A ROLE FOR ARGININE METHYLATION IN DNA REPAIR

by

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ABSTRACT

Arginine methylation is a post-translational modification occurring in higher eukaryotes that results in the addition of one or two methyl group on the nitrogen in the side chain of arginines. The enzymes responsible for protein arginine methylation have been classified in three groups. Type I enzymes promote the formation of both N^G-monomethylated and asymmetric ω-N^G,N^G-dimethylated arginines (aDMA). Type II enzymes catalyze the formation of monomethylated and symmetrical ω-N^G,N'^G-dimethylated arginines (sDMA). The type III enzyme found in yeast catalyzes the monomethylation of the δ-guanidino nitrogen atom of the arginine residue. Although some abundant proteins have been described as being substrates for arginine methyltransferases for some time, there are still few known proteins to bear this modification. The primary goal of the work presented in this thesis was to identify new arginine methylated proteins and functionally characterize the roles of arginine methylation in new cellular processes. First, we generated four arginine methyl-specific antibodies: ASYM24 and ASYM25 are specific for aDMA whereas SYM10 and SYM11 recognize sDMA. Cell extracts were used to purify the protein complexes recognized by each of the four antibodies and the proteins were identified by microcapillary reverse-phase liquid chromatography coupled on line with electrospray ionization tandem mass spectrometry (LC/MS/MS). The analysis of 2 tandem mass spectra for each methyl-specific antibody resulted in the identification of 247 proteins, of which 197 are putatively arginine methylated.

The DNA repair MRE11/ RAD50/ NBS1 (MRN) complex was purified using one of the aDMA specific antibody. Since a role of protein arginine methylation in DNA damage checkpoint control and DNA repair had not been previously reported we chose to investigate the consequence of MRE11 methylation in DNA damage. Our results show that the MRE11 checkpoint protein is arginine methylated as determined by mass

spectrometry and methylarginine-specific antibodies. The glycine-arginine rich (GAR) domain of MRE11 was specifically methylated by protein arginine methyltransferase 1 (PRMT1). Mutation of the arginines within MRE11 GAR domain severely impaired the exonuclease activity of MRE11. Cells treated with methyltransferase inhibitors displayed a DNA damage-resistant DNA synthesis phenotype and prevented the re-localization of the MRN complex to sites of DNA damage. Downregulation of PRMT1 with small interfering RNAs (siRNA) also yielded a damage-resistant DNA synthesis phenotype that was rescued with the methylated MRE11 complex. Taken together, the work presented in this thesis allowed the identification of many new potentially arginine methylated proteins and demonstrated a novel role for arginine methylation in the regulation of DNA repair enzymes and of the intra-S phase DNA damage checkpoint.

SOMMAIRE

La méthylation des arginines est une modification post-traductionnelle qu'on retrouve chez les eucaryotes supérieurs et qui consiste en l'addition d'un ou deux groupements méthylé sur les azotes des arginines. Les enzymes responsables pour la méthylation des arginines dans les protéines ont été divisés en trois groupes. Les enzymes de type I catalysent la formation d'arginines ω-N^G-monométhylées et d'arginines asymétriquement ω-N^G,N^G-diméthylées (aDMA). Les enzymes de type II catalysent la formation d'arginines ω-N^G-monométhylées et d'arginines symétriquement ω-N^G,N'^G-diméthylées (aDMA). Les enzymes de type III qu'on retrouve chez les levures et certaines plantes catalysent la formation d'arginines monométhylées sur l'azote δ-guanidino. Même si quelques protéines abondantes ont été décrites comme étant des substrats des méthyltransferase, il y a jusqu'à maintenant très peu de protéines connues comme étant méthylées sur arginines. Le but principal du travail présenté dans cette thèse consiste à identifier de nouvelles protéines qui sont méthylées sur des arginines et de caractériser le rôle de cette modification dans d'autres fonctions de la cellule. Tout d'abord, nous avons générés quatre anticorps reconnaissant spécifiquement les arginines méthylées: ASYM24 et ASYM25 sont spécifiques pour les aDMAs alors que SYM10 et SYM11 reconnaissent les sDMA. Des extraits cellulaires ont ensuite été utilisé pour purifier des complexes protéiques à l'aide de ces anticorps et les protéines ainsi purifiées ont été identifié par chromatographie liquide à phase reversé sur microcapillaire couplé à un spectromètre de masse en tandem (LC/MS/MS). L'analyse de deux spectres pour chaque anticorps a permis l'identification de plus de 200 nouvelles protéines qui sont potentiellement méthylées sur arginines.

Le complexe MRE11/RAD50/NBS1 (MRN) qui est impliqué dans la réparation de l'ADN a été purifié en utilisant un des anticorps reconnaissant les aDMAs. Un rôle pour la méthylation des arginines dans le contrôle du cycle cellulaire suite à des

dommages à l'ADN et dans la régulation des mécanismes de réparation de l'ADN était jusqu'ici inconnu. Nos résultats démontrent, à l'aide de spectromètre de masse et des anticorps spécifiques aux arginines méthylées que l'exonucléase MRE11 est asymétriquement diméthylé sur des arginines. Le domaine riche en glycines et en arginines (GAR) de MRE11 est spécifiquement méthylé par l'enzyme protéine arginine methyltransferase 1 (PRMT1). La mutation des arginines dans le domaine GAR de MRE11 entraîne une sévère diminution de l'activité exonucléase. Des cellules traitées avec des inhibiteurs de méthyltransférases continuent la réplication de l'ADN malgré la présence de dommages à l'ADN et MRE11 ne peut plus être recruté aux sites de dommages à l'ADN. La diminution de PRMT1 à l'aide de petit ARN d'interférences (siRNA) permet aussi la réplication de l'ADN en présence de dommage à l'ADN et ce phénotype peut être rescapé par la réintroduction du complexe MRN méthylé. Le travail présenté dans cette thèse a permis l'identification d'une multitude de nouvelles protéines potentiellement méthylées sur des arginines et démontra un nouveau rôle pour la méthylation des arginines dans la régulation des mécanismes de réparation de l'ADN.

PREFACE

This Ph.D. thesis was written in accordance with the Guidelines for Thesis preparation from the Faculty of Graduate Studies and Research of McGill University. I have exercised the option of writing the thesis as a manuscript-based thesis. For this, the guidelines state: "...Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. .....The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory. ....The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary. ....In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. .....In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. ....."

As chapters of this thesis, I have included the texts and figures of three original research manuscripts that have been published, accepted or submitted for publication. Each of these chapters (Chapters 2, 3, and 4) contains its own summary, introduction,

materials and methods, results, discussion, and references sections. In addition, a preface is included at the beginning of each chapter in order to introduce and bridge the papers with connecting texts. A general introduction and literature review is presented in Chapter 1, whereas a final discussion is included in Chapter 5. The references for chapters 1 and 5 are included at the end of the thesis.

Papers included in this Thesis:

Introduction Boisvert FM., Chénard, CA. and Richard S. **(2005)** Protein Interfaces in Signaling Regulated by Arginine Methylation. *Science STKE* **271**, 1319-1330.

Chapter 2 Boisvert FM.#, Coté J.#, Boulanger MC. and Richard S. **(2003)** A Proteomic Analysis of Arginine-methylated Protein Complexes. *Molecular and Cellular Proteomics* **2** (12), 1319-1330.

#These authors contributed equally to this work.

Chapter 3 Boisvert FM., Déry U., Masson JY. and Richard S. **(2005)** Arginine methylation of MRE11 by PRMT1 is required for the intra-S-phase DNA damage checkpoint. *Genes and Development* **19**(6), 671-676.

Chapter 4 Boisvert FM., Hendzel MJ., Masson JY. and Richard S. **(2005)** MRE11 and PRMT1 associates at PML nuclear bodies (Submitted to *Journal of Cell Science* 28-03-2005).

Contribution of Authors:

The candidate performed most of the research presented in this thesis and wrote all of the included manuscripts with support from Dr Stéphane Richard. The contribution of other authors to this work is described below:

In Chapter 2, Dr Jocelyn Côté generated the methylarginine specific antibodies and helped with the large scale immunoprecipitations. Marie-Chloé Boulanger provided assistance with the western blots in figure 2.1. Alexandre Forget-Richard helped with identifying proteins from the list of peptides generated through LC/MS/MS using BLAST. In Chapter 3, Ugo Déry and Jean-Yves Masson purified the wild-type and mutant MRN complex from baculovirus-infected insect cells and performed the exonucleases assays. In Chapter 4, Dr Michael Hendzel provided expertise and access to his confocal microscopes. In Chapter 3 and 4, Jean-Yves Masson provided DNA damage expertise and critical analysis of the work.

The different studies were all conducted under the supervision of Dr Stéphane Richard.

Contributions not included in this Thesis:

In addition to the papers included in this thesis, the candidate contributed to the following studies, which have been published or submitted:

Bachand, F., Boisvert, FM., Coté, J., Richard, S., and C. Autexier. **(2002)** The product of the *survival of motor neuron* (*SMN*) gene is a human telomerase-associated protein. *Molecular Biology of the Cell* **13**: 3192-3202.

Boisvert FM#, Coté J#, Boulanger MC, Cléroux P, Bachand F, Autexier C and Richard S. **(2002)** Symmetrical dimethylarginine methylation is required for the localization of SMN in Cajal bodies and pre-mRNA splicing. *The Journal of Cell Biology* **159** (6), 957-969. #These authors contributed equally to this work.

Coté J#, Boisvert FM#, Boulanger MC, Bedford MT and Richard S. **(2003)** Sam68 RNA binding protein is an in vivo substrate for protein arginine N-methyltransferase 1. *Molecular Biology of the Cell* **14** (1), 274-287.

#These authors contributed equally to this work.

Boisvert, FM. and Richard, S. **(2004)** Arginine methylation regulates the cytokine response. *Molecular Cell* **15** (4): 492-494.

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LIST OF ABBREVIATIONS

53BP1	P53 binding protein-1
a.a	Amino acids
aDMA	Asymmetrically dimethylated arginine
Adox	Adenosine dialdehyde
Air1p	Arginine methyltransferase-interacting RING finger protein
A-T	Ataxia-telangiectasia
ATL-D	Ataxia-Telangiectasia like disorder
ATM	Ataxia Telangiectasia Mutated
ATR	ATM kinase related protein
bFGF-2	Basic fibroblast growth factor 2
BRCA1	Breast Cancer Susceptibility gene 1
BRCT	Breast-cancer-associated BRCA-1 protein C-terminal region
BTG	B-cell translocation gene
cAMP	Cyclic adenosine 3',5'-cyclic monophosphate
CARM1	Co-activator methyltransferase
CBP	CREB binding protein
cDNA	Complementary DNA
Chk2	Checkpoint kinase 2
CIRP	Cold-inducible RNA binding protein
CoA	Coenzyme A
CREB	Cyclic-AMP response element binding
DART	Drosophila arginine methyltransferase
DDAH	Dimethylarginine dimethylaminohydrolase
DNA	Desoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase

EBNA	Epstein-Barr nuclear antigen
EGF	Epidermal growth factor
ES Cells	Embryonic stem cells
EWS	Ewing sarcoma
FGF	Fibroblast growth factor
GAR	Glycine- and arginine-rich
GFP	green fluorescent protein
GRIP1	Glucocorticoid receptor interacting protein-1
GSG	GRP33, Sam68, GLD-1
HAT	Histone acetyltransferase
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HMT1	heterogeneous nuclear ribonucleoprotein methyltransferase 1
Hrp1p	Heterogeneous nuclear ribonucleoprotein
HSL7	Histone synthetic lethal-7
HuR	Hu antigen protein R
hnRNP	heterogeneous nuclear ribonucleoprotein
HRMT	HnRNP Arginine Methyltransferase
IFN	Interferon
IgG	Immunoglobulin
ILF3	Interleukin enhancer-binding factor 3
IP	Immunoprecipitation
Ire15	Inositol-requiring phenotype-15
JAK	Janus kinase
JBP1	JAK2 binding protein-1
kb	kilobases

kDa	kilo Dalton
KH	HnRNP K homology
KID	Kinase inducible domain
KIX	Kinase inducible interacting
LC/MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LPS	Lipopolysaccharide
LSm	Sm-like protein
MBP	Myelin basic protein
MDC1	Mediator of DNA damage checkpoint protein 1
MMA	Monomethylated arginine
MRE11	Meiotic Recombination Mutant 11
MRN	MRE11/RAD50/NBS1
mRNA	Messenger RNA
MS	mass spectrometry
MTA	5'-deoxy-5'(methylthio)adenosine
Mtr10p	MRNA transport protein 10
NBS	Nijmegen breakage syndrome
NF-AT	Nuclear factor of Activated T Cells
NGF	Nerve growth factor
NIP45	NF-AT interacting protein of 45 KDa
Npl3	Nuclear protein localization 3
NRS	Normal rabbit serum
PABP	Poly-A binding protein
PAD	Peptidylarginine deiminase
PC3	NGF induced gene 3 in PC12 cells
pICln	Chlorine channel regulator
PIKK	Phosphatidylinositol 3-kinase protein kinase-like

PML	Promyeocytic Leukaemia
PRMT	Protein arginine methyltransferase
RAD50	Radiation sensitive gene 50
RAD51	Radiation sensitive gene 51
RBPs	RNA binding proteins
RDS	Radioresistant DNA synthesis
RHA	RNA helicase A
RNA	Ribonucleic acid
RNAi	RNA interference
RNPs	Ribonucleoproteins
RPA2	Replication protein A2
RRE	Rev response element
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
Sam68	Src-associated-in-mitosis protein of 68 kDa
sDMA	Symmetrically dimethylated arginine
SET	Suvar3-9, Enhancer-of-zeste, Trithorax
Sky1p	SR protein-specific kinase 1
SH2	Src homology 2 domain
SH3	Src homology 3 domain
siRNA	Small interfering RNA
SKB1	Shk1 binding protein 1
Shk1	Ste20 homolog kinase 1
SLM	Sam68-like mammalian proteins
Sm	Small nuclear ribonucleoprotein
SMA	Spinal muscular atrophy
SMN	Survival of motor neuron

SNB	Sam68 nuclear bodies
snRNP	Small nuclear ribonucleoparticle
SPT5	Suppressor of Ty's-5
SRC-2	Steroid receptor coactivator 2
STAR	Signal transduction and activator of RNA
STAT1	Signal transducer and activator of transcription-1
TARPP	Thymocyte cyclic AMP-regulated phosphoprotein
TCL	Total cell lysate
TcPTP	T-cell protein tyrosine phosphatase
TCR	T-cell receptor
TGFβ	Tumor growth factor β
Th	T helper cells
TIF2	Transcriptional mediators/intermediary factors 2
TIS21	Tetradecanoyl phorbol acetate-inducible sequence-21
TNF-α	Tumor necrosis factor-α
TREX	Transcription/export
UV	Ultraviolet
VSV	Vesicular stomatitis virus
WW	Tryptophane Tryptophane
XRS2	X-Ray sensitive gene 2
YY1	Yin Yang 1

Chapter 1

Introduction and Literature Review

1.1 General Introduction

Arginine methylation is a post-translational modification that consists in the addition of methyl group to the nitrogen on the side chain of arginines. Although arginine methylation has been known since 1967 (Paik and Kim, 1967), arginine methylated proteins and the role of this modification are still poorly characterized. The objective of this thesis was to identify new arginine methylated proteins and understand the consequences on the properties and functions of some of those newly identified arginine methylated proteins. The first Chapter will describe the known arginine methylated proteins and discuss the known roles of this post-translational modification in cells and organisms. Chapter 2 will describe a proteomic approach used to identify previously unknown arginine methylated proteins. Chapter 3 will describe a new role for arginine methylation in the regulation of DNA repair, through the modulation of the exonuclease activity of MRE11 by arginine methylation. Chapter 4 covers the localization of PRMT1 and MRE11 within PML nuclear bodies and describes the effect of methylation on the recruitment of MRE11 to sites of DNA damage. Finally, in chapter 5, I will discuss the importance of the findings presented in this thesis.

