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Substrate Specificity of Mammalian N-Terminal α-Amino Methyltransferase NRMT

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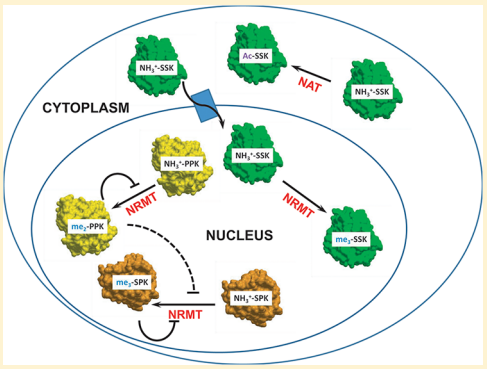
Supporting Information

ABSTRACT: N-Terminal methylation of free α-amino groups is a post-translational modification of proteins that was first described 30 years ago but has been studied very little. In this modification, the initiating M residue is cleaved and the exposed α-amino group is mono-, di-, or trimethylated by NRMT, a recently identified N-terminal methyltransferase. Currently, all known eukaryotic α-amino-methylated proteins have a unique N-terminal motif, M-X-P-K, where X is A, P, or S. NRMT can also methylate artificial substrates in vitro in which X is G, F, Y, C, M, K, R, N, Q, or H. Methylation efficiencies of N-terminal amino acids are variable with respect to the identity of X. Here we use in vitro peptide methylation assays and substrate immunoprecipitations to show that the canonical M-X-P-K methylation motif is not the only one recognized by NRMT. We predict that N-terminal methylation is a widespread post-translational modification and that there is interplay between N-terminal acetylation and N-terminal methylation. We also use isothermal calorimetry experiments to demonstrate that NRMT can efficiently recognize and bind to its fully methylated products.



Modification of free α-amino groups in proteins is a common covalent post-translational modification. The acetylation of protein N-terminal residues is widely observed, with up to 50% of cytosolic proteins estimated to be acetylated at their N-termini in eukaryotic cells.[1] In contrast, the alkylation of α-amino groups appears to be less common and has been studied little. To date, approximately two dozen proteins in various organisms have been reported to be N-terminally methylated, mostly in prokaryotes.[2,3] The function of N-terminal methylation was completely unknown until recently,[3] when it was discovered that α-N-methylation of RCC1 (regulator of chromosome condensation 1) is required for its efficient binding to DNA.[4] However, the functional diversity of proteins reported to be N-terminally methylated suggests this modification likely has additional roles. In bacteria, the known N-terminally methylated proteins are components of large multisubunit complexes, such as the ribosomal proteins (L11, L16, L33, S11, and the associated IF-3),[5] pilins, and the chemotaxis-flagellar apparatus (CheZ protein).[5] There is some evidence that these N-termini are located in regions involved in protein−protein interactions.[5]

Until recently, the enzyme responsible for N-terminal methylation of eukaryotic proteins had not been identified.[6] Apart from RCC1, eukaryotic proteins shown to be methylated at their α-amino group included histone H2B in *Tetrahymena* sp. and *Drosophila* sp., ribosomal protein S25 in *Saccharomyces cerevisiae*, cytochrome c_{557} in *Crithidia* sp., myosin light chains in rabbit,[7] and recently SET and retinoblastoma protein (Rb) in humans.[6]

The first eukaryotic N-terminal methyltransferase, NRMT, has recently been identified.[6,8] The methylation catalyzed by NRMT in eukaryotic cells appears to require a specific consensus on the N-terminus of substrate proteins (M-X-P-K). The N-terminal Met residue must be removed before N-terminal methylation. X can be one of many different residues, but all of the known substrates possess A, S, or P.[6] On the basis of limited mutagenesis studies, P and K residues in the third and fourth positions also appear to be necessary for efficient N-terminal methylation. Using this consensus, several dozen additional proteins are predicted to be α-N-methylated.

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Here, we further address the question of substrate specificity of NRMT by expanding its recognition motif, identifying new substrates, and showing that different recognition motifs tend to have variable affinities for NRMT. We also identify for the first time a substrate that can be either N-terminally methylated or acetylated depending on its tissue of origin.

EXPERIMENTAL PROCEDURES

Constructs and Antibodies. All mammalian expression constructs were designed as previously described.[6] The wild type and the K4Q mutant of ZFP15 and Mina53 vectors were designed using the method used for the RCC1 vectors but using human ZFP15 and Mina53 cDNA.[3] Triple GFP fused with the first 10 residues of human RCC1, followed by either a nuclear localization signal (NLS) or a nuclear export signal sequence (NES), was designed on the basis of the pKGFP vector as described previously.[9] All His-tagged proteins were purified as previously described.[3]

Peptide Synthesis. Synthetic peptides were prepared by using standard Fmoc chemistry in an APEX 396 peptide synthesizer (Advanced Automated Peptide Protein Technologies), resuspended at a concentration of 1 mg/mL in 0.1% (v/v) aqueous acetic acid, and stored at −35 °C until they were used. For sequence confirmation, 5 pmol/μL solutions of each peptide were infused into a linear quadrupole ion trap mass spectrometer (Thermo Finnigan LTQ) and analyzed by electrospray ionization (ESI) and collision-activated dissociation (CAD)/electron transfer dissociation (ETD). The resulting spectra were interpreted manually. Mixtures of 35−45 synthetic peptides (1 nmol of each peptide) were prepared in 0.1% acetic acid, taken to dryness, and stored at −35 °C for in vitro methylation assays.

In Vitro Methylation Assays. In vitro methylation reactions were conducted as described previously.[6] The peptide library was methylated as follows. The mixtures of 35−45 peptides (0.5 nmol of each peptide) were dissolved in 50 mM Tris, 50 mM potassium acetate buffer (pH 8.0) followed by the addition of 300 μM SAM and 2 μM NRMT to a final volume of 50 μL (final concentration of each peptide, 10 μM). The reaction was run at 30 °C for 2 h followed by reapplication to Ni-NTA beads to remove NRMT and microdialysis to remove SAM/SAH (*S*-adenosylmethionine/*S*-adenosylhomocysteine). The microdialysis was conducted using a Spectra/Por Biotech Cellulose Ester (CE) Dialysis Membrane (molecular mass cutoff of 100−500 Da) and 50 μL Dialysis Buttons (Hampton Research) filled with the methylated sample. The buttons were transferred to a NEXTAL hanging drop vapor diffusion crystallization plate with reservoirs filled with 2 mL of 50 mM Tris, 50 mM potassium acetate buffer (pH 8.0). Dialysis was conducted overnight at 25 °C.

