C6-15N2 (Arg10) (Cambridge Isotope Laboratory, Tewksbury, MA) as described previously (18). To inhibit transcription, heavy labeled U2OS SILAC cells were treated with ActD (5 μg/ml) for 1 h, 3 h, 8 h, or 16 h; light control cells were incubated at similar time points with DMSO.

Arginine Methylation Sample Preparation—Cells were harvested with washing with PBS and lysed in 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% Na-deoxycholate, protease inhibitor mixture (Roche, Penzberg, Germany) supplemented with 2 mM Naorthovanadate, 5 mM NaF, 5 mM Glycero-2-phosphate, and 1 mM N-Ethylmaleimide. Lysates were cleared by high speed centrifugation. Proteins were precipitated by adding 4-fold excess volumes of ice-cold acetone and stored at −20 °C overnight. Subsequently, proteins were solubilized in a urea solution (6 M urea/2 M thiourea/10 mM HEPES pH 8.0). The RIPA cell pellets were resuspended in 8 M Urea, sonicated, and after additional centrifugation combined with the al-

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ready solubilized proteins. Protein concentrations in lysates were measured using Bradford assay (Bio-Rad, Hercules, CA).

Next, proteins were reduced by adding 1 mM dithiothreitol, and alkylated with 5.5 mM chloroacetamide (19), digested using endoproteinase Lys-C (1:100 w/w) and modified sequencing grade trypsin (1:100 w/w) after a fourfold dilution in 25 mM ammonium bicarbonate solution. Protease digestion was terminated by addition of trifluoroacetic acid to pH 2. Precipitates were removed by centrifugation for 10 min at 3000 × g. Peptides were purified using reversed-phase Sep-Pak C18 cartridges (Waters, Milford, MA). Peptides were eluted off the Sep-Pak with 50% acetonitrile with subsequent steps of removal of acetonitrile by vacuum. The peptides were dissolved in immunoprecipitation buffer (10 mM sodium phosphate, 50 mM sodium chloride in 50 mM 3-(N-morpholino)propanesulfonic acid pH 7.2). Modified peptides were immunoenriched by addition of 24 μg mono-methyl arginine (100 μl Me-R4–100) and 12 μg mono-methyl arginine (50 μl R*GG) (D5A12) antibodies (#8015 and #8711 Cell Signaling, Danvers, MA) for 4 h at 4 °C unless otherwise stated. 50 μl Protein-A Agarose slurry (Cell Signaling #9863) was added overnight to the immunoprecipitation. The immunoprecipitates were washed three times in ice cold immunoprecipitation buffer followed by three washes in water, and modified peptides were eluted with 2 × 50 μl 0.15% TFA in H2O and subjected to microcolumn-based strong cation exchange chromatography at pH 4.5, 6.5, 8.0, and 11. Peptide eluates were concentrated using a sample concentrator and acidified with 150 μl of 0.1% trifluoroacetic acid before desalting on reverse phase C18 StageTips as described previously (20).

**GFP Precipitation**—Following transcriptional inhibition by Actinomycin D, THRAP3-GFP HeLa cells were lysed in Nonidet P-40 buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 450 mM NaCl, 1 mM EDTA) supplemented with protease inhibitors (Roche, Penzberg, Germany) and phosphatase inhibitors. Immunoprecipitations for GFP-tagged proteins were carried out with GFP-Trap® Agarose beads (Chromotek, Martinsried, Germany) for 2 h at 4 °C. Immunoprecipitated proteins were washed in Nonidet P-40 buffer and boiled in 2x Laemmli sample buffer. Samples were resolved by SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

**Western Blotting**—The following antibodies were used in this study: rabbit polyclonal p53 (#9282, Cell Signaling), rabbit polyclonal phospho p53 (Ser15) (#9284S, Cell Signaling, Massachusetts, US), rabbit polyclonal GAPDH (#Ab9485, Abcam, Cambridge, England), mouse monoclonal Ubiquitin Histone H2B clone 56 (05–1312, Merck Millipore, Darmstadt, Germany), rabbit polyclonal PADI4 (ab50247, Abcam, Cambridge, England), mouse monoclonal GFP (B-2) (sc-9996, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit monoclonal mono-methyl arginine (Me-R4–100) (#8015, Cell Signaling), rabbit monoclonal mono-Methyl-Arginine (R*GG) (D5A12) (#8711, Cell Signaling).

Total cell lysates were resolved on 4–12% gradient SDS-PAGE gels and proteins were transferred onto nitrocellulose membranes. Membranes were blocked using either 5% BSA solution or 5% skim milk solution in PBS supplemented with Tween-20 (0.1%). Secondary antibodies coupled to horseradish peroxidase (Jackson Immuno-Research Laboratories, West Grove, PA) were used for immunodetection. The detection was performed with Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen).