1.2 Arginine methylation: The reaction

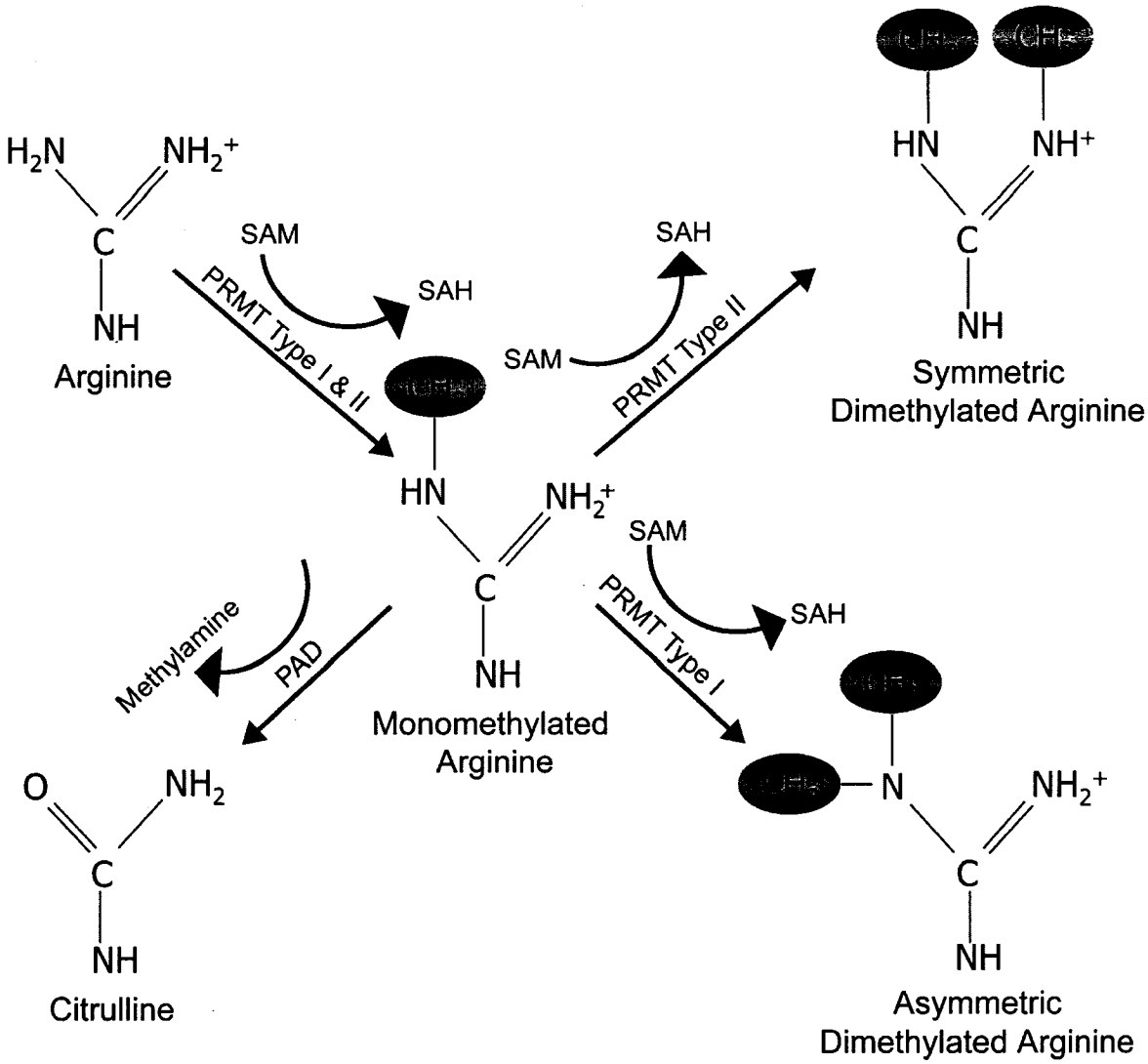
1.2.1 Methylated Arginines

Arginine is a positively charged amino acid that often mediates hydrogen bonding and amino aromatic interactions with proteins and nucleic acids. Arginines may be post-translationally modified to contain methyl groups in a process termed arginine methylation

(Gary and Clarke, 1998a). Arginines can also be cleaved by enzymes such as peptidylarginine deiminases (PADs) and dimethylarginine dimethylaminohydrolase (DDAH) to generate citrulline (Gary and Clarke, 1998a) and ornithine (Tran et al., 2003). Protein arginine methylation is a post-translational modification that adds mono-methyl or dimethyl groups to the guanidino nitrogen atoms of arginine (Gary and Clarke, 1998a) using S-adenosylmethionine (SAM) as the methyl group donor (Atkinson and Murray, 1967). Three main forms of arginine methylation have been identified in eukaryotes: ω-N^G, monomethylarginines (MMA), ω-N^G,N^G-asymmetric dimethylarginines (aDMA), and ω-N^G,N'^G-symmetric dimethylarginines (sDMA) (Figure 1.1). A fourth type of arginine methylation has only been found in yeast and plants and consist of the methylation of the delta nitrogen of the arginine side-chain (Niewmierzycka and Clarke, 1999; Zobel-Thropp et al., 1998). Arginine methylation does not alter the positive charge of the arginine, but rather increases its bulkiness, blocks hydrogen bonding, and increases its hydrophobicity.

Figure 1.1 Methylation of arginines.

Arginine can be methylated on a guanidino nitrogen atom by type I and II protein arginine methyltransferases (PRMT) to become monomethylated. Symmetrically dimethylated arginines result from the addition of a second methyl group to the opposite nitrogen atom by type II enzymes, whereas asymmetrically dimethylated arginines result from the addition of a second methyl group to the same nitrogen with type I enzymes using SAM as a methyl donor releasing S-adenosylhomocysteine (SAH). Monomethylarginines can also be deimidated by peptidylarginine deiminases (PADs) resulting in the formation of a citrulline.


Arginine
SAM
SAH
PRMT Type I & II
Monomethylated Arginine
SAM
SAH
PRMT Type II
Symmetric Dimethylated Arginine
Methylamine
PAD
Citrulline
SAM
SAH
PRMT Type I
Asymmetric Dimethylated Arginine

1.2.2 Protein Arginine Methyltransferases

In humans, protein arginine methyltransferases (PRMT) represent a family of eight enzymes that utilize S-adenosyl methionine (SAM) as a methyl donor (for reviews see (Boisvert et al., 2005a; Gary and Clarke, 1998a; McBride and Silver, 2001b) and Table 1.1). The characteristics of PRMTs include the presence of a highly conserved methyltransferase domain that oligomerizes into a ring-like structure (Weiss et al., 2000; Zhang and Cheng, 2003; Zhang et al., 2000). This domain is subdivided in 4 different conserved regions called I, post I, II and III (Kagan and Clarke, 1994; Schluckebier et al., 1995). Little structure-function analysis has been performed on the PRMTs to identify their regulatory domains. The PRMTs are classified in two groups: type I and type II enzymes. There are five known type I PRMTs that catalyze the formation of MMA and aDMA. They include PRMT1 (Lin et al., 1996b), PRMT2 (Scott et al., 1998a), PRMT3 (Tang et al., 1998a), PRMT4 or CARM1 for co-activator methyltransferase (Chen et al., 1999a) and PRMT6 (Frankel et al., 2002). There exist 2 type II enzymes that catalyze the formation of MMA and sDMA: PRMT5 (Pollack et al., 1999a) and PRMT7 (Lee et al., 2005b). HRMT1L3 (accession number AAF91390), herein called PRMT8 has ~ 80% sequence identity with PRMT1 and is most likely a type I enzyme because of its similarity to PRMT1.

Homo Sapiens		S. Cerevisiae		S. Pombe		D. Melanogaster	
PRMT1	Q99873	HMT1	P38074	PRMT1	NP_594825	DART1	NP_650017
PRMT2	P55345						
PRMT3	O60678			PRMT3	NP_595572	DART3	NP_650434
PRMT4/CARM1	NP_954592					DART4	NP_649963
PRMT5/JBP1	O14744	HSL7	P38274	SKB1	P78963	DART5	NP_477184
PRMT6	Q96LA8					DART8	NP_609478
PRMT7	NP_061896					DART7	NP_611753
PRMT8/HRMT1L3	AAF91390						
PRMT9	Q9DD20						
						DART2	NP_608821
						DART6	NP_650322
						DART9	NP_650321

Table 1.1 Arginine methyltransferases.

1.2.2.1 PRMT1

PRMT1 was the first mammalian methyltransferase identified in a yeast two-hybrid screen for rat cDNA encoding proteins interacting with the negative cell cycle regulators TIS21 (Lin et al., 1996b). There are three known alternatively spliced isoforms of PRMT1 (Scott et al., 1998a), some of which are differentially regulated in breast cancer cells (Scorilas et al., 2000). Mice null for PRMT1 are lethal at the embryonic stage 6.5 and the few embryos analyzed had growth retardation phenotype (Pawlak et al., 2000). However PRMT1 is not required for embryonic stem (ES) cell viability, suggesting a role for PRMT1 in development but not in cellular metabolism (Pawlak et al., 2000). PRMT1 was found to be the major arginine methyltransferase, accounting for 54% of methylated arginines and 85% of asymmetric dimethylargininines in the cell (Pawlak et al., 2000). A growing list of substrates have been identified for PRMT1 (see Table 1.2), most of them consisting of hnRNPs and nucleic acid binding proteins. Thus, it is no surprise that PRMT1 has been involved in many pathways involving RNA biogenesis and regulation.

1.2.2.2 PRMT2

PRMT2 was identified by sequence homology to the rat PRMT1 (Katsanis et al., 1997). It contains a Src homology 3 domain (SH3) that is located at the N-terminal of the methyltransferase domain (Scott et al., 1998a), which has been found to be essential for interaction with the adenovirus early protein E1B-55 kDa (Kzhyshkowska et al., 2001). However, the protein harbors no methyltransferase activity both *in vitro* and *in vivo*, and no protein substrates have been identified.

1.2.2.3 PRMT3

PRMT3 was identified by yeast two-hybrid as a PRMT1 interacting protein (Tang et al., 1998a). PRMT3 is a cytoplasmic methyltransferase that features a C2H2 zinc-finger

domain in its amino terminal that is not required for methyltransferase activity but has been proposed to confer substrate specificity (Frankel and Clarke, 2000). *Schizosaccharomyces pombe* lacking PRMT3 are viable and display an accumulation of free 60S ribosomal subunits, which implies a role for PRMT3 in ribosome biosynthesis and protein translation (Bachand and Silver, 2004a). Consistent with this role, the two known substrates of PRMT3 are the ribosomal protein S2 and the poly-A binding protein II (PABP II) (Bachand and Silver, 2004a; Swiercz et al., 2005).

1.2.2.4 CARM1

Coactivator associated arginine methyltransferase 1 (CARM1/PRMT4) was identified as binding the transcriptional coactivator SRC-2/TIF2/GRIP1-binding protein (Chen et al., 1999a). It has been shown to synergistically stimulate transcription by nuclear receptors in combination with the p160 family of coactivators in a complex including the histone acetyltransferase p300/CBP and the transcriptional co-activator SRC-2/TIF2/GRIP1 (Koh et al., 2001; Lee et al., 2002). CARM1 specifically methylates arginine 17 and 26 in the N-terminal regulatory tail of histone H3 as part of the transcriptional activation process (Bauer et al., 2002a; Daujat et al., 2002). Mice with a targeted disruption of CARM1 die during late embryonic development or immediately after birth, underlining the importance of CARM1 during late embryonic development (Yadav et al., 2003). Apart from histone H3, CARM1 has been shown to methylate p300, CBP, ILF3, PABP, HuR and TARPP on arginines that are not necessarily surrounded by glycines, indicating a different specificity compared to the other methyltransferases (Lee and Bedford, 2002a).

1.2.2.5 PRMT5

PRMT5 was the first type II enzyme shown to catalyze the formation of sDMA (Branscombe et al., 2001). It has been shown to methylate myelin basic protein (MBP) and

histones on arginines *in vitro* (Pollack et al., 1999a). PRMT5 resides in a cytoplasmic 20S complex with pICln called the methylosome and has methyltransferase activity towards SmB, B', D1 and D3 proteins (Brahms et al., 2001a; Friesen et al., 2001b; Meister et al., 2001). It has been proposed that the methylation of Sm proteins in their C-terminus directed the *in vivo* assembly of these proteins into a mature spliceosomal snRNP prior to their nuclear import. This assembly is dictated by a direct, methyl-dependant interaction with SMN (Friesen et al., 2002; Meister and Fischer, 2002). Symmetrical dimethylation has also been shown to be required for SMN localization in Cajal Bodies and is essential for the splicing reaction (Boisvert et al., 2002). SPT5, EBNA-2, p80-Coilin, Sm B, B', D1 and D3, as well as the Sm-like protein LSm4 are the only known substrates for PRMT5 (Baldwin and Carnegie, 1971; Brahms et al., 2001a; Brahms et al., 2000a; Friesen et al., 2001b; Meister et al., 2001).

1.2.2.6 PRMT6

In searching the human genome for protein arginine methyltransferases family members, Frankel and colleagues found a novel gene that encodes an apparent methyltransferase (Frankel et al., 2002). The enzyme was shown to catalyzed the formation of asymmetric dimethylarginines (Frankel et al., 2002). A comparison of substrate specificity demonstrated that PRMT6 had distinct substrate specificity and PRMT6 was shown to be the first arginine methyltransferase that can automethylate itself (Frankel et al., 2002). The HIV-1 transactivator protein Tat is the only known substrate of PRMT6 (Boulanger et al., 2005), indicating a possible role for PRMT6 in the cellular response to viral infection. Interestingly, the methylated arginine is not present in a glycine rich environment, confirming the difference in substrate specificity of this enzyme (Boulanger et al., 2005).