MS Sample Preparation. On-bead samples of proteins immunoprecipitated with anti-FLAG and anti-me3-SPK antibodies were washed three times with 50 μL aliquots of 100 mM ammonium bicarbonate (pH 8.0), suspended in a fourth aliquot, and then reduced, carbamidomethylated, and digested using endoproteinase Glu-C (Roche) at an enzyme:protein ratio of 1:20 as previously described.[10] The resulting peptides were removed from the beads, acidified with glacial acetic acid (pH 3.5), and stored at −35 °C.

MS Analysis. For all analyses by mass spectrometry, peptides were pressure-loaded onto a capillary precolumn (360 μm outside diameter, 75 μm inside diameter) packed with 5−20 μm C18 resin (YMC). The precolumn was washed with 0.1% acetic acid and connected to an analytical column (360 μm outside diameter, 50 μm inner diameter) equipped with an integrated 1 μm emitter tip and packed with 5 μm C18 resin (YMC).[11] Peptides were gradient-eluted into the mass spectrometer at a rate of 60 nL/min using nanoflow, reverse-phase HPLC, and microESI into CAD and front-end ETD-enabled LTQ/Fourier transform hybrid mass spectrometers (LTQ-FTMS and LTQ-Orbitrap, Thermo Scientific) as previously described.[12] Mass analyses were conducted using one high-resolution (60000 at m/z 400) MS scan followed by 5−10 CAD/ETD data-dependent MS2 scans acquired in the linear ion trap. Data resulting from analysis of anti-me3-SPK *Mus musculus* immunoprecipitated proteins were searched with OMSSA (version 2.1.1[13]) using MS/MS peak lists generated by Bioworks Browser (version 3.3.1 SP1) against the human/rat/mouse RefSeq database (downloaded June 2009). These results were used to guide data analysis. All peptide sequences were confirmed by manual interpretation of the spectra. To determine the relative abundances of each peptide, the areas under the extracted ion chromatogram curves for each peptide in all of its charge states and detected forms were summed. The peak area for each peptide was divided by the total area for all forms of the peptide to obtain an estimate of the percent of each form of the modified peptide.[14]

In Vivo Substrate Affinity Assay. Sequences encoding peptides MSPKRIAKRRS, MSPQRIAKRRS, and MPPKRIAKRRS were fused to GFP to create a set of competitor constructs. All constructs were based on vectors published previously.[3] The substrate set was prepared identically but with the FLAG tag on the C-terminus of GFP for efficient immunoprecipitation. Constructs were transfected into 293LT cells using calcium phosphate.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) experiments were performed using an ITC200 microcalorimeter (MicroCal). Recombinant NRMT was dialyzed overnight at 4 °C against methyltransferase buffer [50 mM Tris, 50 mM potassium acetate buffer (pH 8.0)]. Substrate peptides were purchased from GenScript in lyophilized form and dissolved in the same buffer. Product peptides were purchased from American Peptide. The measurements were performed at 25 °C by titrating 100 μM NMRT with an 800−1000 μM peptide solution. Binding curves were analyzed by using Origin (MicroCal).

RESULTS

NRMT Has Broad Substrate Specificity. Our previous RCC1 mutagenesis results suggested that although NRMT can methylate proteins with a variety of residues at the second position in the (M)-X-P-K motif, there is no flexibility allowed at positions 3 and 4. Thus, mutation of K to Q severely lowers the efficiency of methylation. However, the importance of Pro at position 3 had not been thoroughly tested, and we had not investigated any possible supportive role of residue 5. To more rigorously determine the substrate specificity of NRMT, we synthesized a peptide library consisting of the RCC1 N-terminal peptide with varying amino acids at positions 2−5, performed in vitro methylation reactions using recombinant NRMT, removed excess SAM, and subjected the purified peptide mixtures to analysis by MS.

The methylation efficiencies of the different substrate peptides from the peptide library are listed in Table S1 of the Supporting Information. Remarkably, in addition to the expected methylation substrates with (M)-X-P-K motifs, we

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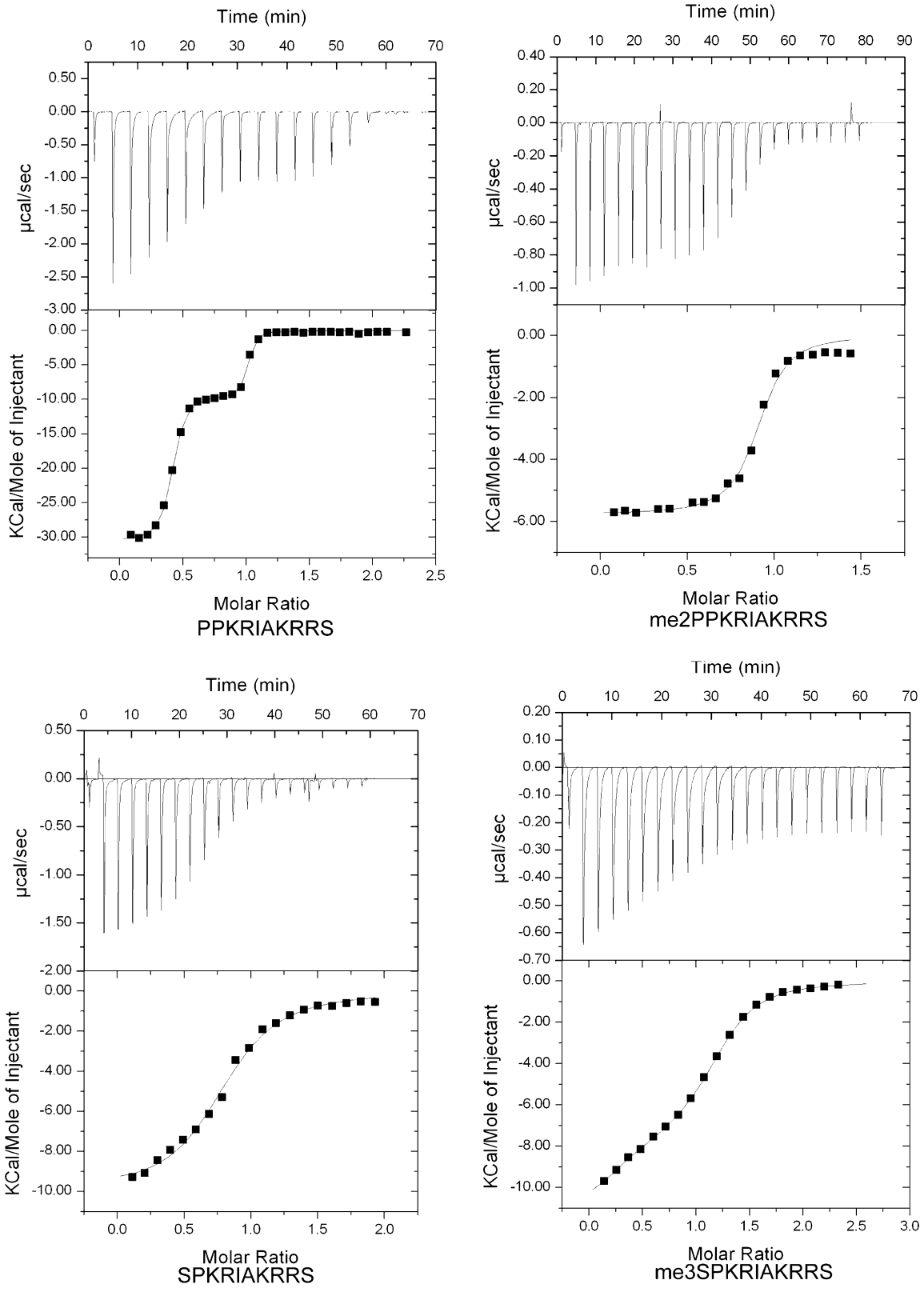