**Mass Spectrometric Analysis**—All MS experiments were performed on a nanoscale UHPLC system (EASY-nLC1000 from Proxeon Bio-systems, Odense, Denmark) connected to an Orbitrap Q-Exactive equipped with a nanoelectrospray source (Thermo Fisher Scientific, Bremen, Germany). Each peptide fraction was auto-sampled and separated on a 15 cm analytical column (75 μm inner diameter) in-house packed with 1.9-μm C18 beads (Reprosil Pur-AQ, Dr. Maisch, Germany) using a 2 h gradient ranging from 5% to 40% acetonitrile in 0.5% formic acid at a flow rate of 250 nl/min. The effluent from the HPLC was directly electrosprayed into the mass spectrometer. The Q Exactive mass spectrometer was operated in data-dependent acquisition mode and all samples were analyzed using previously described ‘sensitive’ acquisition method (21).

**Identification of Peptides and Proteins**—All raw data analysis was performed with MaxQuant software suite (22) version 1.2.6.20 supported by the Andromeda search engine (23). Data was searched against a concatenated target/decoy (24) (forward and reversed) version of the UniProt Human fasta database encompassing 71,434 protein entries (downloaded from www.uniprot.org on 2013–07–03). Mass tolerance for searches was set to maximum 7 ppm for peptide masses and 20 ppm for HCD fragment ion masses. Data was searched with carbamidomethylation as a fixed modification and protein N-terminal acetylation, methionine oxidation, and mono-methylation on lysine and arginine as variable modifications. A maximum of three mis-cleavages was allowed while requiring strict trypsin specificity (25), and only peptides with a minimum sequence length of seven were considered for further data analysis. Peptide assignments were statistically evaluated in a Bayesian model on the basis of sequence length and Andromeda score. Only peptides and proteins with a false discovery rate (FDR) of less than 1% were accepted, estimated on the basis of the number of accepted reverse hits, and FDR values were finally estimated separately for modified and unmodified peptides (26). Protein sequences of common contaminants such as human keratins and proteases used were added to the database. For SILAC quantification a minimum of two ratio-counts was required. Statistical analysis and hierarchical clustering was performed using Perseus (Max-Planck Institute of Biochemistry, Department of Proteomics and Signal Transduction, Munich). Significantly enriched Gene Ontology terms were determined using the Functional Annotation Tool of the DAVID Bioinformatics database (27). Protein interaction networks were analyzed using the interaction data from the STRING database (v. 9.05) (28) and visualized using Cytoscape (v. 2.8.3) (29). Only MMA containing peptides with an Andromeda score above 24 was accepted as positive identifications.

**RESULTS**

**Identification of Endogenous Arginine Mono-methylation (MMA) Sites**—For in vivo identification and site-specific localization of MMA sites, we established an antibody-based peptide-enrichment strategy (16). Briefly, proteins were digested into peptides using trypsin, and MMA containing peptides were subsequently immunoenriched using two commercially available antibodies recognizing mono-methylated arginines located in unspecific or specific motifs (Rme and RmeGG respectively) (Fig. 1A). To further reduce sample complexity of the analyzed sample, the enriched peptides were fractionated into four samples using a microcolumn-based strong cat-ion exchange method (20, 30). Each fractionated sample was subsequently analyzed on a high-resolution Orbitrap mass spectrometer (Q Exactive, Thermo, Bremen, Germany) using a 2-hour LC gradient (21, 31). All peptides were fragmented using HCD, which, combined with detection of all ions in the Orbitrap analyzer, ensured high ppm accuracy on both precursor and fragment ions (32).

To cover the arginine mono-methylome most comprehensively while ensuring optimal throughput for the proteomic analysis, we speculated whether a combined use of the two MMA antibodies would be preferential. To investigate this in more detail, we compared the separate usage of the antibody-
**Fig. 1. Proteome-wide identification of arginine-methylated peptides.** A, Schematic representation of the enrichment strategy for the MMA containing peptides. Modified peptides were isolated using a combination of two MMA antibodies (RMe and RMeGG) and subsequently
ies to their combined one. To this end, we performed three MMA enrichment analyses on 8 mg of peptide material each; for two experiments 12 μg of each antibody was used, and these results were compared with a third sample where we combined the two antibodies (12 μg of each antibody).

Results revealed that the combined use of antibodies identified many more MMA sites in a single-shot experiment as compared with their separate usage (>70% increase; supplemental Fig. S1A). As the combined usage furthermore allows for faster MS analysis, we opted to combine the two antibodies throughout this study. Further investigation revealed that combined antibody amounts corresponding to 24 μg of Rme and 12 μg of RmeGG, respectively, yielded the best results (supplemental Fig. S1B). Notably, the amounts used in this study are still an order of magnitude lower compared to previously published methods using the same antibodies.

Next, we assessed the technical reproducibility of the developed method by performing a triplicate enrichment analysis of MMA-containing peptides from a HEK 293T lysate (24 mg cell lysate divided into three 8 mg samples). Because our investigation did not necessitate extensive pre-fractionation of the investigated sample, the entire replicate analysis only required a total of 12 LC-MS runs (four fractions per replicate sample) allowing for protein arginine methylation studies to be conducted within a short timeframe. In this triplicate analysis, we identified 798 MMA sites on 376 proteins, with 691 MMA sites (86% of total sites) identified with a localization score above 0.75, greatly assisted by the increased fragmentation efficiency of HCD (33). A Venn diagram of overlapping MMA sites between replicate experiments revealed that 62% of all MMA sites with a localization score >0.75 were identified in all three samples, signifying high reproducibility in our established enrichment method (Fig. 1B).