1.2.2.7 PRMT7

PRMT7 was originally found in a genetic screen aimed at identifying proteins conferring resistance to a topoisomerase II inhibitor (Gros et al., 2003). PRMT7 is different then other PRMTs as it contains two S-adenosylmethionine binding domain that are both required for the methyltransferase activity (Miranda et al., 2004a). PRMT7 has been shown to catalyze the formation of MMA (Miranda et al., 2004a) and can also catalyze the formation of sDMA (Lee et al., 2005b), demonstrating that PRMT7 (like PRMT5) is a type II methyltransferase. PRMT7 has been shown to be able to methylate histone H4, myelin basic protein (MBP) and SmD3 *in vitro* (Lee et al., 2005b), but no *in vivo* substrates have been confirmed so far and it remains to be shown why PRMT7 is implicated in the cell resistance to DNA damage.

1.2.2.8 PRMT8

HRMT1L3 (accession number AAF91390), herein called PRMT8 has ~ 80% sequence identity with PRMT1 and is most likely a type I enzyme because of its similarity with PRMT1. However, neither the function nor the activity of PRMT8 has been determined.

1.2.2.9 The Golgi Methyltransferase

An arginine methyltransferase activity was detected in the golgi in a recent proteomic study that identified numerous arginine methylated proteins (Wu et al., 2004). A protein with the accession number Q9DD20 is the enzyme proposed to mediate this activity (Wu et al., 2004). However, its amino acid sequence diverges from that of the known arginine methyltransferase and it remains to be demonstrated whether or not it is a bona fide PRMT.

1.2.2.10 PRMTs in other organisms

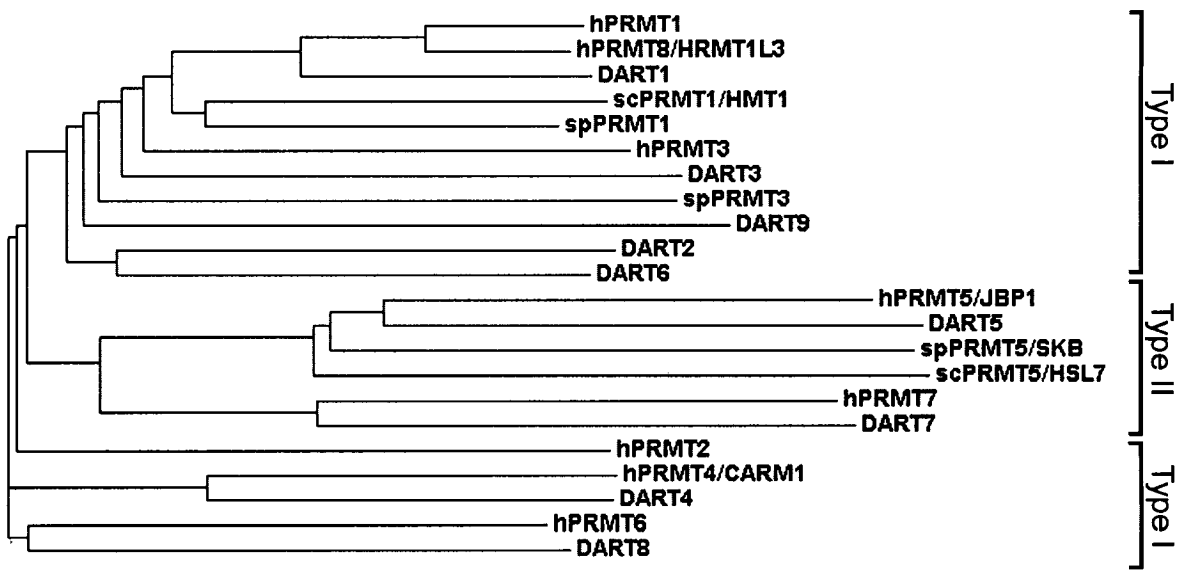
Saccharomyces cerevisiae has one major type I enzyme, hnRNP (heterogeneous nuclear ribo nucleoprotein) methyltransferase 1 (HMT1), that accounts for 85% of all arginine methylation (Gary et al., 1996; Henry and Silver, 1996). HMT1 is the homolog of PRMT1 in human. HMT1 is not essential for cell growth as *hmt1*Δ yeast are viable, but such cells fail to export RNA binding proteins (Green et al., 2002; Shen et al., 1998a; Yu et al., 2004). *S. cerevisiae* also has an enzyme with type II activity called histone synthetic lethal-7 (HSL7) (Lee et al., 2000). Type I and II activities were also identified in *Schizosaccharomyces pombe*. Type I enzymes include the homologs of PRMT1 and PRMT3 herein called spPRMT1 and spPRMT3. *S. pombe* deleted for spPRMT3 are viable and display an accumulation of free 60S ribosomal subunits, implying a role for spPRMT3 in ribosome biosynthesis (Bachand and Silver, 2004a). The role of PRMT3 in ribosomal function has been confirmed in higher eukaryotes, where PRMT3 interacts with and methylates the ribosomal protein rpS2 (Swiercz et al., 2005). *S. pombe* also contains type II activity and the methyltransferase is called SKB1 (Shk1 kinase binding protein), the homolog of PRMT5 (Ma, 2000). Other eukaryotes exhibit type I and type II arginine methyltransferase activity including plants (Disa et al., 1986), the nematode *Caenorhabditis elegans*, the puffer fish *Fugu rubripes,* and the zebrafish *Danio rerio* (Hung and Li, 2004), *Trypanosome brucei* (Pelletier et al., 2001), and the fruitfly *Drosophila melanogaster* (Boulanger et al., 2004). The latter PRMTs are named DART1 to DART9 for *Drosophila* arginine methyltransferases 1-9 (Boulanger et al., 2004).

To compare the evolution of type I and type II PRMTs, we constructed a phylogenetic tree with mammalian, yeast and *Drosophila* enzymes (Figure 1.2). The PRMTs are distributed in three major branches. The first branch (top, Figure 1.2) represents the type I enzymes including hPRMT1, hPRMT8, DART1, HMT1, spPRMT1, hPRMT3, DART3, spPRMT3, DART9, DART2 and DART6. The second branch represents the type II enzymes

including hPRMT5, DART5, SKB1, HSL7, hPRMT7 and DART7. The last branch represents enzymes that have evolved separately from the other PRMTs and include the type I enzymes CARM1, DART4, hPRMT2, hPRMT6 and DART8. Based on the phylogenic tree it appears that DART8 is the homolog of PRMT6. The phylogenic analysis demonstrates that the type I and II enzymes represented by PRMT1 and PRMT5 are conserved in all eukaryotes. The members of the lower branch including CARM1 and PRMT6 are only found in multicellular eukaryotes.

Figure 1.2 Phylogenetic analysis of arginine methyltransferases.

Phylogenetic analysis of yeast, Drosophila and human PRMTs. Multiple sequence alignments were performed using clustalW (http://www.ebi.ac.uk/clustalw/) and a phylogenetic tree analysis of the PRMT amino acid sequences from human (hPRMT1 to hPRMT8), Drosophila (DART1 to DART9), and the yeasts S. cerevisiae [scPRMT1(HMT) and scPRMT5 (HSL7)] and S. pombe (spPRMT1, spPRMT13, and spPRMT5 (SKB)] was plotted.


hPRMT1
hPRMT8/HRMT1L3
DART1
scPRMT1/HMT1
spPRMT1
hPRMT3
DART3
spPRMT3
DART9
DART2
DART6
hPRMT5/JBP1
DART5
spPRMT5/SKB
scPRMT5/HSL7
hPRMT7
DART7
hPRMT2
hPRMT4/CARM1
DART4
hPRMT6
DART8
Type I
Type II
Type I

1.2.3 PRMT consensus sites

The preferred methylation consensus sequence of PRMT1 was determined to be a glycine- and arginine- rich (GAR) motif (Najbauer et al., 1993a). PRMT3 methylates substrates within GAR motifs (Tang et al., 1998a) and also methylates arginines next to small hydrophobic residues in the sequence Arg-Ala-Arg (Smith et al., 1999). CARM1 is not a GAR motif-specific enzyme and the alignment of peptides methylated by CARM1 has defined a loose consensus of Arg-Pro-Ala-Ala-Pro-Arg (Lee and Bedford, 2002a; Lee et al., 2005b). More substrates need to be identified to define an accurate methylation consensus sequence. PRMT5 methylates arginines within GAR motifs and arginines outside the GAR motif (Friesen et al., 2001b; Meister et al., 2001; Pollack et al., 1999a). The specificity of PRMT2 and of PRMT6 through PRMT8 remains to be defined as no physiological substrates have been identified for these enzymes.

1.2.4 PRMT substrates

Techniques to detect methylarginines include mass spectrometry, the incorporation of labeled methyl groups in vivo and in vitro with purified PRMTs, amino acid substitution analysis, and more recently assays with general and site-specific antibodies to methylarginine (Boisvert et al., 2003; Lee et al., 2005a). Over the years many proteins have been demonstrated to contain methylarginines (Table 1.2). In 1967, histones were identified as methylarginine containing proteins (Paik and Kim, 1967; Paik and Kim, 1968a) and a few other proteins were identified before 1990 including the myelin basic proteins (MBP and (Baldwin and Carnegie, 1971)). The number of known methylated proteins increased after 1996, owing to the identification and cloning of the PRMTs. Moreover, proteomic studies using antibodies specific to methylated GAR identified over 200 putative methylated proteins, further demonstrating that arginine methylation may be a broad post-translational

modification implicated in many cellular processes including signal transduction, the cytoskeleton, DNA repair, transcription, translation and apoptosis (Boisvert et al., 2003). It is now known that arginine methylation is an abundant modification, as some of the methylarginine containing proteins identified to date are abundant proteins such as histones, myelin basic protein (MBP) and RNA-binding proteins (Gary and Clarke, 1998a). Most of the known methylated proteins from Table 1.2 are substrates of PRMT1 consistent with this enzyme being the major PRMT in the cell (Tang et al., 2000) and with the fact that the other methyltransferases are less well characterized.

	Substrate	Reference	Enzyme
1	Histone H4	Paik, 1967	PRMT1
2	Fibrillarin	Lischwe, 1985	PRMT1
3	Nucleolin	Lischwe, 1985	PRMT1
4	hnRNP A1, B, D, E, G, H, J, K, P, Q, R, U	Liu, 1995	PRMT1
5	TIS21	Lim, 1998	PRMT1
6	FGF-2	Klein, 2000	PRMT1
7	hnRNP A2	Nichols, 2000	PRMT1
8	Stat1	Mowen, 2001	PRMT1
9	Adenovirus E1B-AP5	Kzhyshkowska, 2001	PRMT1
10	Ewing's sarcoma (EWS)	Belyanskaya, 2001	PRMT1
11	Hepatitis C virus NS3 Helicase	Rho, 2001	PRMT1
12	CIRP2	Aoki, 2002	PRMT1
13	ZF5	Wada, 2002	PRMT1
14	GRY-RBP	Wada, 2002	PRMT1
15	TLS-FUS	Wada, 2002	PRMT1
16	TAFII-68	Wada, 2002	PRMT1
17	SAMT1	Wada, 2002	PRMT1
18	p137GPI	Wada, 2002	PRMT1
19	RBP58	Wada, 2002	PRMT1
20	hnRNP R	Wada, 2002	PRMT1
21	hnRNP K	Wada, 2002	PRMT1
22	Sam68, QKI-5, SLM-1, SLM-2, GRP33	Cote, 2003	PRMT1
23	SPT5	Kwak, 2003	PRMT1
24	NIP45	Mowen, 2004	PRMT1
25	Adenovirus L4-100kDa	Kzhyshkowska, 2004	PRMT1
26	FKBP 12	Chen, 2004	PRMT1
27	RNA Helicase A	Smith, 2004	PRMT1
28	Npl3p	Siebel, 1996	HMT1
29	Hrp1p	Valentini, 1999	HMT1
30	Nab2p	Green, 2002	HMT1
31	Gar1p	Xu, 2003	HMT1
32	Nor1p	Xu, 2003	HMT1
33	Nsr1p	Xu, 2003	HMT1
34	Yra1	Yu, 2004	HMT1
35	PABP II	Smith, 1999	PRMT3
36	Ribosomal S2 protein	Bachand, 2004	PRMT3
37	Histone H3	Paik, 1967	CARM1
38	p300	Xu, 2001	CARM1
39	CBP	Xu, 2001	CARM1
40	ILF3	Lee, 2002	CARM1
41	PABP1	Lee, 2002	CARM1
42	HuR	Li, 2002	CARM1
43	Squid	Boulanger, 2003	DART4
44	Vasa	Boulanger, 2003	DART4
45	TARPP	Kim, 2004	CARM1
46	Histone H2A	Branscombe, 2001	PRMT5
47	Histone H3	Pal, 2004	PRMT5
48	Histone H4	Pal, 2004	PRMT5
49	Sm B, B', D1, D3	Brahms, 2000	PRMT5
50	LSm4	Brahms, 2001	PRMT5
51	p80 Coilin	Boisvert 2002	PRMT5
52	Epstein-Barr Virus EBNA-2	Barth, 2003	PRMT5
53	SPT5	Kwak, 2003	PRMT5
54	PRMT6	Frankel, 2002	PRMT6
55	MBP	Baldwin, 1971	N/D
56	Myosin	Reporterer, 1971	N/D
57	Herpes Simplex Virus ICP27	Mears, 1996	N/D
58	RBP16	Pelletier, 2001	N/D
59	HMGA1a	Sgarra, 2003	N/D
60	Stat6	Chen 2004	N/D
61	Integral membrane protein Tmp21-I (p23)	Wu, 2004	N/D
62	p24B, *cis*-Golgi protein	Wu, 2004	N/D
63	Putative transmembrane methyltransferase	Wu, 2004	N/D
64	TGN38, *trans*-Golgi network protein 1	Wu, 2004	N/D
65	Golgin-84	Wu, 2004	N/D
66	*cis*-Golgi SNARE (p28)	Wu, 2004	N/D
67	Mannoside acetylglucosaminyltransferase 1	Wu, 2004	N/D
68	Mannoayl (-1,3-)-glycoprotein -1,4-*N*-acetylglucosaminyltransferase 2	Wu, 2004	N/D
69	Mannosidase 1, alpha	Wu, 2004	N/D
70	GRASP55	Wu, 2004	N/D
71	Cytochrome P450 2d2	Wu, 2004	N/D
72	Cytochrome P450 2c29	Wu, 2004	N/D
73	Epoxide hydrolase 1	Wu, 2004	N/D
74	Protein disulfide isomerase A3	Wu, 2004	N/D
75	Flavin containing monooxygenase 5	Wu, 2004	N/D
76	Transferrin	Wu, 2004	N/D
77	EMP70, member 2	Wu, 2004	N/D
78	EMP70, member 3	Wu, 2004	N/D

Table 1.2 Known arginine methylated proteins.

