MethylScan®: Affinity Enrichment and Mass Spectrometry Analysis of Protein Methylation

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Introduction

Protein methylation is a post-translational modification that predominantly occurs on arginine and lysine residues. Arginine methylation is catalyzed by protein arginine methyltransferase (PRMT) that can be categorized into two major groups which are both capable of catalyzing the formation of monomethyl arginine (MMA). Type I PRMT is specifically responsible for adding a second methyl group to the same guanidino nitrogen atom of arginine to form asymmetric dimethylarginine (aDMA). Type II PRMT catalyzes the reaction of adding the second methyl group to a different guanidino nitrogen atom to form symmetric dimethylarginine (sDMA) [**Fig. 1**]. Many substrates identified for PRMTs are cytoplasmic and nuclear proteins indicating the potential roles of arginine methylation in RNA processing, transcriptional regulation, DNA damage repairs.

The methylation reaction of sequentially adding three methyl groups to lysine residues forming mono-methyl (K-Me), di-methyl (K-2Me) and tri-methyl (K-3Me) lysine are carried out by the protein lysine methyltransferases (PKMTs) enzyme family [**Fig. 1**]. Lysine methylation is best known for modifying residues of histone proteins, regulating chromatin compaction and gene transcription.

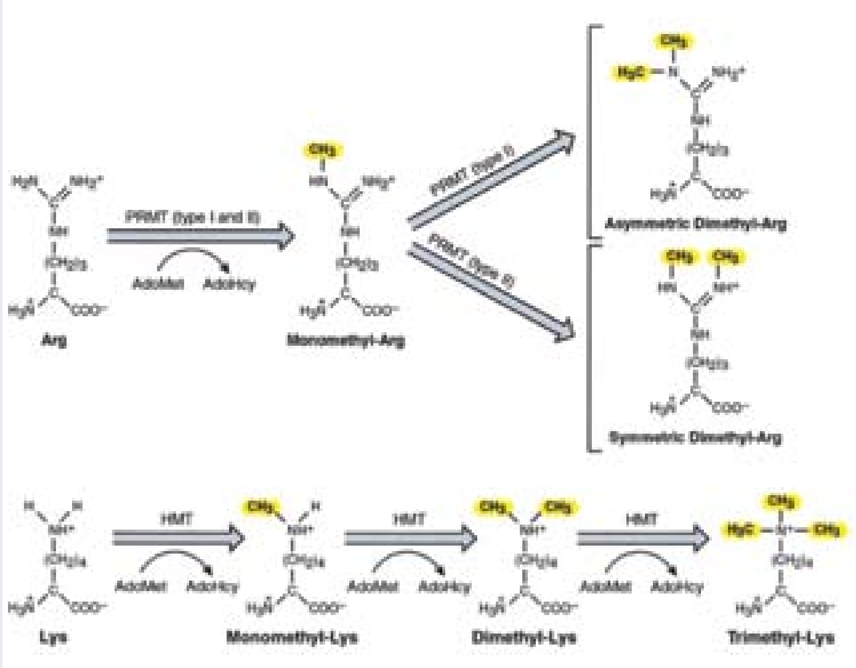


Figure 1: Chemistry of arginine and lysine methylation.

To achieve the quantitative analysis of protein methylation, we have developed a novel technology called MethylScan® utilizing immuno-affinity enrichment at the peptide level using a series of specific monoclonal antibodies against all types of protein methylation followed by LC-MS/MS analysis (IAP-MS). Facilitated by specific enrichment and high-throughput analysis by mass spectrometry, we have identified a large number of novel methylation sites on arginine and lysine from HCT116 cells; we also conducted a binary comparison of arginine methylation in mouse brain and embryo using MethylScan to reveal the quantitative change of over 1,000 sites of arginine methylation in fetal and adult tissues.

METHODS

CELL TREATMENT AND WESTERN BLOTS

HCT116 cells were cultured in DMEM medium with 10% FBS and treated with 20uM of AdOx for 24 hours, followed by another treatment of 20 µM of AdOx in new medium. Cells were lysed by a buffer containing 1% Triton X-100, protease and phosphatase inhibitors. Equal amounts of cleared cell lysate were loaded into SDS-PAGE and transferred to nitrocellulose membrane for western blots using specific methylation antibodies.