Gene Ontology (GO) analysis revealed that 376 MMA containing proteins predominantly are expressed in the nucleus, most notably as part of the spliceosome (Fig. 1C). Moreover, the arginine methylated proteins identified are primarily reported to be involved in various RNA metabolic processes such as RNA splicing, RNA transportation and transcription (Fig. 1D). Mapping the proteins onto the InterPro database reveals that the identified proteins harboring a MMA site also often contain RNA-binding domains (Fig. 1E).

As a further validation of the established method, the data set contains many proteins previously reported to be modified by MMA. These include several known targets of the arginine methylase CARM1, such as the splicing factors CA150/TCERG1, SmB/SNRPB, and PABPC1 (34). Additionally, SAM68/KHDRBS1 is a known target of PRMT1 and our data set confirms the previously reported MMA sites on R291, R320, R331, R340, R346 (35). Furthermore, we find heterogeneous nuclear ribonucleoproteins (hnRNPs) extensively methylated as described in the literature (36). Previous observations report that hnRNPs account for more than 60% of ADMA found in the nucleus (37), whereas in our data set hnRNPs constitute less than 10% of identified MMA sites (supplemental Table S1). This difference may be because of the overall cellular abundance of the various types of arginine methylation, as ADMA is reported to be the most abundant type of arginine methylation, whereas MMA and SDMA make up roughly 50 and 20% of ADMA, respectively (38). These results also suggest that MMA may have functional roles on its own and not only constitutes a transient form of ADMA.

Overall we identified few MMA sites on histones, likely explained by their high arginine/lysine content, which generates very short peptides upon digestion with trypsin. Because of shorter peptides being less specific in database searches, we only allowed MMA peptides with a minimum sequence length of seven amino acids to be considered in our data analysis (See experimental procedure section). As a result, shorter peptides like those often generated from histones are unlikely to be identified. Yet, the histone MMA sites identified in our data set include currently unreported sites in common databases such as Uniprot (H2A-R89, H2B-R88, and H4-R24) (39).

Interestingly, among other MMA sites identified we find several N-terminal methylation sites of PRMT6. Despite being a member of the protein arginine N-methyltransferase family PRMT6 is the only one reported to harbor auto-methylation sites (40). The extent of PRMT6 auto-methylation has not been fully elucidated; however, our data infers that PRMT6 could contain several N-terminal auto-methylation sites (R29, R35, and R37). As the N-terminal regions of other PRMTs previously have been demonstrated to modulate substrate binding specificity and methyltransferase enzymatic activity (41), these MMA sites may function as an autoregulatory mechanism for PRMT6. In support of this, one of the identified sites (R35) was recently confirmed as an auto-methylation site of PRMT6 affecting its methylase activity (42). Apart from PRMT6, MMA sites are identified on several other transferases, including the methyltransferases MLL2, MLL4, and SETD1; the KAT6A acetyltransferases, and the PIMT O-methyltransferase. Moreover, sites are identified on enzymes catalyzing the formation of other PTMs such as E3 ligases (RNF188, RNF12, RNF158, PELI2, TRIM33) and kinases fractionated by strong cat-ion exchange chromatography (pH 4, pH 6.5, pH 8, and pH 11). Each peptide fraction was subsequently analyzed by high resolution LC-MS/MS. B, Venn diagram of identified MMA sites derived from three technical replicates of HEK 293T cell lysates. 62% of all MMA sites with a localization score >0.75 were identified in all three samples. C, GO cellular annotation of proteins harboring MMA sites reveal strong enrichment of nuclear and spliceosomal proteins compared with annotated GO genes across the entire human genome. D, GO functional annotation of MMA proteins shows enrichment of proteins involved in RNA metabolism and transcription. E, InterPro analysis pinpoints that proteins containing a RNA recognition motif are highly overrepresented in our MMA data set.

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(AKAP10, AKAP8, BRSK1, Dyrk2, MLTK, Pak4, SIK3, Smg1), suggesting that protein arginine methylation may be involved in an intricate interplay with other types of PTMs.

Recently, it was reported that PRMT1 is recruited by the estrogen receptor (ER) during estrogen stimulation, hereby mediating extranuclear functions of the receptor and triggering interaction with the p85 subunit of PI3K and Src (43). Because our investigations did not entail estrogen stimulation, we did not observe any arginine methylation of ER, however, the glucocorticoid receptor (NR3C1) was found methylated on R34. Moreover, several known NR3C1 interaction partners, such as SMARCA4 and NCOA6 were identified as MMA substrates.