1.3 The roles of arginine methylation

1.3.1 Arginine Methylation Modulates Protein-Protein Interactions

Post-translational modifications can alter protein-protein interactions. The classical example is binding of the Src-homology domain-2 (SH2) domain to tyrosine residues modified by phosphorylation (Pawson, 2004). Arginine methylation can both negatively and positively regulate protein-protein interactions. Arginines are especially important in hydrogen bonding. For example, a critical aspartic acid within the Src-homology domain-3 (SH3) domain of the tyrosine kinase Fyn mediates an Asp-Arg salt bridge between Fyn and the HIV-1 (Human Immunodeficiency Virus-1) Nef protein (Arold et al., 1997). The methylation of this arginine results in collision with the SH3 interacting surface and blocks the interaction (Bedford et al., 2000a). The Src-associated substrate in mitosis of 68 kDa and adaptor protein Sam68 binds to several SH3 domain- and WW domain (two tryptophan domain)- containing proteins (Figure 1.3H, (Lukong and Richard, 2003)). The methylation of arginines neighboring proline motifs in Sam68 actually prevents association with SH3 domains, but not with WW domains, as demonstrated using blot overlays and BIAcore experiments (Bedford et al., 2000a). These data were further corroborated by using a protein microarray spotted with SH3 and WW domains and probed with methylated and non-methylated peptides (Espejo et al., 2002). Arginine methylation prevents the interaction of the transcription factor STAT1 (signal transducer and activator of transcription-1) with its inhibitor PIAS1 (protein inhibitor of activated STAT1) (Mowen et al., 2001a). Similarly, the interaction of the transcription elongation factor SPT5 (Suppressor of Ty's-5) with RNA polymerase II is stimulated under conditions where SPT5 is hypomethylated (Kwak et al., 2003). The interaction of Npl3 (nuclear protein localization 3) with the TREX (transcription/export) mRNA export machinery is also inhibited by arginine methylation (Yu et al., 2004). The arginine methylation of Ewing sarcoma (EWS) protein decreases its interaction with SMN, the spinal muscular atrophy gene product (Young et al., 2003). In all

these latter examples, the interface and the molecular details of the interaction have not been examined in any detail.

Contrary to the interaction of EWS with SMN, arginine methylation positively regulates the interaction between SMN and the small nuclear ribonucleoproteins SmB/B', SmD1 and SmD3 (Selenko et al., 2001). The Tudor domain of SMN is the likely to mediator of this direct interaction (Buhler et al., 1999; Selenko et al., 2001). The proposed function of SMN is its involvement in ribonucleoprotein complex assembly (Yong et al., 2004). Interestingly, arginine methylation of the C-terminus of the Sm B and D proteins (Brahms et al., 2000a) enhances their association with SMN (Friesen et al., 2001b). Thus a model was proposed where arginine methylation signals the assembly of ribonucleoproteins. Consistent with this model, pre-mRNA splicing reactions were inhibited when using nuclear extracts from HeLa cells treated with methyltransferase inhibitors (Boisvert et al., 2002). Moreover, nuclear extracts incubated with methylarginine-specific antibodies were inefficient in pre-mRNA splicing *in vitro* (Boisvert et al., 2002). Some interactions may be positively regulated by arginine methylation. For example, the interaction between the nuclear factor of activated T cells (NF-AT) and the NF-AT interacting protein of 45 KDa, NIP45, decreased in the presence of methyltransferase inhibitors (Mowen et al., 2004).

Methylation can also selectively impair certain signaling pathways. CREB-binding protein (CBP) is arginine methylated in its CREB kinase inducible interacting KIX domain (Xu et al., 2001) as well as in its N-terminus. CBP, along with p300, function as important transcriptional co-activators in multiple pathways (Figure 1.3E). Arginine methylation of CBP within the KIX domain by CARM1 reduces association of CBP with CREB through its kinase inducible domain (KID domain), resulting in a decrease in cAMP-dependent gene expression (Xu et al., 2001). CBP methylation outside of the KIX domain can also affect both glucocorticoid receptor interacting protein-1 (GRIP1)-dependent and hormone-induced transcriptional activation (Chen et al., 2000; Chevillard-Briet et al., 2002).

1.3.2 Arginine methylation regulates protein localization.

The first indication that arginine methylation may regulate cellular localization came from the observation that higher molecular weight forms of the basic fibroblast growth factor 2 (bFGF-2) that are arginine methylated were found preferentially in the nucleus (Burgess et al., 1991). This was later confirmed after inhibition of the methylation was shown to significantly decrease nuclear accumulation of bFGF-2 (Pintucci et al., 1996). However, the first direct evidence demonstrating that methylation regulated protein localization comes from the observation that two hnRNPs involved in mRNA processing and export, Npl3p and Hrp1p, failed to exit the nucleus in yeast cells lacking Hmt1, the homolog of PRMT1 (Shen et al., 1998a). Methylation of these proteins was later found to be regulated by two RING-finger proteins, Air1p and Air2p, which inhibited methylation of Npl3p resulting in nuclear accumulation of poly-A RNAs (Inoue et al., 2000). Methylation also influenced the nuclear import of Npl3p, by inhibiting Sky1p-mediated phosphorylation which is necessary for the nuclear import of Npl3p through interaction with the nuclear import receptor Mtr10p (Yun and Fu, 2000a).

In mammals, the first report demonstrating a role for arginine methylation in the regulation of hnRNP localization comes from the observation that hnRNP A2 accumulated in the cytoplasm in cells treated with methyltransferase inhibitors or when the methylated arginines were deleted (Nichols et al., 2000a). However, since hnRNP A2 is a shuttling RNA binding protein and since RNA binding is regulated through arginine methylation (Rajpurohit et al., 1994), the cytoplasmic accumulation of hnRNP A2 might be due to its inability to bind RNA in the nucleus. The nuclear localization of KH-domain RNA binding protein, Sam68 was recently found to be regulated by arginine methylation (Cote et al., 2003). Deletion of the methylation site and treatment of cells with methyltransferase inhibitors resulted in Sam68 accumulation in the cytoplasm. Sam68 was also detected in the cytoplasm of PRMT1-deficient embryonic stem cells. Under normal conditions, Sam68 is able to

functionally substitute for the HIV rev protein and export rev response element (RRE) containing RNAs from the nucleus. When cells are treated with Adox, Sam68 can no longer functionally substitute for rev in and RRE-directed reporter gene experiment (Cote et al., 2003). The Ewing Sarcoma (EWS) protein is an RNA binding protein harboring extensive arginine dimethylation in its C-terminal RGG rich RNA binding domain. It was recently shown that inhibition of methylation resulted in loss of nuclear and cell surface localization of EWS protein (Belyanskaya et al., 2003). However, it also resulted in a decrease in the EWS protein expression, making the finding difficult to interpret. XCIRP2, the *Xenopus Laevis* homologue of the cold-inducible RNA-binding protein (CIRP) is a nuclear protein that is overexpressed upon a temperature downshift. XCIRP2 was found to interact with and be methylated by PRMT1 in a domain necessary for nuclear localization (Aoki et al., 2002). Interestingly, methylation by overexpressed *Xenopus Laevis* PRMT1 caused cytoplasmic accumulation of xCIRP2.

RNA helicase A is imported into the nucleus using karyopherin beta and this mechanism is negatively regulated by using methyltransferase inhibitors (Smith et al., 2004). Injection of methylated recombinant protein encompassing the nuclear transport domain allows the fusion protein to localize to the nucleus even in the presence of methylation inhibitors, further confirming the requirement for arginine methylation in the nuclear localization of RNA helicase A (Smith et al., 2004). These results indicate once again a role for arginine methylation in the nucleocytoplasmic transport of proteins.

The majority of sDMA containing proteins were found to localize in the nucleus, mainly in the nucleoplasm and in nuclear foci (Boisvert et al., 2002). Coilin was shown to contain symmetrical dimethylarginines that regulate its binding to SMN and recruits SMN to a nuclear structure called Cajal bodies. Inhibition of methylation or cells harboring hypomethylated coilin have a lower binding of coilin to SMN and this results in the localization of SMN in nuclear structures that are different from Gems (Boisvert et al., 2002;

Hebert et al., 2002a). Thus, arginine methylation of coilin is necessary for intranuclear localization of SMN to Cajal bodies. Moreover, cells derived from patients with spinal muscle atrophy (SMA) harboring a residual level of SMN, displayed mislocalization of sDMA containing proteins (Boisvert et al., 2002), suggesting a role of arginine methylation for proper protein localization.

1.3.3 Arginine methylation regulates transcription.

1.3.3.1 Chromatin

One of the features of eukaryotes is the compaction of a large amount of genomic DNA into the cell nucleus that is only a few microns in diameter. In order to achieve this compaction, cells have evolved a DNA structure unit called the nucleosome. In the nucleosome, approximately one and a half turns of DNA are wrapped around an octameric protein core composed of highly basic nuclear proteins called the histones (Rill et al., 1978). The structural analysis of the histone octamer without DNA revealed that all core histones (H2A, H2B, H3 and H4) share a common structural motif, the "histone fold" (Arents et al., 1991). The presence of such structures along the DNA strands represents an obstacle for enzymes that requires the DNA as a template such as the RNA polymerase or the DNA polymerase during transcription and replication, respectively (van Holde et al., 1992). Thus, it has now become clear that nucleosomal organization represents an important regulatory element and that cells have developed a specific machinery to remodel nucleosomes to accommodate those mechanisms (Felsenfeld et al., 2000). In fact, chromatin reorganization is now well established to be involved in the regulation of replication, transcription and DNA repair (Peterson and Cote, 2004).

1.3.3.2 Histone modifications.

Since the discovery of histone acetylation by Allfrey and coworkers (Allfrey et al., 1964), the post-translational modification of histones has been correlated with chromatin assembly and transcription, DNA repair, replication, silencing and recombination events (Peterson and Laniel, 2004; Stallcup, 2001). Acetylation of histones is a modification found in all higher eukaryotes examined so far (Roth et al., 2001). Histone acetyltransferases (HATs) introduce an acetyl-group from acetyl-CoA into the ε-amine group of lysines and thereby reduce the net positive charge of histones (Roth et al., 2001). The reversal of histone acetylation is catalyzed by a family of enzymes named histone deacetylases (HDACs) (Peterson, 2002). Histones H2A, H2B, H3 and H4 have all been found as substrates for lysine acetylation (Imhof and Wolffe, 1998). Histone acetylation has long been correlated with transcriptional activity, which was confirmed by the identification of HATs as transcriptional coactivators and HDACs as gene silencers (Kuo and Allis, 1998). Histone acetylation acts in the destabilization of higher order structures of chromatin through neutralization of the positive charges on the N-terminal tails of histones, which makes numerous contacts with the negatively charged DNA. Acetylation also signals to regulatory proteins or enzymes that are involved in transcription, replication and DNA repair (Peterson and Cote, 2004).

1.3.3.3 Methylation of histones.

Recent progress with respect to histone methylation has uncovered a whole new aspect of the regulation of histone modifications. Methyltransferases have been found to catalyze the transfer of methyl group to either arginine or lysine residues in histones. Several lysine residues have been shown to be sites of methylation, such as lysines 4, 9, 27, 36 and 79 of histone H3 (Strahl et al., 1999), lysine 20 of histone H4 (Strahl et al., 1999) and lysine 26 of histone H1 (Kuzmichev et al., 2004). Each lysine residue can be mono-, di- or

trimethylated by members of the SET-domain-containing lysine methyltransferase family. These enzymes can be classified into four different families according to their similarities within the SET domain (Kouzarides, 2002).

Arginines residues in the histones N-terminal tails of H3 (arginines 2, 17 and 26) and H4 (arginine 3) can also be methylated by arginine methyltransferases. PRMT1 has been shown to be the enzyme responsible for methylation of histone H4 (Wang et al., 2001a) whereas PRMT4 has been described as catalyzing the methylation of histone H3 (Chen et al., 1999a; Schurter et al., 2001a). The finding that methylation on arginine 3 of histone H4 by PRMT1 facilitates subsequent acetylation of histone H4 by p300 ((Wang et al., 2001a), suggested a potential role for PRMT1 in transcriptional activation. Indeed, the transcription factor Yin Yang 1 (YY1) was able to recruit PRMT1 to DNA, suggesting that YY1 would guide H4 methylation by PRMT1 when the YY1 promoter is activated (Rezai-Zadeh et al., 2003). PRMT4 was originally identified in a yeast two-hybrid screen interacting with GRIP1, a member of the p160 family of transcriptional coactivators (Chen et al., 1999a). CARM1 and p300, a histone acetyltransferase, synergistically enhance transcription of the estrogen receptor (Koh et al., 2001), indicating interplay between acetylation and methylation of histones in the regulation of transcriptional activity (Daujat et al., 2002).

1.3.3.4 The histone code: interplay between acetylation and methylation.

Post-translational modifications within the histone tails are now well established to be responsible for regulation of key event affecting chromatin, but are also affecting the ability of each other's activity. For example, it was shown that methylation of lysine 4 of histone H3 positively affects subsequent acetylation of histone H3 by p300 (Wang et al., 2001a). Moreover, there is clear overlap not only in the function as activators or silencers of transcription, but also on the sites of these modifications within the histone tails. Thus, the interplay of these different covalent modifications (lysine acetylation and methylation,

arginine methylation) reveal a complex regulatory process occurring on the histone tails that has been termed the "histone code" (Jenuwein and Allis, 2001a; Strahl and Allis, 2000). This hypothesis predicts that a pre-existing modification affects subsequent modifications on histone tails and that these modifications serve as marks for the recruitment of different protein complexes to regulate diverse chromatin functions. Also, it proposes that the presence of specific modifications on histones prior to stimulation of specific activities will dictate the response that will occur in those regions of DNA.

1.3.4 The role of arginine methylation in signal transduction.

1.3.4.1 The role of arginine methylation in JAK-STAT signaling.

Cytokine and interferon (IFN) signaling are known to transduce intracellular signals through the JAK-STAT (Janus kinase-STAT) pathway (Shuai and Liu, 2003). The concept that arginine methylation could regulate cytokine signaling was suggested from the observation that a mutation resulting in an inositol-requiring phenotype in yeast (*ire15*) could be rescued by genetic complementation with either the tumor growth factor β (TGFβ) receptor or the HMT1 methyltransferase (Nikawa et al., 1996). A direct link arose from the identification in a yeast two-hybrid screen of an interaction between PRMT1 and the cytoplasmic domain of the IFN-α receptor (Abramovich et al., 1997b). PRMT1 is constitutively associated with the interferon-α (IFN-α) receptor and its expression correlates with IFN-α action (Abramovich et al., 1997b). HeLa cells stably transfected with an expression plasmid encoding PRMT1 antisense were resistant to growth arrest induced by type 1 interferons. These cells no longer mounted an efficient antiviral response when challenged with vesicular stomatitis virus (VSV) (Altschuler et al., 1999).