QUALITATIVE IDENTIFICATION OF METHYLATION SITES

Subconfluent HCT116 cells were harvest and lysed in 9M urea buffer containing phosphatase inhibitors. A general procedure for generating lyophilized tryptic peptides from whole cell lysates can be found in Stokes et al., 2012. Enrichment of methylated peptides was achieved by immunoprecipitation using a corresponding methyl antibody (250ug) from 10mg of tryptic peptides. Enriched peptides were analyzed by LC-MS/MS over a linear gradient of 72 min. MS/MS spectra were collected in data-dependent mode with a Q-Exactive mass spectrometer.

Raw data was searched against the NCBI human database and identified methylated peptides were filtered using a 5% FDR.

RESULTS

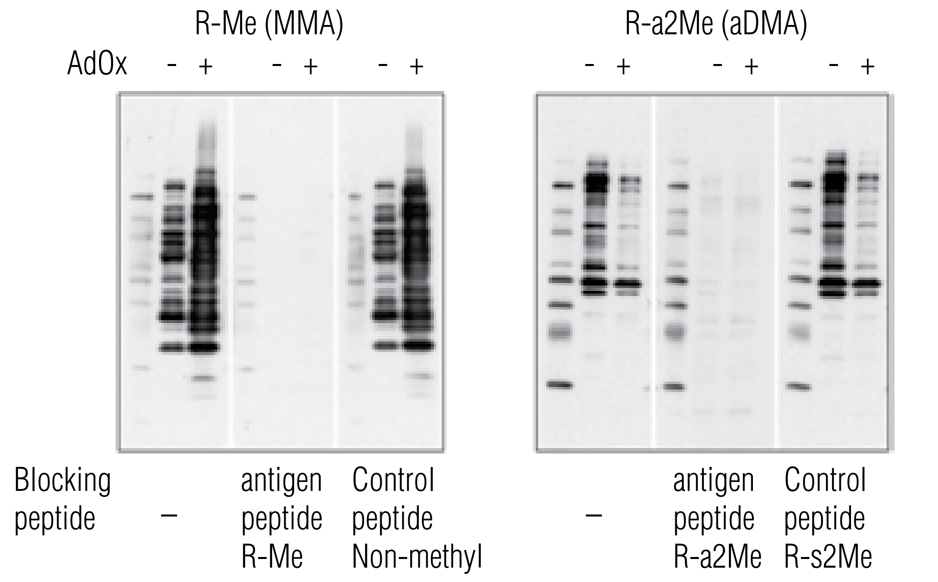


Figure 2: Methyl-Arginine antibodies show good specificities.

R-Me antibody signal can be blocked by antigen peptide but not the control peptide without a methyl group. R-a2Me signal can be blocked by antigen peptide with asymmetric di-methyl or arginine but not the control peptide with symmetric di-methyl on arginine.

Motif	Sites	Peptides	Protein
R-Me	1428	2005	718
R-a2Me	444	495	188
K-Me	245	293	204
K-2Me	99	157	75
K-3Me	43	68	36