Considering that estrogens and glucocorticoids often oppose each other to regulate cellular responses (44), the observed methylation of NR3C1 may reflect an analogous role of protein arginine methylation in glucocorticoid signaling as previously described for estrogen signaling (43). Additionally, we identified Insulin Receptor Substrates 2 & 4 (IRS2 and IRS4) to be modified by MMA, suggesting that arginine methylation may be involved in insulin signaling and glucose metabolism. This is supported by recent discoveries that insulin treatment of L6 myotubes induces translocation of PRMT1 to the membrane fraction (45). However, further analysis will be required to elucidate these theories in more detail. Collectively, we describe a method that allows for extensive analysis of MMA sites in a rapid and reproducible manner. The proteins identified as arginine methylation substrates are primarily nuclear annotated proteins involved in RNA metabolic processes (14). Still, our proteomic analysis identifies a wide range of MMA containing proteins and reveals that MMA may be involved in several other biological processes not previously associated with this PTM. In addition to the biological significance of these observations, the presented data provide a proof-of-principle of the resource potential included in our data set.

Sequence Properties of the Methylated Proteins—In order to further investigate the MMA containing proteins, we examined the properties of the amino acids surrounding the MMA sites. To this end, we compared the frequencies of neighboring residues for modified arginines against nonmodified arginines in the human protein database using IceLogo (46). The analysis revealed a significant preference for glycine residues around the modified arginine. In addition, a weak preference against negatively charged amino acids such as glutamic acid was noticed (Fig. 2A).

As methylated proteins generally harbor RNA-binding properties (Fig. 1D) and identified MMA sites preferentially locate to glycine-rich regions, we investigated whether MMA-containing proteins contain multiple GAR-domains such as the known RGG-box (47). We utilized the Tri-RGG, Di-RGG, Tri-RG, and Di-RG motif terminology recently suggested by Richard and coworkers (48). First, we extracted all human proteins in UniProt harboring the different motifs using ScanProsite (49) and compared their occurrence to our MMA data set. A significant enrichment of MMA-containing proteins harboring Tri-RGG, Di-RGG, and Tri-RG domains were observed (p < 5.24e-09, Fisher exact test), with more than 60% of human Tri-RGG motif containing proteins identified in our data set (Fig. 2B). In contrast to this, Di-RG motif containing proteins were not found enriched, suggesting Di-RG motifs are merely common motifs and do not constitute a representative sequence motif for MMA containing proteins.

Following these results, we investigated whether MMA sites preferentially reside within a RGG-box domain. To this end, we extracted the number of observed MMA sites located within a RGG-box motif and compared these to the proteome-wide distribution of regular arginine residues residing in the same motif (Fig. 2C). Notably, the RGG-box motif contains three consecutive RGG sequences separated by random amino acids. Thus, as a control experiment, we investigated how many MMA sites reside in a sequence of similar size to the RGG-box and that randomly contains three arginines and six glycine residues (referred to as R3G6). Again, the distribution of MMA sites residing in R3G6 to that of regular arginine residues was compared. Interestingly, these results demonstrated that MMA sites do not significantly reside in RGG-box domains but merely follow the proteome-wide occurrence of arginine residues in these motifs (p < 0.4818, Fisher’s exact test). Similarly, we investigated whether MMA sites specifically locate to RNA-binding motifs as compared with the natural distribution of arginine residues (Fig. 2D). Although more than 30% of MMA sites localize to RNA-binding domains, a comparison to the general occurrence of arginine residues within these domains reveals that MMA sites preferentially are located outside RNA-binding regions (p < 1.27e-13, Fisher’s exact test). Although the role of arginine methylation in RNA metabolism is widely known, these results support the notion that MMA prominently is involved in biological processes taking place outside RNA-binding regions. Moreover, the strong preference for extended glycine-rich regions beyond the previously reported RG and RGG motifs (Fig. 2A), and the localization preferences against RNA-binding domains may reflect a currently overlooked sequence preference for MMA sites. Thus, we speculated whether the sole requirement for mono-methylation of an arginine residue could be the amino acid localization in strong glycine-rich regions.

To investigate this further, we extracted all possible 19-mer sequence windows from UniProt that contained one arginine residue and between 1 and 12 randomly located glycine residues. The size of the sequence window was chosen to match the size of the RGG-box motif. Next, we assessed how many of the 19-mer sequences contained a MMA sites in our data set (Fig. 2E). The analysis confirms that MMA sites predominantly reside on arginine residues located in highly extended
Fig. 2. Analysis of MMA peptides reveals strong preference for glycine-rich regions. A, IceLogo analysis shows that MMA sites are strongly associated with glycine-rich regions. B, Identified MMA substrates are enriched for Tri-RGG, Di-RGG and Tri-RG, but not Di-RG domains. C, MMA sites are not located within RGG-box motifs in comparison to the regular distribution of arginine residues. D, MMA sites are prominently located outside RNA-binding domains in comparison to the proteome-wide distribution of arginine residues, with 32% of MMA sites located outside these domains.
glycine-rich regions (Fig. 2A), with more glycine residues surrounding the modification sites as the more preferred sites for mono-methylation (Fig. 2E). Conversely, a similar analyses for a 19-mer sequence windows with varying number of glycine residues revealed no effect on MMA site preferences (data not shown).