Evidence for the involvement of arginine methylation in the JAK-STAT signaling pathway also comes from the identification of JBP1 (JAK2-binding protein 1) as a JAK2-

interacting protein in a yeast two-hybrid assay (Pollack et al., 1999a). JBP1 is the mammalian homolog of SKB1; it is a type II enzyme and was renamed PRMT5 (Branscombe et al., 2001). PRMT5 binds to a protein known as pICln (Chlorine channel regulator) (Krapivinsky et al., 1998) and is part of a large complex dubbed the methylosome (Friesen et al., 2001b; Meister et al., 2001). Although PRMT5 was identified as a JAK2 interacting protein, there is no functional data linking PRMT5 with the JAK protein kinases or other tyrosine kinases. Moreover, a link between arginine methylation by PRMT5 and tyrosine phosphorylation has yet to be established.

Another link between arginine methylation and the JAK-STAT pathway was revealed in studies showing that STAT1 is asymmetrically dimethylated by PRMT1 on arginine 31 (Mowen et al., 2001a). Hypomethylation of this arginine results in a decrease in transcriptional activation by STAT1 of an interferon responsive gene in cells stimulated with IFNα (Mowen et al., 2001a). Arginine methylation blocks the association of STAT1 with PIAS1 (protein inhibitor of activated STAT1) but not other properties of STAT1 such as DNA binding activity, tyrosine phosphorylation, or nuclear translocation (Mowen et al., 2001a). Arginine methylation is also implicated in the regulation of STAT6 function. Inhibition of methylation of Arg^{27} of STAT6 results in reduction of STAT6 tyrosine phosphorylation and failure of STAT6 to translocate to the nucleus and bind DNA (Chen et al., 2004). There is no known PIAS1 equivalent for STAT6. However a mutant form of STAT6 containing an amino acid substitution of Arg^{27} to alanine, was less stable than its methylated wild-type counterpart, suggesting that methylation may enhance stability of this protein (Chen et al., 2004). Because all STAT family members contain a conserved Arg at the N-terminus (Arg^{31} for STAT1 and Arg^{27} for STAT6) it is possible that all STATs can be methylated. Nevertheless, the finding that arginine methylation of STAT1 and STAT6 has distinct consequences indicates that arginine methylation may have multiple roles in the JAK-STAT signaling cascades (Figure 1.3D). The methylation of STAT1 on Arg^{31} has been

called into question (Meissner et al., 2004) by studies in which no peaks corresponding to methylated STAT1 were detected by mass spectrometry and no incorporation of radioactive methyl groups could be detected in STAT1 immunoprecipitated after methylation in vivo (Meissner et al., 2004). In these experiments, treatment of cells with the methyltransferase inhibitor MTA (5'-deoxy-5'(methylthio)adenosine) diminished IFN-induced phosphorylation of STAT1, which is contradictory to results mentioned above showing that the methylation status of STAT1 does not affect the levels of its phosphorylation (Mowen et al., 2001a). Further studies are required to clarify these discrepancies.

Arginine methylation of STAT1 and signaling through the JAK-STAT pathway are exploited in chronic viral infection. The hepatocytes of transgenic mice expressing the entire hepatitis C virus (HCV) open reading frame and patients infected with chronic HCV show reduced IFN-α gene expression. This signaling defect results from increased expression level of the catalytic subunit of protein phosphatase 2A and the hypomethylation of STAT1 Arg*31* (Duong et al., 2004). These observations suggest arginine methylation may have anti-viral effects and that viruses such as HCV may neutralize this line of defense to help establish a chronic infection.

On the basis of these observations, it would be predicated that PRMT1$^{-/-}$ and PRMT5$^{-/-}$ mice would be defective in interferon and cytokine signaling. PRMT1$^{-/-}$ mice have been generated but die during early embryogenesis (Pawlak et al., 2000). PRMT5$^{-/-}$ animals also die as embryos (S. Richard, unpublished observations of the mice derived from the BayGenomics clone # RRA014). Thus elucidation of physiological role of PRMT1 and PRMT5 in the JAK-STAT pathway will have to await the generation of mice with conditional alleles of PRMT1 and PRMT5.

1.3.4.1 Regulation of the NF-AT Pathway by Methylarginines

T lymphocytes incubated with methyltransferase inhibitors have a reduced ability to stimulate cytokine release after activation (Mowen et al., 2004). T cell receptor (TCR) stimulation increases the abundance of mRNAs of PRMT1 and CARM within 1 hour. These studies suggest that increased arginine methylation may be associated with TCR activation, pending further investigation. The NF-AT pathway [which results in activation of the transcription factor NF-AT (nuclear factor of activated T cells)] is one regulated cascade that appears to be modulated by arginine methylation (reviewed in (Boisvert and Richard, 2004)). T cell receptor signaling triggers a cascade of events that leads to increased cytokine gene expression. The secreted cytokines then bind their receptors through a paracrine or autocrine loop and induce STAT-dependent gene activation (Figure 1.3C). NF-AT transcription factors likely determine the spectrum of cytokines that are produced by stimulated T helper cells (Rengarajan et al., 2002). The NF-AT coactivator NIP45 is arginine methylated by PRMT1 and the NF-AT-NIP45 interaction is enhanced with arginine methylation by an unknown mechanism (Mowen et al., 2004). Differentiated T helper cells (Th1 and Th2) had increased levels of PRMT1 and CARM1 compared to T helper progenitors and upon stimulation with anti-CD3 and anti-CD28, PRMT1 transcripts and protein level increased. Overexpression of PRMT1 resulted in an increase in transcription of the IFNγ gene and of NF-AT mediated transcription of the interleukin 4 gene (Mowen et al., 2004). The importance of CARM1 in T cell development was observed in $CARM1^{-/-}$ mice, which die shortly after birth because of breathing problems. These animals have T lymphocyte defects because thymocytes remain at an early progenitor stage (Kim et al., 2004). Collectively, these studies represent early work in the area of T cell signaling, and future studies may reveal other roles of arginine methylation in key regulatory steps in lymphocytes.

1.3.4.2 Regulation of other signaling cascades.

PRMT1 was identified in a yeast two-hybrid screen for proteins that interact with the immediate early B-cell translocation gene (BTG) products BTG1 and BTG2 (BTG2 is also called PC3 (NGF induced gene 3 in PC12 cells) or TIS21 (tetradecanoyl phorbol acetate-inducible sequences)) (Lin et al., 1996b). The human antiproliferative BTG1 gene was cloned from a chromosomal translocation of a B-cell chronic lymphocytic leukaemia (Rimokh et al., 1991) and BTG2 was identified as an immediate early gene expressed in response to nerve growth factor (NGF) in PC12 cells (Altin et al., 1991). BTG2 expression is increased by p53 in cells exposed to agents that cause DNA damage. Such cells, but not cells lacking functional BTG2, undergo cell cycle arrest, suggesting that BTG2 might function in control of the cell cycle (Rouault et al., 1996). BTG1 and BTG2 activate PRMT1 activity in vitro (Figure 1.3G) (Lin et al., 1996b), but the PRMT1 interacting domain alone of BTG1 or BTG2 (the BoxC domain) has an inhibitory effect (Berthet et al., 2002). BTG1 is a target gene for the forkhead box transcription factor Foxo3a during erythroid differentiation. The increased expression of BTG1 during erythroid differentiation coincided with increased arginine methylation, whereas inhibition of methylation blocked erythroid differentiation (Bakker et al., 2004). These results demonstrate that PRMT1 activity may be modulated by changes in expression of BTG1 and BTG2, and that regulation through these proteins may influence cell differentiation. Considering that NGF causes a change in subcellular localization of the p53 tumor suppressor protein and an increase in the overall amount of the protein (Eizenberg et al., 1996; Gollapudi and Neet, 1997), and that BTG2 is a p53-transcriptional target (el-Ghissassi et al., 2002), it seems possible that the increased expression of p53 in response to NGF and its subsequent stimulation of BTG2 synthesis, which can cause an increase in methylation, could explain the increase in methylation seen upon neuronal differentiation (Figure 1.3F). However, it is still unclear what mechanism or which specific target proteins account for the observed effects. Lipopolysaccharide (LPS),

cytokines, and T-cell receptor activation can all increase the expression of BTG1 (Kolbus et al., 2003; Lee et al., 2003; Shires et al., 2001) and the fact that cytokines increase the expression of BTG2 (Bradbury et al., 1991; Yin and Yang, 1993) suggests an additional mechanism that explains the increase in methyltransferase activity observed after activation of cytokine signaling pathways.

Amounts of cellular arginine methylation increase after stimulation of PC12 cells with NGF (Figure 1.3F) (Cimato et al., 1997; Kujubu et al., 1993). Treatment of cells with methyltransferase inhibitors or in vivo delivery of the BoxC domain, of BTG1 or BTG2, which have an inhibitory effect on arginine methyltransferases, inhibited NGF- (Berthet et al., 2002), but not epidermal growth factor (EGF) induced differentiation of PC12 cells (Kujubu et al., 1993). Therefore, arginine methylation appears to be required during cellular differentiation of PC12 cells.

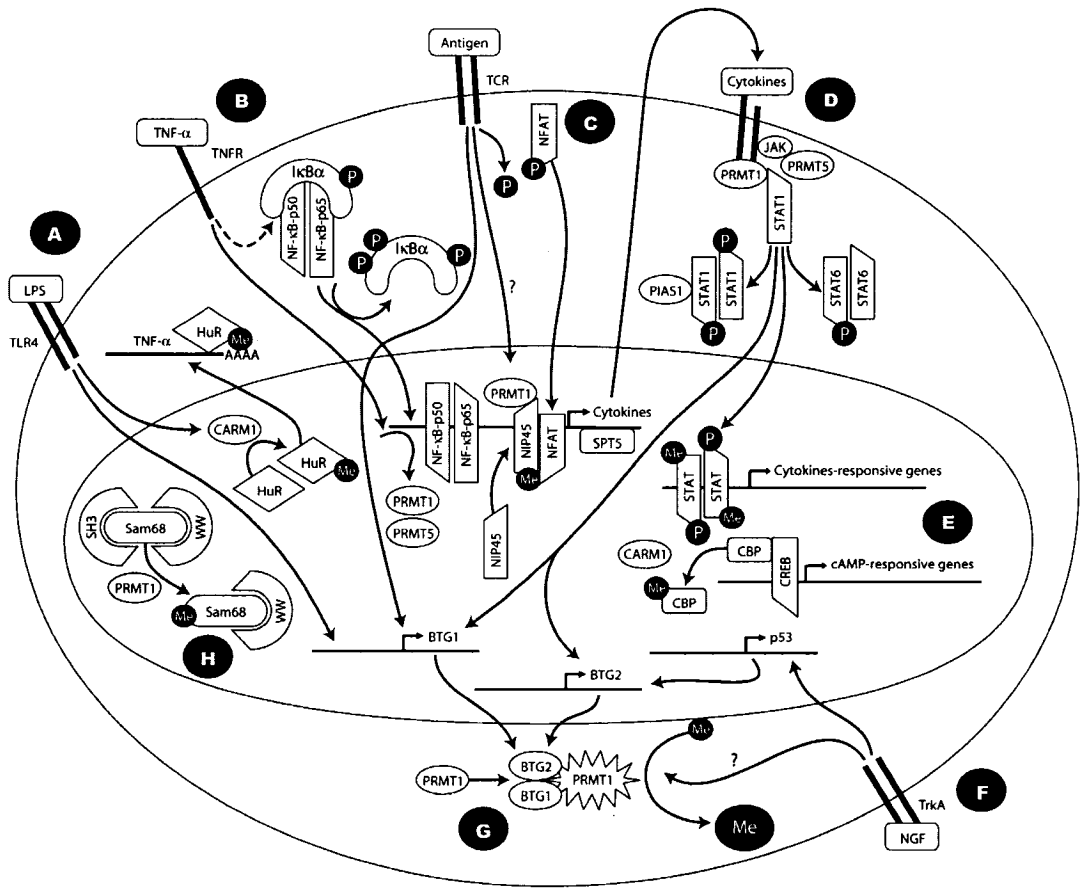
A role for arginine methylation was observed in cells exposed to LPS. The RNA-binding protein HuR (Hu antigen protein R) was observed to be methylated on Arg^{217} by CARM1 in response to LPS treatment (Li et al., 2002). HuR binds AU-rich elements and stabilizes mRNAs in response to cellular signals including cytokines and LPS (Brennan and Steitz, 2001) and how methylation regulates these functions is unknown. Liver macrophages pretreated with methyltransferase inhibitors had a decrease in TNF-α and NF-κB promoter activity in response to LPS (Figure 1.3A-B) (Veal et al., 2004; Watson et al., 1999). Taken together these findings suggest that methylation on arginines may influence transcriptional and post-transcriptional events that modulate TNF-α and NF-κB function.

In several cases, phosphorylation and methylation events appear to be co-regulated (Lukong and Richard, 2004a). For example arginine methylation of the Npl3p RNA binding protein prevents its phosphorylation and nuclear import (Yun and Fu, 2000a). Methylation of STAT6 also influences its phosphorylation and activation (Chen et al., 2004). In contrast,

STAT1 phosphorylation was not altered by methylation, but rather its activity is regulated by PIAS1 (Mowen et al., 2001a). However, arginine methylation of STAT1 controls the rate of STAT1 dephosphorylation by the nuclear TcPTP (T-cell protein tyrosine phosphatase) protein tyrosine phosphatase (Zhu et al., 2002).

Figure 1.3 Arginine methylation in signalling cascades.

(**A**) Binding of lipopolysaccharide (LPS) to the toll-like receptor 4 (TLR4) receptor results in increased methylation of HuR by CARM1, which in turn stabilize the mRNA of TNF-α. (**B**) TNF-α then activates the NF- κB pathway, resulting in transcriptional activation of several genes (including genes encoding cytokines) through transcriptional activation by NF-κB and through the elongation factor for polymerase II–mediated transcription SPT5. (**C**) Binding of antigen to the TCR, also results in transcriptional activation of cytokine genes through an increase in the amount of PRMT1 and CARM1 coactivators. Methylated NIP45 stimulates cytokine gene expression by associating with NF-AT. (**D**) Activation of the JAK-STAT pathway by cytokines depends on arginine methylation of STAT1 and STAT6 by PRMT1 in order to achieve proper gene expression responses. (**E**) Arginine methylation of the transcriptional coactivator CBP by CARM1 reduces its association with CREB, resulting in a decrease in cAMP-dependent gene expression. (**F**) Amounts of cellular arginine methylation are increased in response to NGF. (**G**) LPS, TCR, and cytokines increase expression of BTG1, and cytokines increase expression of BTG2. BTGs increased PRMT1 activity. (**H**) The RNA binding protein and Src adaptor protein, Sam68, binds to SH3 and WW domains. Arginine methylation of Sam68 by PRMT1 inhibits its binding to SH3, but not to WW, domains.