Figure 1. Affinity of substrate and product peptides for NRMT measured using isothermal titration calorimetry. The wild-type 10-residue SPK-RCC1 N-terminal peptide (bottom left) showed efficient binding to NRMT with a K_a of $1.0 \times 10^5 \pm 1.10 \times 10^4$ M^{-1} (K_d = 10 μM). A PPK-RCC1 N-terminal peptide showed very efficient binding (top left) with a K_{a1} of $(6.60 \pm 2.0) \times 10^8$ M^{-1} and a K_{a2} of $(7.3 \pm 2.3) \times 10^6$ M^{-1} (K_d values of 1.5 and 140 nM, respectively). The 10-residue me3-SPK-RCC1 ($K_a = 4.8 \times 10^5 \pm 6.75 \times 10^4$ M^{-1}) and me2-PPK-RCC1 [$K_a = (7.70 \pm 1.5) \times 10^5 M^{-1}$] N-terminal peptides (right) showed efficient binding to NRMT with K_d values of 2.0 and 1.3 μM, respectively.

identified an unexpectedly large number of other positives: APR, GNK, GPK, GPR, MGKK, MNK, MNR, MPK, MPRR, SAKR, SEKR, SMKR, SNKR, SPRR, SQKK, SQKR, GGKK, GGKR, GSK, and SSKR. This peptide methylation assay reveals

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Table 1. Summary of ITC Results

peptide	N	K_d	ΔH (cal/mol)	ΔS (cal mol^{-1} °C^{-1})
SPKRIAKRRS	0.8	10 μM	−9943 ± 207	−10.5
PPKRIAKRRS	0.4/0.6	1.5 nM/140 nM	$-3.1 \times 10^4 \pm 221/-9428 \pm 192$	−62.2/−0.2
me3SPKRIAKRRS	1.2	2.0 μM	$(-4333 \pm 1.2) \times 10^3$	11.5
me2PPKRIAKRRS	0.9	1.3 μM	−5768 ± 93	7.6

that the promiscuity of NRMT in terms of position 3 is quite high. Residues on the third position that appear to promote N-terminal methylation in the pool of tested peptides are P, A, E, M, N, Q, G, and S. Arginine, lysine, and residues with aromatic side chains did not result in efficient methylation when they were at position 3. Similarly, as we have shown previously,[6] K at position 4 is absolutely crucial for efficient methylation but can be substituted with R in certain situations (Tables S1 and S2 of the Supporting Information).

Another interesting outcome of the peptide library methylation assays is that different substrates appear to be methylated with different efficiencies in vitro and vary in the total percentage of mono-, di-, and trimethylation (or full methylation) (Table S1 of the Supporting Information). PPK peptides were fully dimethylated with 100% efficiency in contrast to other known NRMT substrates. SPK-RCC1 peptides were mostly mono-, di-, and trimethylated (up to 60, 3, and 0.8%, respectively). These results are in agreement with the ITC experiments, in vivo substrate affinity assays, and in vitro product inhibition assays described below. They suggest that PPK substrates are the best targets for NRMT and that there is a wide range of substrate affinities for NRMT.

On the basis of the results described above, we were able to predict how abundant each N-terminal methylation motif is in the human genome (see the Supporting Information) and to divide all potential NRMT substrates into functional groups. A total of 308 potential substrates were identified on the basis of sequence identity to those successfully methylated in the peptide library. The functional analysis of NRMT-predicted targets was completed using CDD.[15]

Of these targets, 32 (∼10%) are defined by the CDD database as chromatin/DNA binding, 6 (∼2%) as ribosomal proteins, 27 (∼9%) as zinc finger-containing proteins (not included in the chromatin/DNA binding group), 12 (∼4%) as RNA binding/processing proteins, and 10 (∼3%) as myosins.

To test if some of the novel motifs are N-terminally methylated in vivo, we have used mouse spleen lysates as a source of N-terminally methylated proteins. It was shown previously that mouse spleen lysates contain a large number of methylated proteins in comparison to other tissues.[6] The rabbit anti-trimethylated RCC1 antibody was used to immunoprecipitate (IP) N-terminally methylated proteins.[6] Three of the coprecipitated proteins were myosin light chain (MLC) isoforms, MLC9, MLC12B, and MLCB-like, which begin with an SSKR or SSKK N-terminal sequence. Subsequent MS analysis confirmed that these MLCs are methylated at their N-termini in vivo (Figure S3D,E of the Supporting Information).