As it is becoming more evident that arginine methylation participates in other processes besides RNA processing (50–52), a sequence preference based upon an increasing number of glycine residues would allow for arginines located in vastly different protein regions to become methylated by the same PRMTs. This would constitute an elegant enzymatic solution to maintain the wide-spread regulatory role of arginine methylation in various biological processes using a small number of PRMTs.

**Quantifying Arginine Mono-methylations Under Transcriptional Inhibition**—As the majority of MMA-containing proteins identified in our data set are involved in RNA metabolism, we next investigated the functional roles of MMA in more detail. To this end, we analyzed the expression profiles of arginine methylation in human cells under transcriptional inhibition of RNA Polymerase I and II using Actinomycin D (ActD). Experimentally we employed stable isotope labeling by amino acids in cell culture (SILAC) leaving light SILAC cells untreated, whereas heavy SILAC cells were treated with ActD (Fig. 3A). Protein lysates from the two individual SILAC states were digested to peptides, enriched for MMA containing peptides and arginine methylation sites were subsequently identified by LC-MS/MS. Because heavy labeled SILAC cells were exposed to ActD, only MMA-containing peptides affected by the ActD treatment should exhibit an altered heavy/light (H/L) SILAC ratio. To establish the temporal changes for individual MMA sites, we performed a time-course analysis in which heavy SILAC cells were exposed to ActD for 1, 3, 8, and 16 h, respectively. As a result, the entire analysis required five quantitative experiments, one for each time point and an additional control experiment to assess reproducibility of the transcriptional arrest (Fig. 3B; supplemental Fig. S1C).

To ensure our observations are caused by transcriptional inhibition, we verified several cellular markers of transcriptional arrest by Western blot (Fig. 3C). As previously described in the literature, Histone 2B ubiquitylation decreased following ActD treatment (53), and the tumor suppressor p53 accumulated because of a feed-back loop involving enhanced synthesis and protein stability (54, 55). Moreover, ActD is known to intercalate with DNA and hereby induce double-strand breaks (56). We confirmed this through increased Sirt15 phosphorylation levels of p53 (57), although it should be noted that the overall abundance of p53 similarly increased. However, we did not identify any regulated MMA sites on known methylated proteins involved in the DNA damage response (58).

Consequently, we conclude that the observed regulation of MMA levels primarily is caused by the cellular responses related to transcriptional arrest.

A box-plots analysis of quantified MMA sites (H/L SILAC ratios) confirmed a temporal regulation of MMA sites upon transcriptional inhibition. Minor changes were observed after 1 h of ActD treatment with an increasing regulation throughout the time-course (Fig. 3D). Following this, we investigated the extent of temporally regulated MMA sites in more detail. Generally, the experiments revealed a strong down-regulation of MMA sites within the first three time-points (1, 3, and 8 h) as compared with up-regulated sites. In fact, the number of up-regulated MMA sites upon ActD treatment decreased within the first 8 h of the experiment, and only reached a similar level as down-regulated sites after 16 h (Fig. 2E).

To investigate the distribution of regulated MMA sites in more detail, we performed a hierarchical cluster analysis of all MMA sites identified and quantified throughout the ActD experiments (Fig. 4A). We analyzed the regulated MMA sites using the Graphical Proteomics Data Explorer (GProX) suite (59), which revealed that MMA sites in general can be clustered into three distinct categories; MMA sites with a protracted down-regulated expression throughout the time-course experiment (Cluster 1; Fig. 4B), MMA sites down-regulated only after prolonged (16 h) ActD treatment (Cluster 2; Fig. 4C) and MMA sites exhibiting late up-regulation upon ActD treatment (Cluster 3; Fig. 4D).

When investigating the differences between the three clusters, we found that proteins harboring down-regulated MMA sites were more prone to cluster in glycine-rich regions than up-regulated ones (Fig. 4E). These differences could point toward up- and down-regulated MMA sites being involved in distinct biological processes during transcriptional arrest. Following this notion, we find that 42% of proteins with an early down-regulated MMA site (belonging to Cluster 1) are uniquely involved in transcriptional regulation (Fig. 4F).