Antigen
TCR
Cytokines
B
C
D
TNF-α
TNFR
NFAT
JAK
PRMT1
PRMT5
STAT1
IκBα
NF-κB-p50
NF-κB-p65
A
LPS
TLR4
PIAS1
STAT6
HuR
AAAA
CARM1
SPTS
Cytokines-responsive genes
E
SH3
Sam68
WW
CBP
CREB
cAMP-responsive genes
NIP45
BTG1
BTG2
p53
H
G
F
TrkA
NGF
Me

1.4 Demethylases: Reversing Arginine Methylation.

Arginine methylation was recently demonstrated to be reversible (reviewed in (Zhang, 2004)). Peptidylarginine deiminase 4 (PAD4) converts MMA into citrulline and releases methylamine (Cuthbert et al., 2004b; Wang et al., 2004b). Thus there may exist an equilibrium between methylation and demethylation, much like the equilibrium between kinases and phosphatases. PADs were identified decades ago (Kubilus et al., 1980). However, their demethylating activity may have eluded discovery because of their inability to reverse a dimethylarginine residue, the major type of arginine methylation that was studied over the past decades. Interestingly, PADs only convert MMA and not DMA to citrulline (Cuthbert et al., 2004b; Wang et al., 2004b). Therefore, the conversion of MMA to citrulline by PADs would prevent its methylation into aDMA and sDMA by PRMTs. Also, the methylation of MMA by PRMTs into DMA would protect these residues against PAD activity. Therefore, PADs and PRMTs may have antagonistic activities. Further studies will better elucidate the interplay between methylation and demethylation.

1.5 Arginine Methylation and DNA repair.

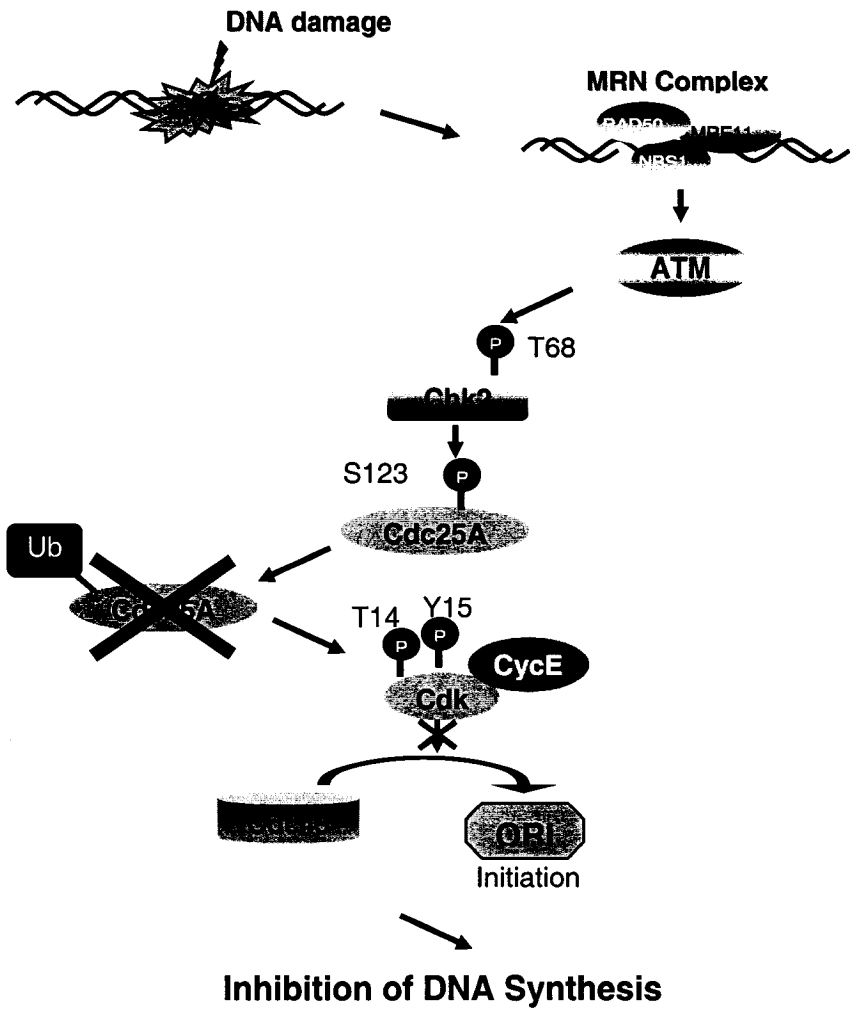
1.5.1 Mechanisms of DNA repair

All cell types must ensure the fidelity of transmission of genetic information. Simple errors that can occur in the genomic DNA sequence can be fatal either for the cell, or for the organism in which the cell is present. DNA double-strand breaks are a common form of DNA damage. Such damage can occur because of a block during the replication of the DNA at the replication fork, but can also occur due to external factors such as ionizing radiation or chemicals. Cells have evolved mechanisms to monitor genome integrity. They respond to DNA damage by activating a complex DNA-damage-response pathway that includes cell-

cycle arrest to allow the cells to repair their DNA (Figure 1.4) (Lisby and Rothstein, 2004). They can then determine whether to continue proliferation, if proper DNA repair has been achieved, or whether to trigger a programmed cell death, if repair of DNA damage has failed. An inability to respond properly to, or to repair, DNA damage leads to genetic instability, which in turn may enhance the rate of cancer development. Indeed, failures in DNA-damage signaling and repair pathways are fundamental to the etiology of most, if not all, human cancers (Hartwell and Kastan, 1994).

Figure 1.4 Inhibition of DNA synthesis following DNA damage.

DNA damage activates the ATM kinase through recognition of DNA breaks by the MRN complex, which results in the phosphorylation of Chk2. Phosphorylation of Chk2 activates its kinase activity, resulting in phosphorylation of the phosphatase Cdc25A, and targeting it to degradation through the ubiquitin pathway. The absence of Cdc25A keeps Cdk2/CycE in an inactive, phosphorylated form, resulting in the inhibition of Cdc45 loading on the origins of replication. This pathway results in the inhibition of DNA synthesis, in response to DNA damage. (Adapted from Falck et al., 2002).


DNA damage
MRN Complex
ATM
P
T68
S123
P
Cdc25A
Ub
T14
Y15
P
P
CycE
Cdk
ORI
Initiation
Inhibition of DNA Synthesis

1.5.2 DNA repair and diseases

Several cancer-prone syndromes reflect defects in the DNA damage response. Description of the genetic defects underlying the chromosomal instability syndromes ataxia-telangiectasia (A-T) (Savitsky et al., 1995b), Ataxia-Telangiectasia like disorder (A-TLD) (Stewart et al., 1999) and Nijmegen breakage syndrome (NBS) (Carney et al., 1998; Varon et al., 1998) has provided important insight regarding the mammalian DNA damage response. A-T, A-TLD and NBS exhibit hypersensitivity to ionizing radiation, immunodeficiency and an increased predisposition to the development of malignancies (reviewed by (Shiloh, 1997)). These phenotypic outcomes indicate that ATM, MRE11 and NBS1, the gene products deficient in A-T, A-TLD and NBS respectively, play an important role in maintaining genomic integrity in the face of intrinsic as well as extrinsic DNA damage (Carney et al., 1998; Savitsky et al., 1995b; Stewart et al., 1999; Varon et al., 1998). A clear biochemical link between double-strand break (DSB) repair and mammalian cellular responses to DNA damage was uncovered by the observation that NBS1 functions in a complex with the highly conserved DSB repair proteins MRE11 and RAD50 (Carney et al., 1998). Interestingly, certain phenotypic features of the yeast mutants affecting the *S. cerevisiae* MRE11/RAD50/XRS2 protein complex are reminiscent of both A-T and NBS; in addition to recombination DNA repair deficiency, these mutants exhibit genomic instability in the form of increased allelic recombination, increased frequency of chromosome loss, and sensitivity to DNA damaging agents (Ajimura et al., 1993; Bressan et al., 1998).

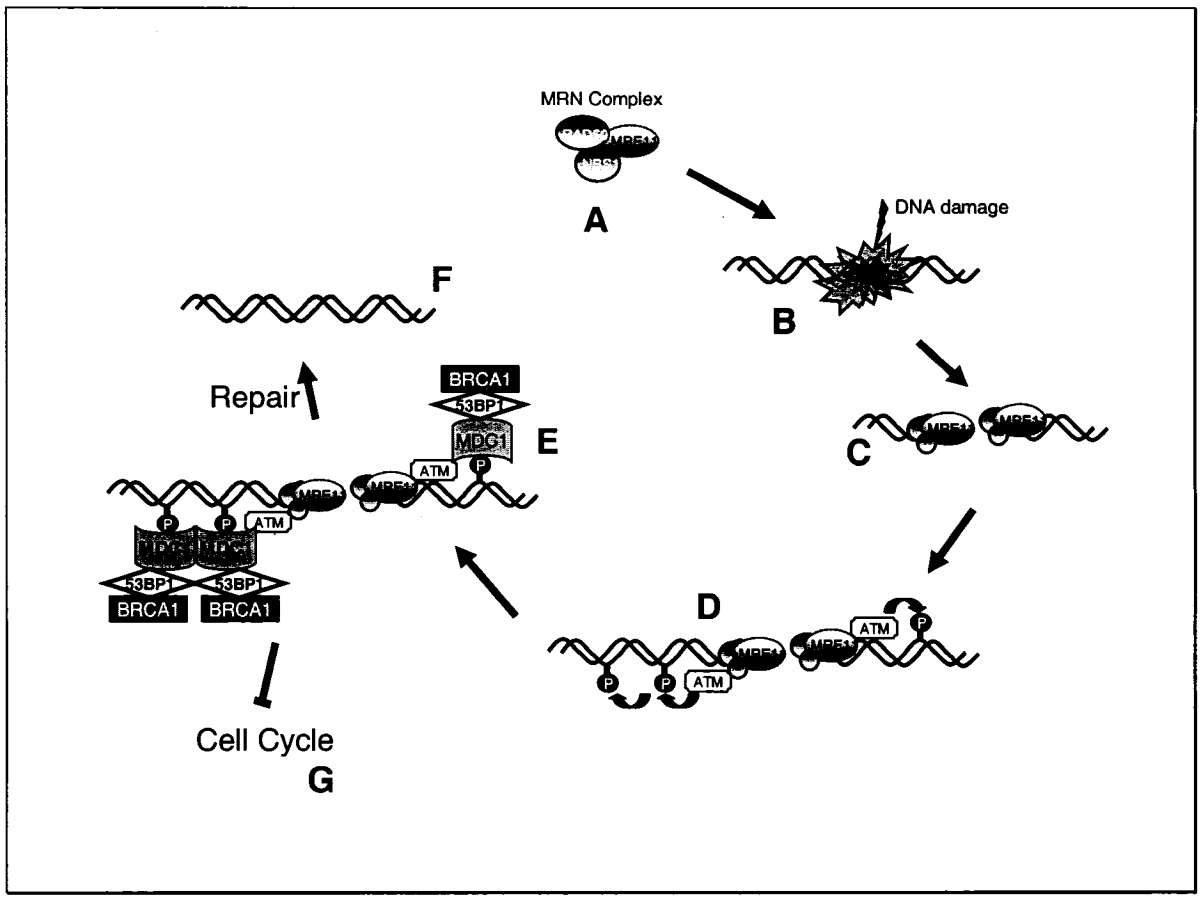
1.5.3 Recruitment of DNA damage response factors to DNA breaks

The detection and repair of DNA double strand breaks requires the orderly recruitment of a plethora of factors including protein kinases, enzymes and adaptors molecules that will mediate the repair and signaling of DNA damage (Rouse and Jackson, 2002a). One of the first steps of the DNA damage response consists in the phosphorylation of

a multitude of targets by the phosphatidylinositol 3-kinase protein kinase-like (PIKK) family members ATM, ATR and DNA-PK (Shiloh, 2003a). Immediately following DNA damage, H2AX becomes phosphorylated by PIKK kinases on serine 139 surrounding the DNA break (Rogakou et al., 1999), which is followed by the recruitment of several DNA damage response factor such as BRCA1, 53BP1, MDC1, Rad51 and the MRE11/RAD50/NBS1 complex (MRN complex) (Celeste et al., 2003a; Celeste et al., 2002; Fernandez-Capetillo et al., 2002; Lou et al., 2004; Lukas et al., 2004; Maser et al., 1997a; Stucki and Jackson, 2004). Although the exact order of events has proven almost impossible to decipher, H2AX deficient mice have demonstrated that phosphorylation of H2AX is one of the earliest events (Celeste et al., 2002). Indeed, the recruitment of several DNA repair proteins such as NBS1, 53BP1, MDC1 and BRCA1 to the site of DNA damage requires the presence of H2AX (Celeste et al., 2003b). This was further confirmed by the fact that NBS1, 53BP1 and MDC1 could directly interact with phosphorylated H2AX (Kobayashi et al., 2002; Morales et al., 2003; Peng and Chen, 2003; Shang et al., 2003; Ward et al., 2003; Xu and Stern, 2003). In contrast, H2AX phosphorylation happened normally in 53BP1 deficient mice (Fernandez-Capetillo et al., 2002), but downstream events such as Chk2 activation was impaired (Ward et al., 2003), placing 53BP1 downstream of H2AX in the response to DNA damage. The MRN complex associates with chromatin during DNA replication and acts as a sensor of DNA double strand breaks after exposure to radiation (van den Bosch et al., 2003). Although the recruitment to sites of DNA damage of NBS1 is impaired in H2AX deficient mice (Celeste et al., 2003b), the MRN complex was shown to be able to sense breaks in DNA and relays this information to ATM (Costanzo et al., 2004a; Lee and Paull, 2004; Lee and Paull, 2005), which in turn activates pathways for cell cycle checkpoint activation. Thus the activity of the MRN complex appears to be the first event in the recognition of DNA damage (Carson et al., 2003a; Theunissen et al., 2003a; Uziel et al., 2003).

Figure 1.5 Model of the double-stranded-break response cycle.

(**A**) The MRE11/RAD50/NBS1 (MRN) complex is present in the nucleus. (**B**) Induction of a DNA double-stranded break (DSB). (**C**) Recruitment of the MRN complex to sites of DNA damage. (**D**) The MRN complex recruits and activates ATM, resulting in H2AX phosphorylation in the chromatin surrounding the sites of DNA damage. (**E**) Recruitment of mediators (mediator of DNA damage checkpoint protein 1 (MDC1), p53-binding protein 1 (53BP1) and breast-cancer-associated protein 1 (BRCA1)) to the growing DNA damage focus. (**F**) Disassembly of the focus, ATM inactivation and chromatin remodeling and (**G**) signaling of the presence of DNA damage to the cell cycle machinery.