The Affinity of NRMT for Its Substrates Depends on the Substrate Consensus Sequence. To further characterize NRMT substrate specificity, we employed ITC to measure the affinity of recombinant NRMT for RCC1 peptides. The following 10-residue RCC1 N-terminal peptides were used for the measurements: SPKRIAKRRS, me3SPKRIAKRRS, PPKRIAKRRS, and me2PPKRIAKRRS. The four peptides that contain Lys at position 4 all bound to recombinant human NRMT with high affinity (Figure 1). The two peptides with Gln at the fourth position (PPQRIAKRRS and SPQRIAKRRS) demonstrated very low affinity binding [in the millimolar range (data not shown)], which is in accordance with previous results.[6]

The SPKRIAKRRS peptide represents the wild-type N-terminal sequence of human RCC1, a native substrate of NRMT. This peptide demonstrates efficient, binding with a K_d of 10 μM (Table 1). The binding of peptide PPKRIAKRRS, representing the N-terminus of mouse RCC1, reproducibly produced a biphasic titration curve. Surprisingly, ∼40% of the protein binds the peptide with a K_d of 1.5 nM, while the other 60% binds with a K_d of 140 nM. We postulate that the PPKRIAKRRS peptide exhibits this behavior because of very slow isomerization of the *cis*/*trans* peptide bond in amino acid sequences containing a Pro-Pro dipeptide, as has been reported previously.[16−18] The SPKRIAKRRS peptides (both non-methylated and methylated forms) did not show biphasic binding. Interestingly, binding studies of the methylated me2PPKRIAKRRS peptide also did not yield a biphasic curve. To investigate possible explanations for this difference between the methylated and unmethylated PPK peptides, we generated a model of dimethylated tetrapeptide PPAA using ICM-Pro (Internal Coordinate Mechanics Professional).[19] Using this model, we determined the 10 lowest-energy conformers, calculated by Marvin Suite version 5.9.2, and found that all have the *trans* conformation of the Pro-Pro peptide bond. There are no energetically favorable conformations for the *cis* isomer, as calculated by Marvin and ICM-Pro. The search for the lowest-energy conformers for the non-methylated tetrapeptide PPAA (for a subset of 10 lowest-energy conformers) yielded six of the calculated conformers in the *trans* conformation, and four were found to be *cis*. We conclude that the addition of a methyl group to the N-terminus of PPKRIAKRRS makes it more likely to adopt a *trans* conformation (Figure S1A,B of the Supporting Information), while the *cis* conformation is not favored because of steric hindrance. As a result, me2PPKRIAKRRS no longer can form a stable mixture of *cis* and *trans* isomers. Despite the complication of this biphasic titration curve, the data clearly indicate that the affinity of NRMT for its substrates is influenced by the N-terminal consensus sequence.

NRMT Can Also Bind Its Methylated Products. Interestingly, both me3SPKRIAKRRS and me2PPKRIAKRRS peptides, which are products of the enzymatic reaction catalyzed by NRMT, also efficiently bound to recombinant NRMT with K_d values of 2.0 and 1.3 μM, respectively (Table 1). This is a surprising result, as interactions between an enzyme and its product are usually of very low affinity. It predicts that methylated products would remain bound to NRMT and inhibit further substrate methylation. To test whether such interactions can be detected in vivo, we expressed two target proteins, VRK2 and RCC1, as FLAG-tagged constructs in 293LT cells and asked if we could immunoprecipitate endogenous NRMT with these proteins. From previous