One of the proteins identified with an early regulated MMA site is the transcription elongation factor SUPT5H/SPT5, previously reported to be methylated in vitro and in vivo on arginines 681, 696, and 698 by PRMT1 and PRMT5 (60). Mutational studies of these sites revealed that arginine methylation affects SPT5s association with RNA polymerase II and enhances the ability of SPT5 to mediate DRB-inhibited transcription (61). Thus, reduced arginine methylation of SPT5 affects its promoter association and transcriptional elongation. Considering we find other down-regulated MMA sites residing on proteins involved in transcriptional regulation (supplemental Fig. S3), this could constitute a regulatory trend of transcriptional regulators. However, more detailed experiments would be required to investigate this and is beyond the scope of this report.
Fig. 3. Transcriptional inhibition perturbs the regulation of MMA sites. A, Schematic representation of MMA regulation analysis by transcriptional inhibition in a SILAC based set-up. Heavy labeled cells were treated with transcriptional inhibitor Actinomycin D. Separate pull-downs of SILAC-encoded lysates were performed and peptide eluates were combined equally and fractionated by strong cat-ion exchange. B, Table illustrating the SILAC experimental set-ups with Actinomycin D added to ‘heavy’ U2OS cells for 1 h, 3 h, 8 h, and 16 h compared with ‘light’ counterparts with DMSO added for equal amount of time. C, Validation of transcriptional inhibition shows decrease in
Contrary to the transcriptional regulators, MMA sites on the RNA helicases DDX5 and DDX17 were found significantly up-regulated upon prolonged treatment with ActD (supplemental Table S1). The two sites, R478 on DDX5 and R555 on DDX17, are conserved between the two proteins and located in the N-terminal part of the transactivation domain. Notably, DDX5 and DDX17 are the only members of the DEAD box helicase family that contain a transactivation domain, and the only members identified with MMA sites in our data set (supplemental Fig. S3). Both DDX5 and DDX17 were recently confirmed by Western blot to harbor arginine methylation (62), yet the exact amino acid location of these modified sites was not mapped. As an example of a protein harboring regulated MMA sites that do not conform to our cluster analysis, we find the RNA-processing factor THRAP3/THRAP150 to be modified with MMA at position R66. This MMA site is located within ubiquitylation of histone 2B and accumulation of p53 and phosphorylated p53 on Western blots. D, Box plot analysis indicating the quantitative regulation of MMA sites after 1 h, 3 h, 8 h, and 16 h of ActD treatment. Regulation increases over the time period indicating a temporal regulation of the MMA sites. E, Bar-plot illustrating the total number of significantly regulated MMA sites after ActD treatment. During the first 8 h of treatment substantially more MMA sites are down-regulated compared to up-regulated sites.

Fig. 4. Hierarchal clustering groups the majority of MMA sites into three specific clusters with diverse dynamic regulation. A, Hierarchal cluster analysis of all identified and quantified MMA sites in the ActD experiment. B, GPProx suite clusters the regulated MMA sites into 3 categories dependent on their temporal regulation. Cluster 1, exhibits continual down-regulation of the MMA sites. C, Cluster 2, shows down-regulation of the MMA sites only after prolonged (16 h) ActD treatment, whereas MMA sites in Cluster 3 D, are up-regulated after 16 h of ActD treatment. E, Significantly more glycine residues are present in seven amino acids sequence windows surrounding down-regulated MMA sites compared with up-regulated sites. F, GO term analysis for clustered proteins reveals that down-regulated MMA sites uniquely reside on proteins involved in transcription.
the domain region that is required for the mRNA splicing activation of THRAP3 (63). Strikingly, we observed this MMA site to be up-regulated upon 1 h of ActD treatment with a subsequent decreasing abundance throughout the time course. This time-course profile correlates nicely with observations describing the mRNA degradation capabilities of THRAP3 during transcriptional inhibition (63), suggesting that MMA at R66 potentially plays a regulatory role in THRAP3 activity. We additionally confirmed the mono-methylation of THRAP3 by immunoprecipitation of GFP-tagged THRAP3 (supplemental Fig. S4A), however, because of THRAP3 harboring several methylation sites the Western blot analysis only monitors the total methylation signal.

Collectively, these data demonstrate the ability of our proteomics methodology to identify individual MMS sites and dissect their individual methylation changes in response to cellular stimuli. Moreover, these results position MMA as a dynamic modification during transcriptional arrest.

Arginine Mono-methylation (MMA) is Dynamically Regulated—In our experiment we observe regulation of MMA sites already after 1–3 h of ActD treatment. In contrast, the average protein turnover rate for the investigated cell line is ~20 h (64)), indicating that the MMA regulation is unlikely caused by protein degradation and re-synthesis. Hence, the observed regulation of MMA sites may reflect either increased conversion of MMA to ADMA/SDMA, reduced activity of MMA methyltransferases, or the presence of an active MMA demethylase. To elucidate the most likely event, we analyzed the proteome level changes of all ActD-treated samples used for mapping MMA sites (supplemental Table S2; supplemental Fig. S2). Although proteome expression levels were measured for all time-points, we initially focused our data analysis on the sample treated with ActD for 3 h to ensure limited effects of protein turnover. The analysis revealed that the majority of down-regulated MMA sites do not undergo any changes in their overall protein expression levels during 3 h of ActD treatment (supplemental Fig. S2; supplemental Table S2), in full agreement with the previously established and much longer protein turnover rates (Table I).