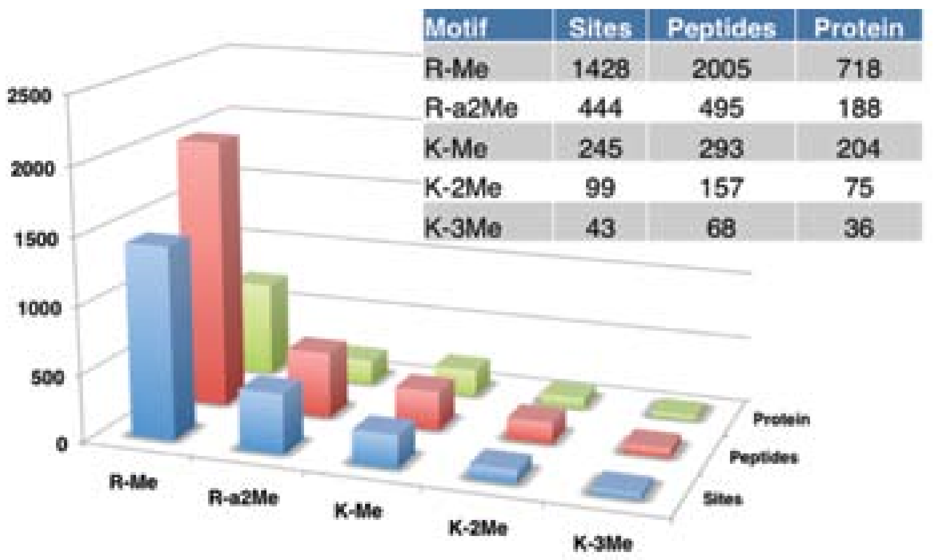


Figure 3: Statistical Summary of Sites, Peptides and Proteins with Methylation Identified by MethylScan from HCT116 Cells.

A 5% FDR was applied to filter the SORCERER results.

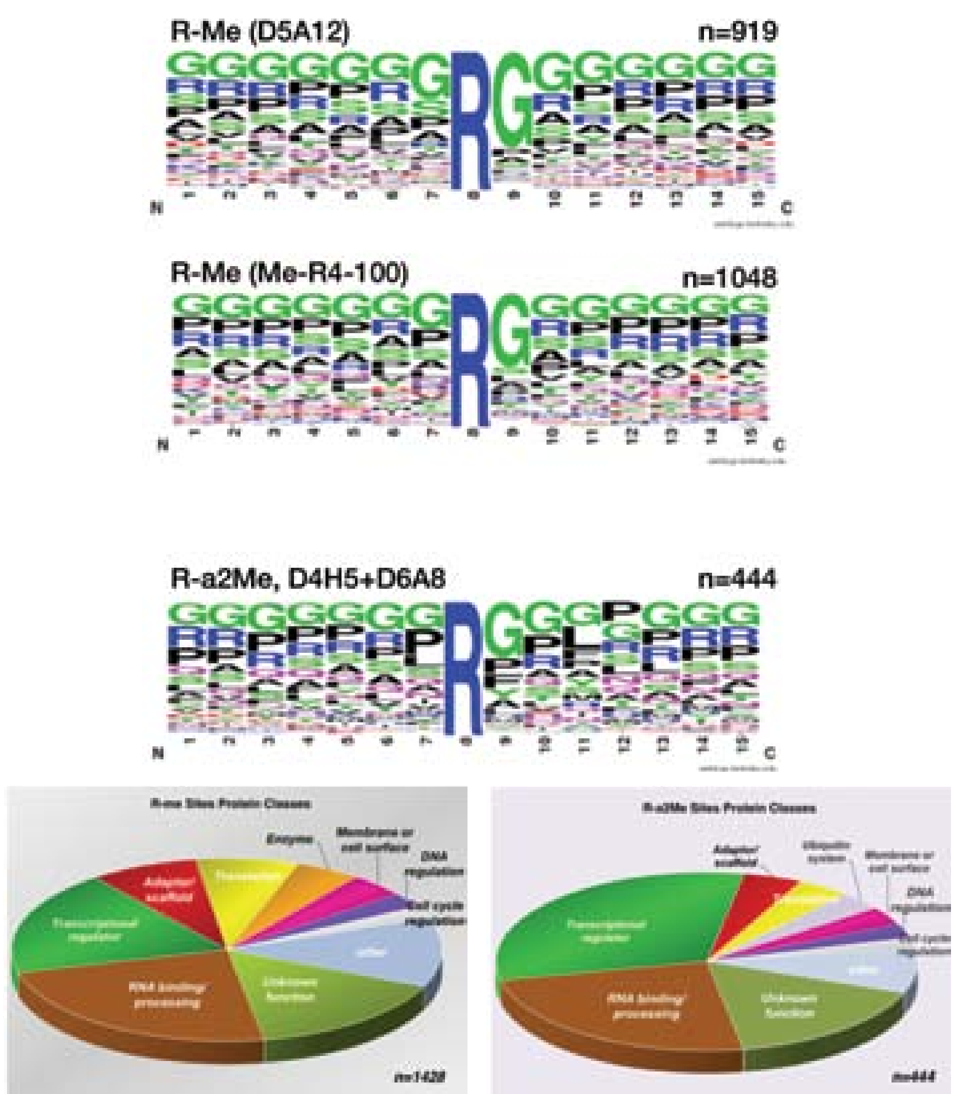


Figure 4: Motif Analysis and Functional Distribution of Arginine Methylation Identified in HCT-116 cell.