MRN Complex
A
DNA damage
B
C
D
ATM
P
E
BRCA1
53BP1
MDC1
F
Repair
G
Cell Cycle

1.5.4 The MRN complex

The MRE11/RAD50/NBS1 complex is thought to play a key role in the sensing, processing and repair of DNA double-stranded breaks. RAD50 is an ATP-dependent DNA binding protein that will recognize DNA ends (Hopfner et al., 2000b). It then recruits MRE11, a conserved protein with an N-terminal nuclease domain that has both 3'-to-5' exonuclease and endonuclease activity (Paull and Gellert, 1998) as well as a C-terminal DNA binding region (de Jager et al., 2001b; Hopfner et al., 2002). An intact MRE11 C-terminus, deleted in A-TLD1/2 cells, is also required to assemble signaling proteins at sites of DNA damage (Costanzo et al., 2004a). Finally, NBS1 seems to be involved in controlling the activity of this complex and is regulated upon phosphorylation by the kinase ATM (Gatei et al., 2000; Paull and Gellert, 1999). Although MRE11 has been proposed to also be a target of ATM (Kim et al., 1999), ATM deficient cells still harbor phosphorylated MRE11 (Gatei et al., 2000). It was shown that upon exposure to ionizing radiation the MRE11/RAD50/NBS1 complex becomes rapidly associated with the DNA DSBs and remains at these sites until the damage is repaired (Nelms et al., 1998). Loss of functional NBS1 in NBS patients prevents the formation of the radiation-induced MRE11/RAD50 nuclear foci (IRIF) (Carney et al., 1998). Similarly, the formation of the MRE11/RAD50/NBS1 radiation-induced foci is abnormal in A-T cells, but the defect observed is less severe than in NBS cells (Maser et al., 1997a). Since NBS1 and ATM deficiencies abrogate specific DNA damage-dependent cell cycle checkpoints, the association of the MRE11/RAD50/NBS1 complex with DSBs suggests that the DNA damage recognition functions of the complex are linked to the signal transduction pathway(s) required to activate ATM-dependent cell cycle checkpoints.

Objectives and Hypothesis

The objective of this thesis was to identify proteins that were previously unknown to be arginine methylated using newly generated methylarginine-specific antibodies in a large-scale proteomic identification approach. We hypothesized that discovering new arginine methylated proteins would lead to a better characterization and understanding of the possible roles of that post-translational modification. The major protein complexes that were identified include components required for pre-mRNA splicing, polyadenylation, transcription, signal transduction, the cytoskeleton and DNA repair. The second objective of this thesis project was to further characterize selected proteins identified in the proteomic study to define new roles for arginine methylation. We decided to investigate the role of arginine methylation in DNA repair, mainly because such a role had not been demonstrated before. In addition, MRE11, the DNA repair protein identified in the proteomic study, had a defined and well-studied role and a measurable biochemical activity that would allow us to address the cellular roles of arginine methylation.

Chapter 2

A proteomic analysis of arginine methylated protein complexes

2.1 Preface

Prior to this project, abundant RNA binding proteins, the myelin basic protein and histones were the only few known arginine methylated proteins. In order to discover new roles for arginine methylation in different cellular functions, we generated a collection of methylarginine specific antibodies. These antibodies proved useful in characterizing roles for arginine methylation in splicing and regulation of RNA binding proteins such as the spliceosomal Sm proteins and Src-associated in mitosis protein of 68 kDa, Sam68 (Boisvert et al., 2002; Cote et al., 2003). To discover new arginine methylated proteins, we used these methylarginine specific antibodies in a proteomic experiment to identify proteins that were previously not characterized as arginine methylated. This study led us to the identification of several new arginine methylated proteins and to propose new roles for this post-translational modification.

2.2 Abstract

Arginine methylation is a post-translational modification that results in the formation of asymmetrical and symmetrical dimethylated arginines (aDMA, sDMA). This modification is catalyzed by type I and II protein arginine methyltransferases (PRMT), respectively. The two major enzymes PRMT1 (type I) and PRMT5 (type II) preferentially methylate arginines located in RG-rich clusters. Arginine methylation is a common modification but the reagents for detecting this modification have been lacking. Thus less than 20 proteins have been identified in the last 40 years to contain dimethylated arginines. We have previously generated four arginine methyl-specific antibodies: ASYM24 and ASYM25 are specific for aDMA whereas SYM10 and SYM11 recognize sDMA. All these antibodies were generated by using peptides with aDMA or sDMA in the context of different RG-rich sequences. HeLa cell extracts were used to purify the protein complexes recognized by each of the four antibodies and the proteins were identified by microcapillary reverse-phase liquid chromatography coupled on line with electrospray ionization tandem mass spectrometry (LC/MS/MS). The analysis of 2 tandem mass spectra for each methyl-specific antibody resulted in the identification of over 200 new proteins that are putatively arginine methylated. The major protein complexes that were purified include components required for pre-mRNA splicing, polyadenylation, transcription, signal transduction and the cytoskeleton and DNA repair. These findings provide a basis for the identification of the role of arginine methylation in many cellular processes.

2.3 Introduction

Protein arginine methylation is a post-translational modification that adds mono-methyl or dimethyl groups to the guanidino nitrogen atoms of arginine (Gary and Clarke, 1998b). The enzymes responsible for protein arginine methylation have been classified in two major classes: type I enzymes promote the formation of asymmetrical ω-N^G,N^G-dimethylated arginines (aDMA) and type II enzymes catalyze the formation of symmetrical ω-N^G,N'^G-dimethylated arginines (sDMA) (Gary and Clarke, 1998b). ω-N^G-monomethylarginine (MMA) is thought to be an intermediate formed by both enzyme types. The metabolic cost of methylation is high, requiring the use of 12 ATP molecules per methylation event (Gary and Clarke, 1998b). The fact that evolution has retained such an "expensive" reaction underscores the biological importance of this post-translational modification (McBride and Silver, 2001a). There is now at least five type I protein arginine methyltransferases in mammals, PRMT1 (Lin et al., 1996c), PRMT2 (Scott et al., 1998b), PRMT3 (Tang et al., 1998b), CARM1 (PRMT4) (Chen et al., 1999b), PRMT6 (Branscombe et al., 2001) and one type II, PRMT5 (Pollack et al., 1999b). Recently, a new arginine methyltransferase was identified and by homology is likely a type I PRMT (Gros et al., 2003).

Myelin basic proteins (MBP) and histones are among the first proteins shown to contain dimethylated arginines (Brostoff and Eylar, 1971; Paik and Kim, 1968b). MBP has been shown to contain sDMA but the enzyme or the function of this post-translational modification remains unknown. Histones have been shown to be methylated by PRMT1 and CARM1 *in vivo* (Bauer et al., 2002b; Ma et al., 2001; Schurter et al., 2001b; Strahl et al., 2001; Wang et al., 2001b). Histone arginine methylation is thought to contribute to the histone code (Jenuwein and Allis, 2001b). Another class of dimethylated proteins include RNA binding proteins (Liu and Dreyfuss, 1995; Najbauer et al., 1993b). Arginine glycine

rich sequences have been suggested and shown to contribute to the RNA binding activity (Burd and Dreyfuss, 1994; Chen et al., 2001; Darnell et al., 2001). Thus, the methylation of these arginines would be predicted to alter or regulate their RNA binding activity, but the evidence for this has been lacking. It has been shown that arginine methylation regulates protein localization (McBride and Silver, 2001a). It was first shown by Shen and coworkers that the removal of the yeast methyltransferase Hmt1p causes the nuclear retention of two hnRNP proteins Npl3p and Hrp1p (Shen et al., 1998b). Subsequently it was shown that arginine methylation regulates the import of Npl3p (Yun and Fu, 2000b) and the protein localization of hnRNP A2 (Nichols et al., 2000b), Sam68 (26) and p80-coilin (Boisvert et al., 2002; Hebert et al., 2002b).

Arginine methylation has been shown to regulate protein-protein interactions (Bedford et al., 2000b). The discovery that Sm proteins are methylated (Brahms et al., 2000b) and the discovery that the product of the spinal muscular atrophy gene product SMN associates with methylated Sm proteins (Friesen et al., 2001a), led to the proposal that arginine methylation may be a signal that targets proteins. In the case of small nuclear ribonucleoprotein particle (snRNP) assembly, arginine methylation by the PRMT5 methylosome (Friesen et al., 2001c; Meister et al., 2001) has been proposed to be the signal for the recognition and targeting to the SMN protein complexes (Friesen et al., 2001a).

Although the role of arginine methylation in signal transduction was proposed in 1998 (Aletta et al., 1998), few signaling proteins have been identified to be arginine methylated. The inhibition of PRMT1 by anti-sense quenches the interferon signaling (Abramovich et al., 1997a) and similarly arginine methylation of STAT1 has been shown to be required for interferon signaling (Mowen et al., 2001b). Moreover, the methylation of Sam68 peptides prevents their association with SH3 domains (Bedford et al., 2000b).

In the present manuscript, we utilized 4 arginine dimethyl-specific antibodies to purify arginine methylated protein complexes. The proteins were identified by LC/MS/MS

and the protein sequences searched for neighboring RG sequences which match the epitopes used to generate the peptide antibodies. We report the identification of over 200 new proteins that are putatively arginine methylated. In addition to RNA binding proteins and transcription factors, we identified proteins involved in polyadenylation, signal transduction, the cytoskeleton and DNA repair. These data will help elucidate the role of arginine methylation in many cellular processes.

2.4 Experimental Procedures

2.4.1 Antibodies

SYM10 and ASYM24 have been described previously (Cote et al., 2003; Boisvert et al., 2002) and are distributed by UBI Inc (Upstate, NY). SYM11 and ASYM25 were derived from SmD3 and from a proposed consensus site (Gary and Clarke, 1999) and generated by immunizing rabbits with the following peptides KAAILKAQVAAR^{sDMA}GR^{sDMA}GR^{sDMA}GMGR^{sDMA}G and KFGGR^{aDMA}GGGR^{aDMA}GGGR^{aDMA}GGFGGR^{aDMA}GGR^{aDMA}G, respectively. Polyclonal antibodies were generated by using New Zealand White rabbits injected with peptides coupled to keyhole limpet hemocyanin (Sigma-Aldrich, St. Louis, MO).

2.4.2 Mass Spectrometry

HeLa-S3 (5 X 10^8) cells were obtained from Biovest International Inc./National Cell Culture Center (Minneapolis, MN) and lysed in a buffer containing 1% Triton X-100 (Roche), 20 mM Tris pH 7.4, 150 mM NaCl, 1 μg/ml Aprotinin, 1 μg/ml Leupeptin and 0.01% phenylmethanesulfonyl fluoride. Endogenous methylated proteins were immunopurified from the cell lysate using 1 mg of the respective polyclonal methyl-specific antibody coupled to 1 g of protein A-Sepharose (Sigma-Aldrich). After extensive washings with lysis buffer and PBS pH 7.4, the bound proteins were eluted with 500 μl of 1X PBS containing 250 μM of the corresponding immunogenic peptide. Eluted proteins were dialyzed against water overnight, lyophilized and identified using trypsin digestion and LC/MS/MS sequencing of peptides. Specifically, proteins were directly digested (not reduced or alkylated) with 20:1 protein:trypsin (mass ratio) for 16 h at 37 °C. LC/MS experiments were conducted on a Qstar Pulsar *i* instrument, configured with a Protana nanospray source, to which was coupled a Ultimate LC system (LC Packings/Dionex). A 75 micron i.d. by 15 cm reverse phase C18 PepMap™ column (LC Packings) operated at a flow rate of 200 nl/min,

with a gradient of 5-15%B (0-5 min), 15-50% B 5-50%B (5-40 min), 50-80%B (40-50 min). Solvent A: 0.05% aqueous formic acid. Solvent B: 0.05% formic acid in acetonitrile. All solvents were HPLC grade (Fisher Scientific). An information-dependent acquisition (IDA) experiment was conducted, using a 1 second survey scan and two, 2-second pendant MS/MS scans incorporating mass-dependent ion-bunching for sensitivity enhancement (pulsar mode). Some of the eluted proteins were resolved by SDS-PAGE and revealed by Coomassie Blue R-250 staining. Bands corresponding to appropriate molecular weights were excised, in-gel digested with trypsin, and analysed on a Voyager DE-STR reflectron MALDI-TOF, using HCCA as the matrix in a standard dried droplet sample preparation protocol.

2.5 Results

To identify dimethylated arginine containing cellular proteins, immunoprecipitations were performed with dimethyl-specific antibodies. SYM10 is an antibody that we generated previously against peptide $R^{sDMA}GR^{sDMA}GR^{sDMA}GR^{sDMA}G$. Using ELISA, we have shown that SYM10 could effectively distinguish sDMA versus aDMA, even at low dilutions of 1:500 (Boisvert et al., 2002). Further analysis by ELISA helped us define the SYM10 epitope as at least two, preferentially non-contiguous, sDMA-Gs in a given peptide, which is consistent with the fact that SYM10 does not recognize a peptide derived from MBP that harbors a singly sDMA-G (Boisvert et al., 2002). The epitope for SYM10 diminishes in PRMT5 siRNA treated cells demonstrating that PRMT5 is an enzyme that contributes to the SYM10 epitope (Boisvert et al., 2002). We have shown previously that one of the complexes immunoprecipitated by SYM10 was the spliceosomal snRNP core proteins, which include known sDMA-containing proteins Sm B/B', D1 and D3 (Boisvert et al., 2002). Among the additional proteins present in the SYM10 immunoprecipitate, we identified p80-coilin as being an sDMA-containing protein (Boisvert et al., 2002). A second antibody, SYM11 was generated against $KAAILKAQVAAR^{sDMA}GR^{sDMA}GR^{sDMA}GMGR^{sDMA}G$ and also specifically recognizes sDMA containing peptides (data not shown), as determined by the methodology described above for SYM10. SYM10 and SYM11 each recognize specific protein patterns as determined by immunoblotting (Figure 2.1). The recognition patterns are distinct but partially overlap as the Sm proteins are both recognized by these antibodies (Figure 2.1). ASYM24 was generated by using the following peptide as an antigen $KGR^{aDMA}GR^{aDMA}GR^{aDMA}GR^{aDMA}GPPPPPR^{aDMA}GR^{aDMA}GR^{aDMA}GR^{aDMA}G$ as previously reported (Côté et al. 2003). ASYM24 recognizes aDMA specifically and was shown to recognize Sam68, an RNA binding protein (Côté et al. 2003). The epitope for ASYM24 diminishes significantly in PRMT1-/- cells demonstrating the major enzyme that contributes to ASYM24 epitope is PRMT1 (26). ASYM25 was generated by immunizing rabbits with