Besides measuring the protein expression changes during ActD treatment, our proteome approach allowed for an investigation of regulated arginine di-methylations under the same conditions (supplemental Table S3). This is primarily because of the abundant expression of particularly ADMA in human cells (38), which allows for identification and quantification of ADMA sites from intact proteome analysis. Indeed, for several

### Table I

| Gene names | Position | SILAC ratio MMA | SILAC ratio Protein | SILAC ratio Dimethyl | SILAC ratio PADI4 | Protein turn-over (hours) | Novel site |
|------------|----------|----------------|-------------------|---------------------|-----------------|--------------------------|------------|
| TRIP6      | R236     | 0.38           | 1.04              | 0.67                | 14.13           |                         |            |
| HNRNP1A    | R218     | 0.27           | 0.93              | 0.85                | 25.66           |                         |            |
| HNRNP1D    | R408     | 0.29           | 1.25              | 1.04                | 0.98            | 27.08                    |            |
| HNRNP1A    | R225     | 0.30           | 0.93              | 0.98                | 25.66           |                         |            |
| HNRNP1U2   | R684     | 0.32           | 0.97              | 1.22                | 1.01            | 22.34                    |            |
| FUBP1      | R361     | 0.32           | 0.97              | 0.81                | 12.11           |                         |            |
| YLFM1      | R1303    | 0.37           | 1.12              | 1.70                | 23.03           |                         | Y          |
| HNRNP1A    | R196     | 0.38           | 0.93              | 0.76                | 25.66           |                         | Y          |
| HNRNP1A    | R206     | 0.39           | 0.93              | 0.79                | 1.01            | 25.66                    |            |
| HNRNP3A    | R224     | 0.41           |                   | 0.95                | 23.84           |                         |            |
| MEPCE      | R86      | 0.42           | 0.93              | 1.24                | 27.22           |                         |            |
| HNRNP1D    | R296     | 0.43           | 1.17              | 1.11                | 0.84            | 23.10                    | Y          |
| DDX5       | R516     | 0.47           | 0.95              | 0.41                | 13.25           |                         |            |
| SYNCRIP    | R526     | 0.47           | 1.06              | 25.43               |                |                         | Y          |
| CCDC9      | R121     | 0.47           |                   | 0.68                |                |                         |            |
| HNRNP1A    | R739     | 0.48           | 1.07              | 0.81                | 24.36           |                         | Y          |
| RPS2       | R22      | 0.48           | 0.94              |                    | 35.83           |                         |            |
| CHF8       | R172     | 0.48           |                   | 0.62                |                |                         | Y          |
| NCL        | R281     | 0.50           | 1.05              | 25.36               |                |                         | Y          |
| HSPA1B     | R483     | 0.51           | 1.01              |                    |                |                         |            |
| C11orf95   | R15      | 0.53           |                   | 0.51                |                |                         | Y          |
| GBPB1      | R87      | 0.53           |                   | 0.82                |                |                         | Y          |
| QKI        | R242     | 0.54           | 0.74              |                    | 10.19           |                         | Y          |
| COG5       | R309     | 0.55           | 0.90              | 0.86                | 10.34           |                         | Y          |
| API5       | R500     | 0.55           | 1.01              | 0.83                | 10.07           |                         | Y          |
| HNRNP1D    | R272     | 0.56           | 1.17              | 0.74                | 1.03            | 23.10                    | Y          |
| TSSC4      | R139     | 0.57           |                   | 0.87                |                |                         |            |
| SYDE1      | R640     | 0.57           |                   | 0.89                |                |                         | Y          |
| QKI        | R256     | 0.58           | 0.74              | 0.56                | 10.19           |                         | Y          |
| PHPF3      | R191     | 0.59           | 0.96              | 0.68                | 11.64           |                         | Y          |
| API5       | R504     | 0.60           | 1.01              | 1.05                | 10.07           |                         | Y          |
Proteomic Analysis of Arginine Methylation Sites

A

ASRGGGNHQNNYQPY

B

ASRGGGNHQNNYQPY

C

VFVGGISPDTSEEQIK

D

Log2 SILAC ratio

E

Hours after DOX induction

Proteome

MMA

ADMA

Padi4

Gapdh

Light SILAC

Heavy SILAC

Methyl group (PTM)
of the regulated MMA sites we were able to identify the corresponding arginine di-methylation site as not being regulated upon ActD treatment (Table I).

As an example, we plotted the peptide abundance for the MMA containing peptide (ASRmeGGGNHQNNYQPY) derived from the protein hnRPDL, which is 3-fold down-regulated on R408 after 3 h of ActD treatment (Fig. 5A). Notably, the corresponding di-methylated peptide (ASRme2GGGNHQNNYQPY) was not altered in expression within the proteome experiment (Fig. 5B). Likewise, the overall protein level of hnRPDL was not affected as demonstrated by the unique peptide sequence VFVGGISPDTSEEQIK (Fig. 5C), in full agreement with the previously reported 27 h turnover rate of hnRPDL (64). As a result, our data reveals that the observed down-regulation of MMA on hnRPDL is not because of an increased conversion of MMA into ADMA/SDMA (increased PRMT activity) or altered protein turnover.

These results are particularly interesting considering the short experimental timeframe (3 h treatment), and that several of the MMA sites already are down-regulated after 1 h of ActD treatment (supplemental Table S1). Analyzing the same expression profiles throughout the entire ActD experiment revealed that the MMA on hnRPDL (R408) is constitutively down-regulated, whereas both the protein level of hnRPDL and di-methylation on R408 is not regulated at all (Fig. 5D). Consequently, our data suggests the presence of an active MMA demethylase as being responsible for the dynamic regulation of MMA sites during transcriptional arrest.