Both clones of R-Me antibodies (D5A12 and Me-R4-100) and R-a2Me antibody prefer binding methylated peptides with glycine enriched motif. Functional analysis shows methylations are abundantly involved in the regulation of transcription and RNA binding/processing.

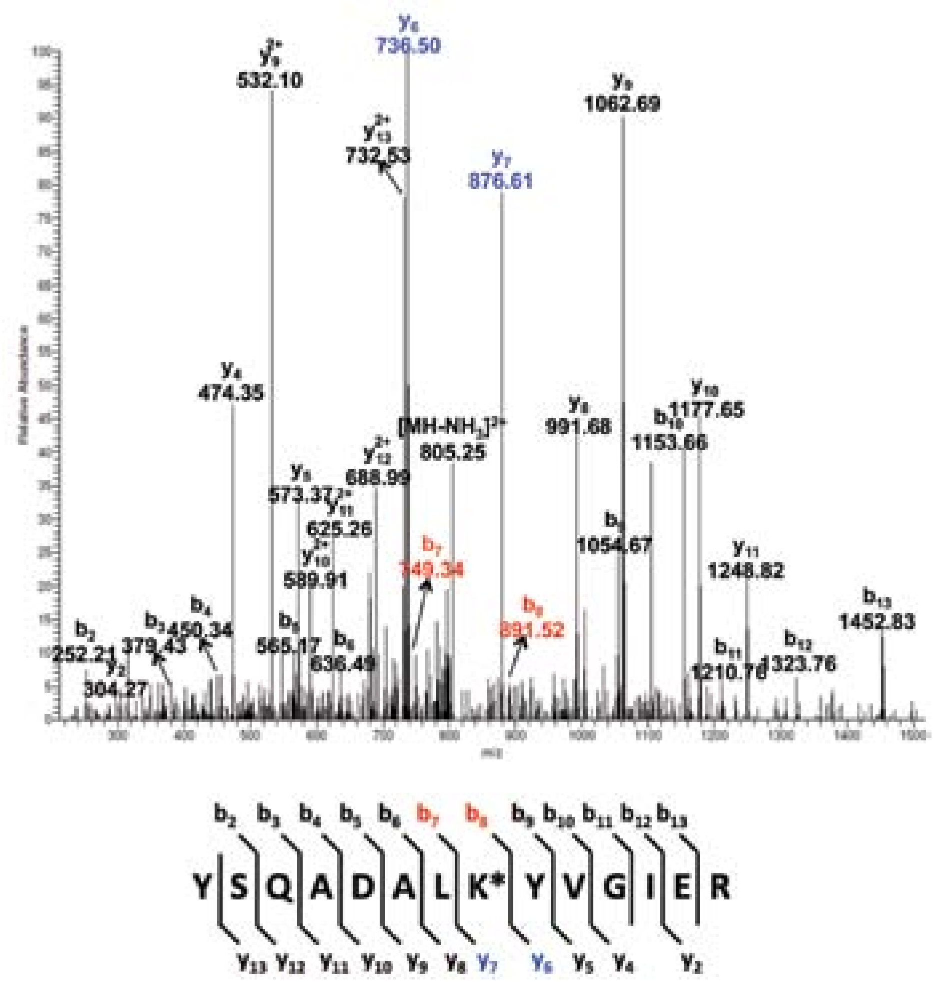


Figure 5: MS/MS spectrum of mono-methylation on K736 of Histone-lysine N-methyltransferase (EZH1).

The presence of both b and y ions flanking the modification site confidently localized the position of the methylation (highlighted in red and blue text).