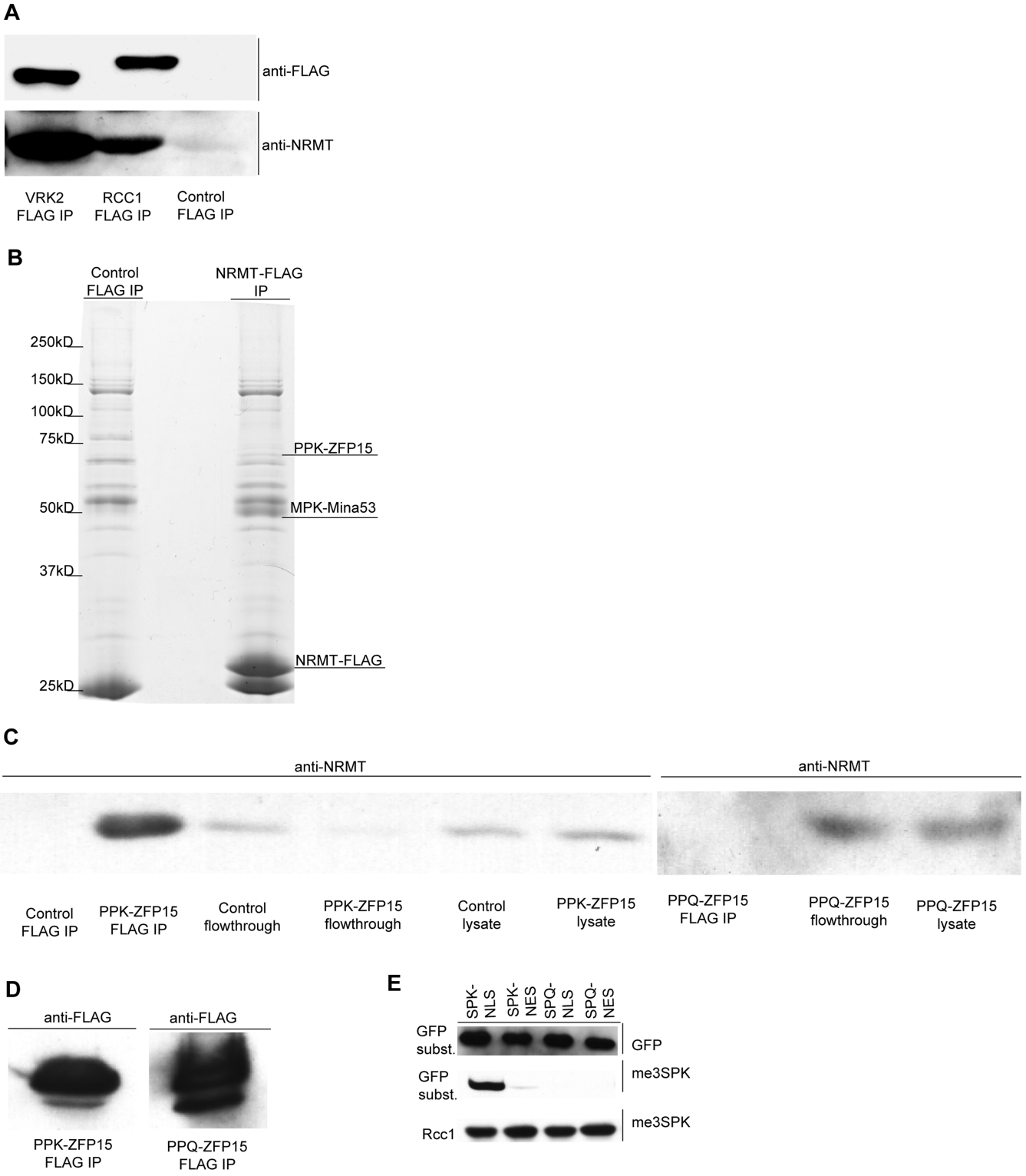

Figure 2. NRMT can bind to its fully methylated substrate proteins. (A) NRMT is coprecipitated with C-terminally FLAG-tagged substrates (RCC1 and VRK2). (B) C-Terminally FLAG-tagged NRMT coprecipitates with endogenous ZFP15 and Mina53. IPs were run on the 10% polyacrylamide gel and stained with Coomassie blue. (C) C-Terminally FLAG-tagged wild-type ZFP15 coprecipitates with endogenous NRMT. The PPQ nonmethylatable mutant of ZFP15 does not bind endogenous NRMT. (D) Both PPK- and PPQ-ZFP15 FLAG-tagged proteins are efficiently expressed and immunoprecipitated. (E) N-Terminal methylation activity is localized in the nucleus. NLS-fused SPK-GFP3 substrates are efficiently methylated, in contrast to NES-fused SPK-GFP3.

MS studies, we expect that the vast majority of the target proteins will be fully methylated. As shown in Figure 2A, both VRK2-FLAG and RCC1-FLAG efficiently captured endogenous NRMT. Consistent with our in vitro data, VRK2, which possesses a P-P-K motif, captured more NRMT than RCC1, which possesses an S-P-K motif.

Next, we took advantage of this unusually strong product binding to help identify new NRMT substrates. To this end, we

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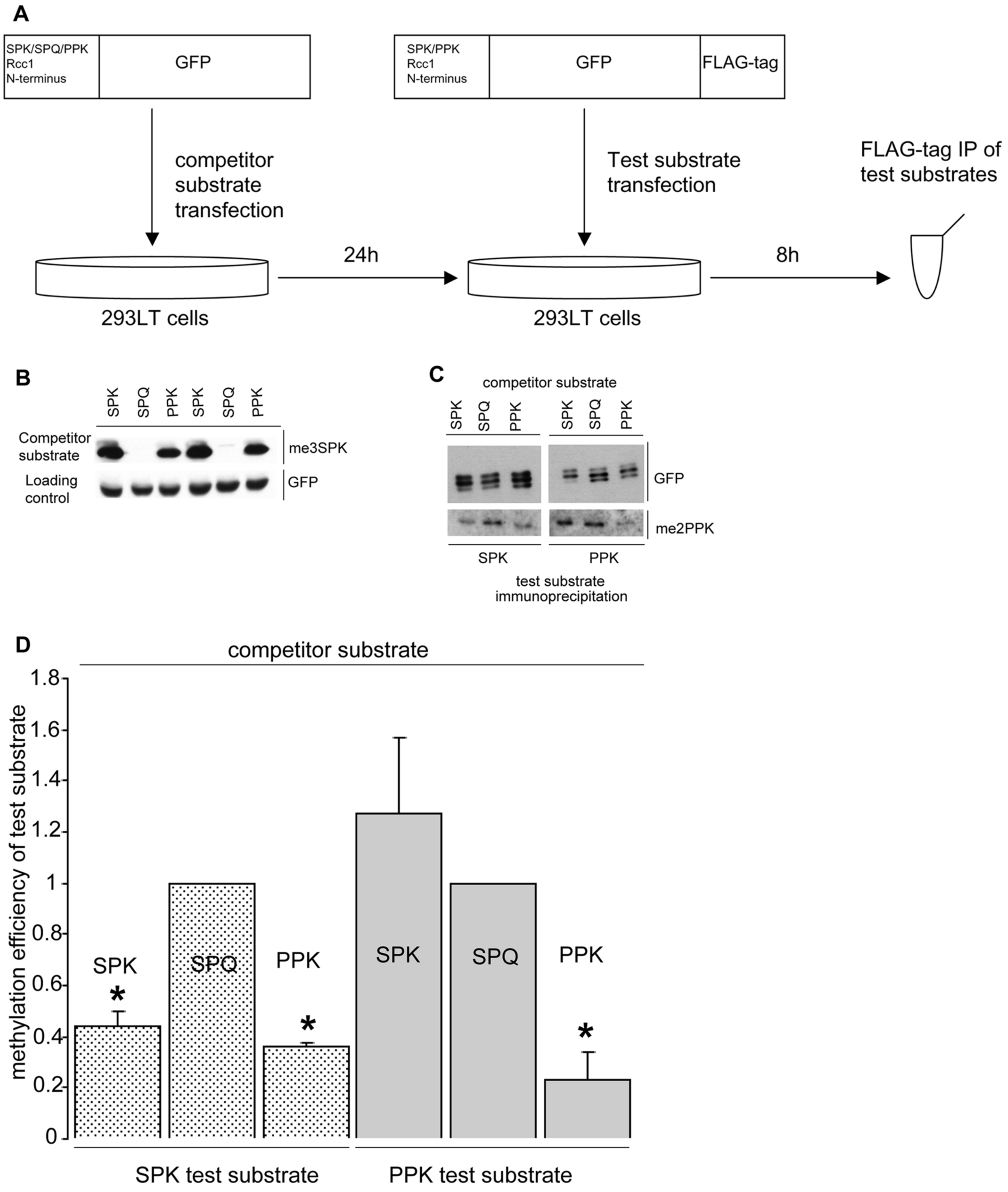

Figure 3. In vivo substrate affinity assay. (A) Schematic of the in vivo substrate affinity assay. (B) Competitor substrate set of SPK-, SPQ-, and PPK-GFP efficiently expressed and N-terminally methylated after expression for 32 h following calcium phosphate transfection. (C) The efficiency of N-terminal methylation of immunoprecipitated SPK- and PPK-GFP-FLAG test substrates is variable and depends on the presence of a cotransfected competitor substrate. (D) Quantification of the efficiency of N-terminal methylation of SPK- and PPK-GFP-FLAG test substrates in the presence of an SPK-, SPQ-, or PPK-GFP competitor. Data were normalized and compared to the level of N-terminal methylation of test substrates coexpressed in the presence of an unmethylatable SPQ-GFP competitor substrate by two-tailed independent t tests. An asterisk indicates $P < 0.01$. Three independent repetitions were conducted per combination. Error bars are one standard deviation.