Quantification of PADI4 Demethylation—One of the early descriptions of an arginine demethylase came from a study by Chang and coworkers, who reported that JMJD6 was able to demethylate arginine residues (H3R2me2 and H4R3me2) (65). The authors incubated a peptide containing the dimethylated arginine residues with JMJD6 and immuno-precipitated with a MMA-specific antibody revealing loss of one methyl group for the dimethylated peptide species. Recently, JMJD6 has been reported to be a bifunctional enzyme able to catalyze both demethylation and lysyl-hydroxylation reactions (66). A similar demethylase of MMA has so far not been reported. However, MMA residues may be converted into citrulline by peptidyl-arginine deiminases (PADIs), which hydrolyze the side-chain of arginine residues releasing methylamine (supplemental Fig. S4B). Currently, five PADI enzymes have been described in human cells, whereas the conversion of MMA into citrulline has only been demonstrated for PADI4 (67, 68). Because no enzyme capable of converting citrulline back to arginine has been described, it appears that citrullination of MMA sites might efficiently block re-methylation of arginine residues (67), although investigations into the demethylase activity of PADI4 has indicated that MMA is a poor substrate for PADI4 (69, 70).

Nonetheless, deimination of potential MMA sites represents a relevant mechanism for regulation of protein arginine methylation and PADI4 could still play a pivotal role in MMA regulation although the identified sites might not be direct targets of deimination. In conclusion, our data reveal that MMA is a dynamic modification with the observed regulation most probably because of increased activity of a hitherto uncharacterized MMA demethylase.

DISCUSSION

In this study, we have established a stream-lined method for identification of in vivo MMA sites directly from trypsin digested human proteins, which we combined with SILAC-based mass spectrometry to quantify the cellular changes of MMA sites in response to the transcriptional inhibitor ActD. Our approach entails several advantages over recent studies aimed at mapping arginine methylated proteins through MS-
The present study is substantially larger than any recent proteomics analysis and illustrates potential novel roles of MMA in regulation of cellular processes. A, Close to 80% of previously reported proteins are confirmed in our MMA screen, whereas novel MMA substrates are increased more than 6-fold. B, Network interaction analysis of MMA substrates was performed using interaction information from STRING database. Proteins modified by MMA were grouped using associated GO biological processes, and reveals that methylated proteins prominently are involved in RNA processing, RNA transportation, chromatin remodeling and transcription.
Proteomic Analysis of Arginine Methylation Sites

based proteomics, including 10-fold lower amounts of antibody (12–24 μg) for the immunoenrichment steps (10, 62, 72). Notably, our described methodology does not entail use of any methanol-based sample preparation, which previously has been described to induce artificial mono-methylations on glutamic acids (67), and potentially could give rise to incorrect identifications of protein arginine methylation sites during database searches.

Using this newly established method, we identified 1027 MMA sites on 494 proteins corresponding to more than two MMA sites per protein on average. In the presented data set we confirm a substantial number of known MMA containing proteins while concurrently identifying many more novel ones. As a result, these data expand the current knowledge of arginine methylated proteins and site-specific localization of MMA (Figure 6A). Our resource data furthermore reveals that a large number of MMA containing proteins are involved in known biological functions related to RNA processing, RNA transport, chromatin remodeling and transcription (Fig. 6B).

To assess the regulatory role of MMA, we performed a quantitative analysis of human cells treated with transcriptional inhibitor ActD. Our results reveal that MMA is strongly affected upon transcriptional inhibition, most prominently observed through down-regulation of several MMA sites just few hours after ActD treatment. Strikingly, the corresponding dimethylation sites and protein levels show no changes in expression under same treatment and time point. This suggests that a specific MMA demethylase is activated upon transcriptional arrest and that MMA sites contain physiological relevant functions independent from arginine di-methylation.

Currently, only PADI4 has been reported to entail such MMA demethylase activity and our data demonstrate that cells treated with DOX-inducible PADI4 does not exhibit expression changes of the MMA sites regulated during transcriptional arrest. As a result, we conclude that the observed MMA changes may be because of a hitherto uncharacterized MMA demethylase being activated upon transcriptional arrest. Hence, our data demonstrate that MMA is a dynamic modification similar to other PTMs such as lysine methylation, phosphorylation, and ubiquitylation.

In summary, the MMA sites identified in this study will serve as a valuable resource for functional characterization of proteins modified by arginine methylation. Moreover, investigations of the biological role that protein arginine methylation plays in human diseases has only begun (73, 74), but will undoubtedly increase dramatically with increased knowledge and improved methodologies for studying arginine methylation. As the methodology presented here is applicable to any cell or tissue type, and allows for site-specific quantitative characterization of MMA upon cellular perturbations, we expect it to become a valuable technology for both basic science and biomedical research.

All mass spectrometric data have been deposited to the ProteomeXchange Consortium (http://proteomexchange.org) via the PRIDE partner repository (75) with the data set identifier PXD000559 and username review65637 and password dFFyYfnK. Upon publication the proteomics data will be made available for all readers.

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