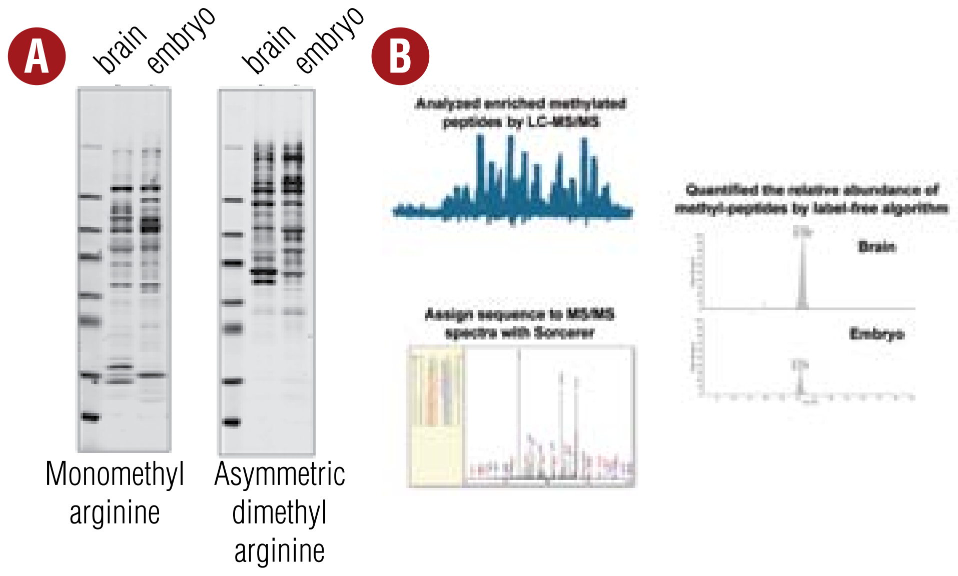
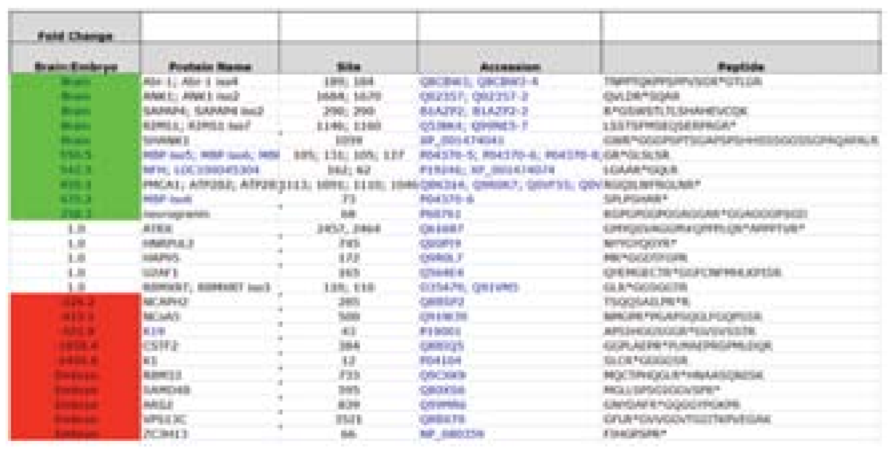

A
brain
embryo
brain
embryo
Monomethyl arginine
Asymmetric dimethyl arginine
B
Analyzed enriched methylated peptides by LC-MS/MS
Quantified the relative abundance of methyl-peptides by label-free algorithm
Assign sequence to MS/MS spectra with Sorcerer
Brain
Embryo

Figure 6: Quantification of mono-methylated peptides from mouse brain and embryo. (**A**) Different patterns of overexpression of arginine monomethylation and asymmetric dimethylation were observed by western blots using corresponding antibodies; (**B**) Scheme of quantification of relative abundance of methyl-peptides by in-house label free quantitation algorithm (LFQ): peptide sequence were identified by searching the raw MS/MS spectra against NCBI mus musculus database; filtered searching results containing arginine-methylation peptides were used for generating extracted ion chromatograms (EICs) from raw files; relative abundance ratio of particular arginine methylation between mouse brain and embryo was calculated using either peak height or peak area of the corresponding EICs.