screened for proteins that were coprecipitated with FLAG-tagged NRMT expressed in 293LT cells. One of the precipitated proteins was identified as ZFP15, which possesses a PPKKQAQ N-methylation motif (Figure 2B). In a reverse precipitation, we also found that FLAG-tagged ZFP15 could efficiently capture endogenous NRMT (Figure 2C,D). We confirmed that a majority of precipitated ZFP15 is dimethylated on the N-terminal proline residue, as shown by MS (Figure S3A of the Supporting Information). As expected, a PPQ nonbinding/nonmethylatable mutant of ZFP15 did not capture endogenous NRMT, suggesting the interaction between NRMT and N-methylated ZFP15 occurs through the methylated N-terminus (Figure 2C,D and Figure S3B of the Supporting Information).

An additional protein that coprecipitated with FLAG-tagged NRMT, Mina53, possesses a potential N-terminal methylation motif, MPKKAKPT.[6] Previously, the MPK N-terminal motif was suggested to be one recognized by NRMT. However, in a reverse precipitation, C-terminally FLAG-tagged Mina53 was unable to capture endogenous NRMT (Figure S2B,C of the Supporting Information). Subsequent mass spectrometric analysis of both samples demonstrated the initial Met residue was cleaved in both cases with 100% efficiency (data not shown), effectively destroying the N-terminal methylation motif and preventing methylation and NRMT binding. We hypothesize that a certain fraction of proteins can escape MAP (methionine amino peptidase) activity and retain the initial

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methionine, which in turn can be subjected to N-terminal methylation.

Evidence of NRMT Substrate Competition in Intact Cells. To test whether the high-affinity substrates and products of NRMT can outcompete other substrates within the intact cell, we designed an experiment in which a test substrate was transiently expressed in cells that had previously been transfected with a potential competitor protein. Eight hours after the introduction of the substrate plasmid, the cells were lysed and analyzed for methylation of the substrate (Figure 3A). The competitor proteins consisted of SPK-, SPQ-, or PPK-RCC1 N-termini fused to GFP. The test substrates consisted of SPK- and PPK-RCC1 N-termini fused to GFP followed by a FLAG tag (Figure 3A). 293LT cells were transfected with the competitors and 24 h later were retransfected with the substrates. This yielded six different combinations of competitors and substrates. The competitor and substrate were expressed together for 8 h. The cells were then lysed; the FLAG-tagged substrates were immunoprecipitated, and immunoprecipitates were blotted for N-methylation and GFP. The results were consistent with the in vitro ITC results. In the presence of either an SPK or PPK competitor, the level of methylation of the SPK substrate was decreased, as compared to the SPQ competitor control (Figure 3B–D). The SPQ competitor is incapable of being methylated (Figure 3B) and does not bind to NRMT; thus, it cannot compete with substrate for methylation and is used as a reference control of endogenous NRMT activity.

When the PPK substrate was coexpressed with the SPK competitor, the level of methylation of the PPK substrate was comparable to the SPQ competitor control, again consistent with the ITC data showing PPK binds NRMT with a higher affinity than SPK. However, in the presence of the PPK competitor, the level of PPK substrate methylation was substantially reduced (Figure 3C,D). Together, these data suggest that different substrates and products of NRMT can compete with one another for access to the enzyme, which could result in differential methylation of low-affinity versus high-affinity substrates.

NRMT Is Inhibited in Vitro by me3SPK and me2PPK Product Peptides. The experiment described above cannot distinguish whether the methylated or unmethylated competitor was most effectively outcompeting the substrate proteins. Therefore, to test whether the methylated products feedback inhibit NRMT, we monitored the efficiency of N-terminal methylation of SPK- and PPK-RCC1 proteins in vitro in the presence of increasing concentrations of me2PPK or me3SPK product peptides. For the SPK-RCC1 substrate, both me3SPK and me2PPK peptides achieved efficient inhibition at 1.3 μM, which is consistent with the ITC data (Figure 4A,B). However, as predicted by the ITC studies, inhibition of PPK-RCC1 was not observed (Figure 4A,B). Together, these data support the concept that methylated NRMT target proteins can efficiently outcompete some types of substrate, thereby limiting their N-methylation. This effect might reduce the number of N-methylated proteins in the cell or allow them to be acetylated instead.

N-Methylation Is Restricted to Proteins with Access to the Nuclear Compartment. A second mechanism that can potentially limit the number of substrates that are N-methylated by NRMT is location. The enzyme is predominantly nuclear, which suggests that cytoplasmic proteins will not be efficiently methylated except during mitosis. Cytoplasmic targets might therefore be either methylated in a cell cycle-dependent manner or not methylated at all. To determine whether substrate location does determine methylation efficiency, we expressed in unsynchronized 293LT cells a triple GFP fused with the first 10 residues of human RCC1, followed by either a nuclear localization signal (NLS) or a nuclear export signal sequence (NES). After 24 h, only fusion proteins with an NLS sequence were efficiently methylated (Figure 2E). During this period, all of the cells are expected to have cycled through at least one mitosis. Therefore, access to the nuclear compartment is essential for N-methylation, and the M phase is not sufficient to allow α-N-methylation of cytoplasmically restricted substrates.