Fold Change Brain:Embryo	Protein Name	Site	Accession	Peptide
Brain	[illegible]	[illegible]	[illegible]	[illegible]
Brain	[illegible]	[illegible]	[illegible]	[illegible]
Brain	[illegible]	[illegible]	[illegible]	[illegible]
Brain	[illegible]	[illegible]	[illegible]	[illegible]
Brain	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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1.0	[illegible]	[illegible]	[illegible]	[illegible]
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Embryo	[illegible]	[illegible]	[illegible]	[illegible]
Embryo	[illegible]	[illegible]	[illegible]	[illegible]
Embryo	[illegible]	[illegible]	[illegible]	[illegible]
Embryo	[illegible]	[illegible]	[illegible]	[illegible]
Embryo	[illegible]	[illegible]	[illegible]	[illegible]

Table 1: Representative arginine-methylation peptides from quantitative analysis.

Fold change for each methylation site is calculated by converting the ratio of peptide ion abundance from brain and embryo. "Brain" and "Embryo" in the fold change column indicate the methylation specifically existed in the tissue. The full table of quantitative analysis can be downloaded from www.cellsignal.com/services/methylation.html.

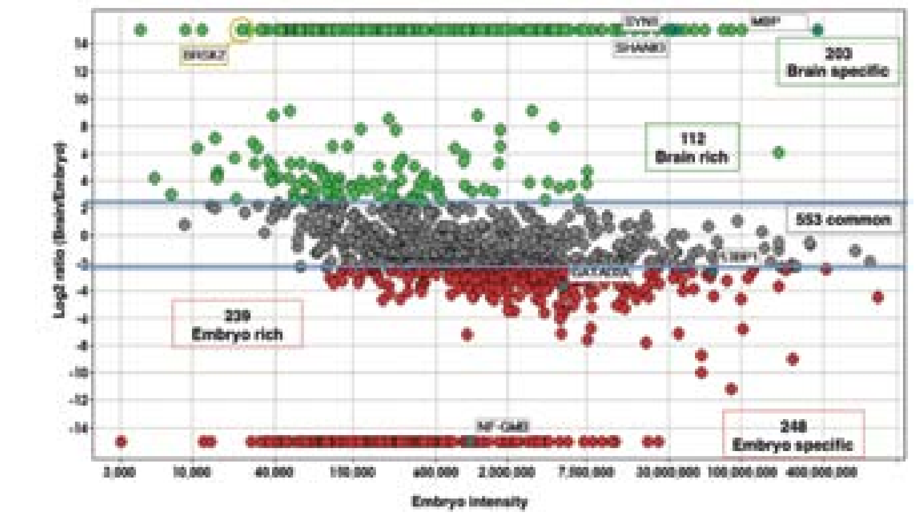

Figure 7: Scatter plot showing the log2 ratio of the abundance of the arginine-methylated peptides quantified from brain and embryo

Top region in green and bottom region in red are the peptides that are abundantly rich in brain and embryo, respectively. Tissue specific methylated peptides are assigned with the log2 ratio of 15 and -15 for brain and embryo.