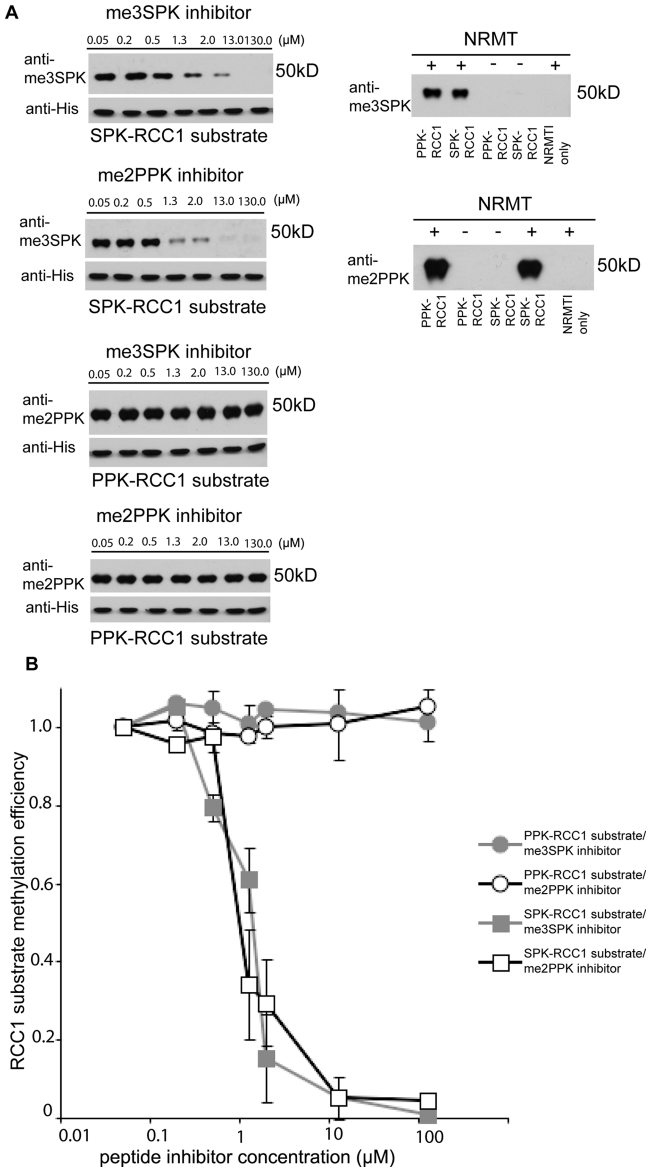

Figure 4. (A) NRMT is inhibited in vitro by me3SPK and by me2PPK product peptides with variable efficiency dependent on the affinity of the enzyme for the substrate. (B) Quantification of inhibition by me3SPK and me2PPK product peptides. Data were normalized and compared to the level of N-terminal methylation of SPK-RCC1 or PPK-RCC1 substrates methylated by recombinant NRMT in the presence of 0.05 μM me3SPK or me2PPK product peptide by two-tailed independent t tests. $P < 0.01$. Two independent repetitions were conducted per reaction. Error bars show the range.

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DISCUSSION

The majority of enzymes exhibit low affinities for their products, which promotes efficient catalysis. Strong binding of product peptides and fully methylated proteins (like ZFP15, VRK2, and RCC1) to NRMT is, therefore, a surprising result. The molecular basis for this high affinity is not immediately apparent. However, we noticed that the active site of the NRMT crystal structure contains a conserved triad of aromatic residues (Y19, W20, and H141), the spatial arrangement of which is similar to that found in chromo domains, which bind to methylated lysine residues (Figure S2A of the Supporting Information). Binding specificity results from a cation−π interaction between the tertiary amine group and the aromatic triad.[20−22] In NRMT, the stoichiometric methylation of the N-terminus places a stable positive charge on the amino group, making the interaction with the side chain rings of the aromatic triad very favorable. A product inhibition assay using the peptide from histone H1 that contains a trimethylated lysine residue (K26) (Figure S2D,E of the Supporting Information) failed to inhibit N-terminal methylation of SPK- or PPK-RCC1. This result suggests that me3 lysine is not recognized by the NRMT aromatic cage.

The high affinity of methylated peptides for NRMT suggests the possibility of feedback inhibition of NRMT by its own products. Indeed, fully methylated product peptides in vitro inhibit N-terminal methylation of SPK-RCC1. This inhibition is achieved at concentrations of inhibitory product peptides consistent with the K_d values obtained from ITC experiments. We have shown that fully methylated FLAG-tagged substrates can bind endogenous NRMT, consistent with the possibility of a product feedback inhibition by methylated substrates as one of the regulatory mechanisms of NRMT. Future experiments will address this possibility.

Diverse substrate and product affinities toward NRMT might be another means by which it is regulated. PPK consensus proteins are much better substrates than others as shown by ITC, in vitro and in vivo methylation assays, and the peptide library screen. PPK peptides are also the only ones that achieve 100% methylation. Additionally, methylation of PPK-RCC1 cannot be inhibited in vitro by fully methylated products. These differences in efficiency of methylation imply some substrate preference of NRMT in vivo.

One surprising result indicates interplay between N-terminal methylation and N-terminal acetylation in different tissues. When we immunoprecipitated MLC12B, MLC B-like, and MLC9 from mouse spleen extract, we found them to be N-terminally methylated. However, when we overexpressed one of the isoforms (MLC9) in 293LT cells, it was not N-terminally methylated but instead was 100% N-terminally acetylated (Figure S3C of the Supporting Information). When FLAG-tagged on the C-terminus and overexpressed in 293LT cells, N-terminally acetylated MLC9 also cannot IP endogenous NRMT (Figure S3F of the Supporting Information). This is in agreement with the MS data, but different from the results with other tested NRMT substrates (Figure 2A,B). These results suggest that N-terminal methylation is a tissue-specific post-translational modification and can be interchanged with N-terminal acetylation. Apart from MLC9, which is a novel protein to be N-terminally acetylated, there are other proteins, which could be potentially modified in these two different ways. PMI40 (SNK-N-terminus), MRP23 and MYP2 (SQK-N-terminus), TSNAX protein (SNK-N-terminus), ENSA protein and GBGC (SQK-N-terminus), TTC37 (SSK-N-terminus), protein tyrosine phosphatase PTPCAAX2, PWP1 (MNR-N-terminus), and RP42 (MNK-N-terminus) have all been reported to be N-terminally acetylated in yeast and mammals.[23,24] In our in vitro peptide library study, peptides with these N-terminal sequences were partially methylated by NRMT. Future studies will address the importance of the possible interplay between various N-terminal post-translational modifications.

Subcellular localization is another means by which the number of NRMT targets could be regulated. We have previously shown endogenous NRMT localizes in the nucleus,[6] and that all N-terminal methylation in the asynchronous 293LT cell culture is achieved in the nucleus. Compartmentalization of N-terminal methyltransferases and/or exclusion of substrate proteins from the nucleus would, therefore, constrain the number of proteins in the cell that will be efficiently methylated and allow for certain substrates to be acquire competing N-terminal post-translational modifications. Abnormal localization of NRMT could then lead to deleterious effects, as it would have access to previously sequestered targets and perhaps alter their normal regulation.