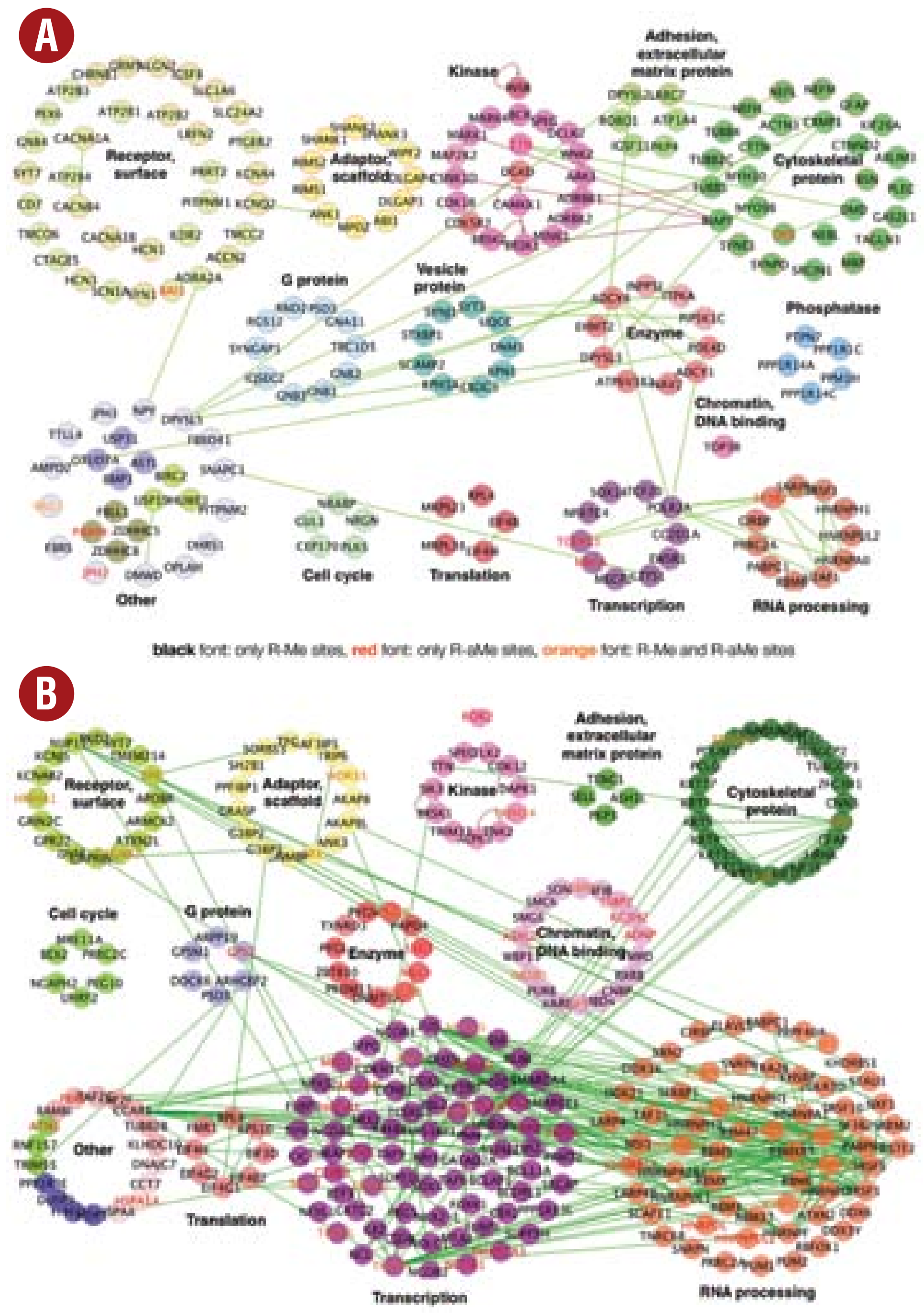
A
Receptor, surface
Adaptor, scaffold
Kinase
Adhesion, extracellular matrix protein
Cytoskeletal protein
G protein
Vesicle protein
Enzyme
Phosphatase
Chromatin, DNA binding
Other
Cell cycle
Translation
Transcription
RNA processing
black font: only R-Me sites, red font: only R-aMe sites, orange font: R-Me and R-aMe sites
B
Receptor, surface
Adaptor, scaffold
Kinase
Adhesion, extracellular matrix protein
Cytoskeletal protein
Cell cycle
G protein
Enzyme
Chromatin, DNA binding
Other
Translation
Transcription
RNA processing

Figure 8: Bioinformatic analysis of interactions between proteins with arginine methylation specifically enriched in mouse brain (A) and embryo (B).

Summary

:: A panel of monoclonal antibodies for protein methylation with high specificity was developed and validated.

:: A robust method for qualifying methylation by antibody affinity enrichment and Lc-MS/MS was established.

References

1. Zhang Y. et al. (2001) *Genes Dev.* 15: 2343–2360.
2. Stokes M. et al. (2012) *Mol. Cell Proteomics* 11:187–201.

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