ASSOCIATED CONTENT

Supporting Information

Supporting information and methods, list of predicted NRMT substrates, Tables S1 and S2, and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

NRMT, N-terminal RCC1 methyltransferase; SAM, *S*-adenosylmethionine; RCC1, regulator of chromosome condensation 1; ITC, isothermal titration calorimetry.

REFERENCES

(1) Tsunasawa, S., Stewart, J. W., and Sherman, F. (1985) Amino-terminal processing of mutant forms of yeast iso-1-cytochrome c. The specificities of methionine aminopeptidase and acetyltransferase. *J. Biol. Chem. 260* (9), 5382−5391.

(2) Stock, A., Clarke, S., Clarke, C., and Stock, J. (1987) N-terminal methylation of proteins: Structure, function and specificity. *FEBS Lett. 220* (1), 8−14.

(3) Chen, T., Muratore, T. L., Schaner-Tooley, C. E., Shabanowitz, J., Hunt, D. F., and Macara, I. G. (2007) N-terminal α-methylation of RCC1 is necessary for stable chromatin association and normal mitosis. *Nat. Cell Biol. 9* (5), 596−603.

(4) Hao, Y., and Macara, I. G. (2008) Regulation of chromatin binding by a conformational switch in the tail of the Ran exchange factor RCC1. *J. Cell Biol. 182* (5), 827−836.
(5) Stock, A. M., and Stock, J. B. (1987) Purification and characterization of the CheZ protein of bacterial chemotaxis. *J. Bacteriol. 169* (7), 3301−3311.
(6) Tooley, C. E., Petkowski, J. J., Muratore-Schroeder, T. L., Balsbaugh, J. L., Shabanowitz, J., Sabat, M., Minor, W., Hunt, D. F., and Macara, I. G. (2010) NRMT is an α-N-methyltransferase that methylates RCC1 and retinoblastoma protein. *Nature 466* (7310), 1125−1128.
(7) Henry, G. D., Winstanley, M. A., Dalgarno, D. C., Scott, G. M., Levine, B. A., and Trayer, I. P. (1985) Characterization of the actin-binding site on the alkali light chain of myosin. *Biochim. Biophys. Acta 830* (3), 233−243.
(8) Webb, K. J., Lipson, R. S., Al-Hadid, Q., Whitelegge, J. P., and Clarke, S. G. (2010) Identification of protein N-terminal methyltransferases in yeast and humans. *Biochemistry 49* (25), 5225−5235.
(9) Brownawell, A. M., Kops, G. J., Macara, I. G., and Burgering, B. M. (2001) Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. *Mol. Cell. Biol. 21* (10), 3534−3546.
(10) Schroeder, M. J., Shabanowitz, J., Schwartz, J. C., Hunt, D. F., and Coon, J. J. (2004) A neutral loss activation method for improved phosphopeptide sequence analysis by quadrupole ion trap mass spectrometry. *Anal. Chem. 76* (13), 3590−3598.
(11) Martin, S. E., Shabanowitz, J., Hunt, D. F., and Marto, J. A. (2000) Subfemtomole MS and MS/MS peptide sequence analysis using nano-HPLC micro-ESI fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem. 72* (18), 4266−4274.
(12) Syka, J. E., Coon, J. J., Schroeder, M. J., Shabanowitz, J., and Hunt, D. F. (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A. 101* (26), 9528−9533.
(13) Geer, L. Y., Markey, S. P., Kowalak, J. A., Wagner, L., Xu, M., Maynard, D. M., Yang, X., Shi, W., and Bryant, S. H. (2004) Open mass spectrometry search algorithm. *J. Proteome Res. 3* (5), 958−964.
(14) Ong, S. E., and Mann, M. (2006) A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat. Protoc. 1* (6), 2650−2660.
(15) Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H., Geer, L. Y., Geer, R. C., Gonzales, N. R., Gwadz, M., Hurwitz, D. I., Jackson, J. D., Ke, Z., Lanczycki, C. J., Lu, F., Marchler, G. H., Mullokandov, M., Omelchenko, M. V., Robertson, C. L., Song, J. S., Thanki, N., Yamashita, R. A., Zhang, D., Zhang, N., Zheng, C., and Bryant, S. H. (2011) CDD: A Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res. 39* (Database Issue), D225−D229.
(16) Gesquiere, J. C., Diesis, E., Cung, M. T., and Tartar, A. (1989) Slow Isomerization of Some Proline-Containing Peptides Inducing Peak Splitting during Reversed-Phase High-Performance Liquid-Chromatography. *J. Chromatogr. 478* (1), 121−129.
(17) Grafl, R., Lang, K., Wrba, A., and Schmid, F. X. (1986) Folding mechanism of porcine ribonuclease. *J. Mol. Biol. 191* (2), 281−293.
(18) Lang, K., and Schmid, F. X. (1990) Role of two proline-containing turns in the folding of porcine ribonuclease. *J. Mol. Biol. 212* (1), 185−196.
(19) Abagyan, R., Totrov, M., and Kuznetsov, D. (1994) Icm: A New Method for Protein Modeling and Design: Applications to Docking and Structure Prediction from the Distorted Native Conformation. *J. Comput. Chem. 15* (5), 488−506.
(20) Nielsen, P. R., Nietlispach, D., Mott, H. R., Callaghan, J., Bannister, A., Kouzarides, T., Murzin, A. G., Murzina, N. V., and Laue, E. D. (2002) Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9. *Nature 416* (6876), 103−107.
(21) Jacobs, S. A., and Khorasanizadeh, S. (2002) Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. *Science 295* (5562), 2080−2083.
(22) Blus, B. J., Wiggins, K., and Khorasanizadeh, S. (2011) Epigenetic virtues of chromodomains. *Crit. Rev. Biochem. Mol. Biol. 46* (6), 507−526.
(23) Helbig, A. O., Gauci, S., Raijmakers, R., van Breukelen, B., Slijper, M., Mohammed, S., and Heck, A. J. (2010) Profiling of N-acetylated protein termini provides in-depth insights into the N-terminal nature of the proteome. *Mol. Cell. Proteomics 9* (5), 928−939.
(24) Polevoda, B., and Sherman, F. (2003) N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. *J. Mol. Biol. 325* (4), 595−622.