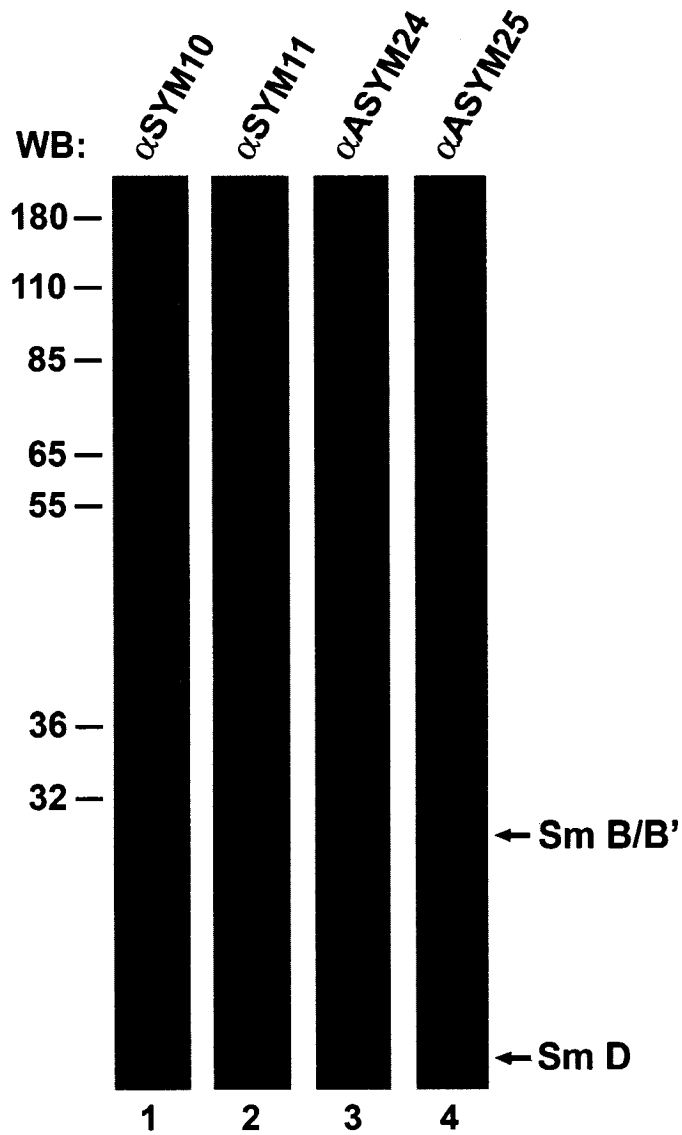
the following peptide KFG GR^{aDMA}GGGR^{aDMA}GGGR^{aDMA} GGFGGR^{aDMA}GGR^{aDMA}G. ASYM25 is aDMA specific and has a different specificity than ASYM24 as demonstrated by immunoblotting (Figure 2.1).

At least two large scale immuno-purifications were performed with HeLa-S3 cell extracts using each of the dimethylarginine-specific antibody. One control immunoprecipitation was performed using the protein A-Sepharose resin alone (data not shown). The bound protein complexes were eluted with the respective immunogenic peptides. The eluted proteins were digested with trypsin and the peptides separated and identified by LC/MS/MS, which is a mass spectrometry technique particularly suitable to the analysis of complex protein mixtures. In the case of SYM10, predominant bands visualized by SDS-PAGE were also identified by using MALDI-TOF mass spectrometry. The resulting peptide mass maps were internally calibrated using three molecular weight standards and were submitted to the Mascot (Matrix Science Ltd, UK) search algorithms (with a 30 ppm mass tolerance with acrylamide adduction chemistry for cysteines) to query the NCBI-nr database. These results were then confirmed using the Protein Prospector tools at UCSF (http://prospector. ucsf.edu) and the identifications giving MOWSE score greater than 1000 were considered positive. All proteins that were scored positive using this procedure were also identified using LC/MS/MS, validating this approach. Hence, only LC/MS/MS was used for the remainder of the analysis. LC/MS/MS data were searched using Mascot against the NCBI-nr database (release 2003-04-05) with a peptide mass tolerance of 100 ppm and a fragment mass tolerance of 0,1 Da as well as allowing for two missed cleavages. A protein was scored positive by LC/MS/MS if it was not found in the experiment performed with protein A-Sepharose alone and either 1) it was identified in more than one analysis, 2) the number of unique peptides was greater than 3, or 3) the amino acid sequence contained two RG repeats separated by less than 10 amino acids. The identified proteins were grouped according to their known or putative functions (Table 2.1-4). The proteins that contain the

RG repeats represent the epitopes for our DMA-specific antibodies and are likely to contains sDMA or aDMA (shown in bold character in Tables 2.1-4). The proteins that are devoid of RG motifs are likely co-purifying proteins.

Figure 2.1 Western blot using the methylarginine specific antibodies.

HeLa cell lysates were immunoblotted with the indicated methyl-arginine specific antibodies. The migration of the molecular mass markers is shown on the left. The migration of SmB/B' and Sm D1-D3 is shown.


WB:
αSYM10
αSYM11
αASYM24
αASYM25
180
110
85
65
55
36
32
Sm B/B'
Sm D
1
2
3
4

2.5.1 A) Pre-mRNA processing

2.5.1.1 SYM10 and SYM11

A fraction of the purified proteins with SYM10 and SYM11 include proteins associated directly or indirectly with pre-mRNA splicing (Tables 2.1, 2.2). The Sm B, B', D1/D3, and U6 snRNA-associated Sm-like proteins 4 and 8 contain RG repeats that have been shown to contain sDMA (Brahms et al., 2001b; Brahms et al., 2000b) and are known epitopes for SYM10 (Boisvert et al., 2002) and predicted epitopes for SYM11. The entire spectrum of core Sm proteins (U1A, U2A', SmB/B', U2B', SmD1-3, SmE/F/G) and several snRNP-specific factors were affinity purified indicating that all assembled snRNPs are likely purified with SYM10 (Table 2.1). Several snRNP co-purifying proteins were obtained including U1-70K, SAP155, U5-102K, U5 snRNP-specific protein, U5 snRNP 200 kD helicase, and Prp8 (Jurica and Moore, 2003). The serine-arginine (SR) kinase hPrp4 and SR proteins ASF/SF2, 9G8, SRm300 and SRp20 were likely co-purified with the snRNPs (Hastings and Krainer, 2001). However, 9G8, SRm300 and U1-70K contain RG repeats that are candidate epitopes for arginine methylation and SYM10 and SYM11 (Tables 2.1 and 2.2).

The SYM10 and SYM11 antibodies purified the splicing factors KH-type splicing regulatory protein (Min et al., 1997), homologs of zipcode binding proteins (Farina et al., 2003; Ross et al., 1997). The presence of RG repeats suggest that they contain sDMAs. The gene for *TLS* (Translocated in LipoSarcoma) or *FUS* is rearranged in human myxoid liposarcoma (Crozat et al., 1993). The TLS protein has been shown to function as a splicing factor and is also implicated in DNA repair (Hallier et al., 1998; Hicks et al., 2000). TLS was shown to be a substrate of PRMT1 (Lee and Bedford, 2002b) and shown to contain aDMA (Rappsilber et al., 2003). Thus TLS most likely represents a co-purifying protein for SYM11. Another gene product often associated with chromosomal translocation was identified namely the Ewing sarcoma protein EWS (May et al., 1993). The EWS proteins has been

shown to contain dimethylated arginines, however, it was not shown whether the modification was symmetric or asymmetric (Belyanskaya et al., 2001). Our data suggest that EWS contains sDMA because of its presence in SYM10 immunoprecipitations (Table 2.1). P80coilin is the marker for Cajal bodies and this nuclear structure is associated with snRNP assembly and thus splicing (Ogg and Lamond, 2002). We and others have shown that the RG repeats of p80coilin are symmetrically dimethylated on arginines (Boisvert et al., 2002; Hebert et al., 2002b).

Function	Accession	Protein	SYM10 epitope	a.a
Pre-mRNA Processing	4759156	U1 A	-	282
	4507121	U2 A'	-	255
	134037	Sm B/B'	PGRGGPPPPMGRGAP	240
	4507123	U2 B"	-	225
	5902102	Sm D1	AGRGRGRGRGRGRGRGRGG	119
	4759158	Sm D2	-	118
	4759160	Sm D3	ARGRGRGMGRGN	126
	312005	Sm E	-	81
	4507131	Sm F	-	86
	4507133	Sm G	-	76
	6912486	U6 snRNA-associated Sm-like protein 4, LSm4	KGRGMGGAGRGVFGGRGRGIPGTGRGQ	139
	7706425	U6 snRNA-associated Sm-like protein, LSm8	-	96
	4507119	U1-70K	HKRGERGSERGRDEARG	614
	11360330	U4/U6-associated RNA splicing factor	-	682
	2708305	hPrp4	-	522
	2463577	Prp8	-	2335
	24212088	U5 snRNP-associated 102 kD protein, U5-102K	-	941
	4759280	U5 snRNP-specific protein, 116 kD	-	972
	12643640	U5 snRNP 200 kD Helicase	-	1701
	338043	Splicing Factor ASF/SF2	-	278
	3929380	Splicing Factor 9G8	RGRRYSRSRSRSRGRR	238
	4506901	Splicing Factor SRp20	-	164
	6912654	Splicing Factor 3b, Subunit 1 (SAP155)		1304
	4504865	KH-type splicing regulatory protein, KSRP / ZBP2	GRGRGRGQG	711
	585632	p80-Coilin	GRGMRGRGRGRG	576
	544261	RNA-binding protein EWS	RGGRGGGRGG	656
	448295	TLS protein	FNRGGGNGRGGRGRGGP	260
Protein Translation	5901926	CPSF5, 25 kD subunit	-	227
	5901928	CPSF6, 68 kD subunit	PQGGRGRGRFPGA	551
	18203334	CPSF, 100 kDa subunit	-	579
	3183544	Polyadenylate-binding protein 1 (PABP1)	-	636
	4505575	Polyadenylate-binding protein 2 (PABP 2)	-	633
	346208	eIF-4 gamma		1396
DNA Transcription	5901962	MYST histone acetyltransferase 2	-	611
	107932	Transcription Factor TFEB	RGGRGSGRGADGGREGR	514
	4827071	Zinc finger protein 9	GGGRGRGMRSRGRGG	177
Receptors and Signalling	219406	Alpha2CII-adrenergic receptor	RRGALRRGGRR	458
	9506745	Urotensin II Receptor	LRGRVRGPGSGGGRGP	389
	4502371	Breast cancer antiestrogen resistance 3, RasGEF	-	825
	6740102	Crk-associated substrate p130Cas	-	870
	28373065	Potassium voltage-gated channel	SGRGRVLLNSAAARGD	932
Cytoskeletal Proteins	4502961	Alpha 1 type VII collagen precursor	PRGERGEPGIRGE	2944
	22652113	Alpha 1 type XXII collagen	ERGEKGTRGEKGERGL	1626
	5902122	Spectrin, beta, non-erythrocytic 2	PRGERQTRTRGP	2390
	1346343	Keratin, type II cytoskeletal 1	ARGGGRGSGF	644
	4508019	Bassoon	ARGPHGGPSQPTGPRGL	3926
Apoptosis	7019477	HtrA2	-	458
	2134780	Apoptosis Inhibitor IAP	-	497
	292059	Mortalin-2 (Mitochondrial Heat Shock Protein 70 kD 9B)	-	679
Enzymes	13623199	ATP citrate lyase	-	1101
	21361331	Carbamoyl-phosphate synthetase 1	-	1500
	23503239	NAD-dependent aldehyde dehydrogenase	GRGLDGAVDMGARGA	802
Others	2342526	IgE autoantigen	ERGGERGSGRRGA	757
	6912454	Extra Spindle Poles like 1	RRGTASRGRGRA	1795
	21740275	Hypothetical Protein	ARGAGRGSARAARRARRG	459
	7243183	KIAA1401	GGRHRGRGSAQRDGKGR	853
	20520991	KIAA0294	RGTRGTRGTRGTAGNG	1405
	3882183	KIAA0731	FRGRGRGRGRGRGRGRGGT	1096

Table 2.1 Proteins identified with the SYM10 sDMA-specific antibody.

Function	Accession	Protein	a.a.	SYM11 epitope
Pre-mRNA Processing	36495	SmB/B'	218	PGRGGPPPMGRGAP
	5902102	Sm D1	119	KREAVAGRGRGRGRGRGRGRGRGRGPRR
	4759160	Sm D3	126	QGSGAGRGKAAILKAQVAARGRGRGMGRGN
	88959	U1- 70k	614	HKRGERGSERGRDEARG
	1710627	HnRNP A3	379	GSQRGRGGGSGNCMGHRGNF
	27481414	Similar to hypothetical RNA- binding protein KIAA0117	425	RGSPPEDYRLGQVASSLFRGEHHSRGGTGR
	4504865	KH-type splicing regulatory protein KSRP	711	GMPPGGRGRGRGQGNW
	19923466	Splicing coactivator subunit SRm 300	2752	RGRSHSRSPATRGRSRSRTPARRGR
Protein Translation	25453474	Eukaryotic translation elongation factor 1 delta isoform 1	647	ARRGRRDRRGRN
DNA & Transcription	22094135	Histone methyltransferase DOT1L	1537	ARGDCVPSHGQDSRRRGRRK
	4505117	Methyl-CpG binding domain protein 2 isoform 1	411	GRGGGVCGRGRGRGRGRGRGRGRGRGRPP
	22653669	Transcription terminator interacting protein 5	1878	ITGKRGRPRNTEKAKTKEVPKVKRGRGRPP
	2723380	DSIF p160 / transcription elongation factor	1087	PSAGGGRGGFGSPGGGSGGMSRGRGR
	16554587	TFIIF-associating CTD phosphatase I	961	SAAGGRGPRGHKR
	13540590	C/EBP induced protein	453	MAGAAAGGRGGGAWGPGRGGAGGLRRGCSP
	4758600	Interleukin enhancer binding factor 1 (ILF1)	655	GTTRARGRGRGAGGGSRRALGRGPPGGPRV
	24234747	Interleukin enhancer binding factor 2 (ILF2)	390	MRGDRGRGRGGRFGSRGGPG
	256299	p98=Rel / NF-kappa B p105 homolog	900	-
Receptors and Signaling	4506569	Roundabout 1 isoform a; ROBO1	1651	EEEDERGPTPPVRGAASSP
	1351829	Alpha-2A adrenergic receptor	450	RGPRGKGKARASQVKPGDSLPRRG
	30353923	Prostate derived STE20- like Kinase PSK	1462	RGGFPGPPLRKGESRGRGGK
	4504795	Inositol 1,4,5-triphosphate receptor, type 3	2671	VGNRGTFIRGYKAMV
	4507157	Mosaic receptor SorLA / LRII	2214	WARGDARGAS
	1497931	Ataxia- telangiectasia / ATM	3058	-
Cell Cycle	940536	P1 Cdc21 protein	923	RGSRRGRATPAQTPRSEDARSSPSQRRRGE
Cytoskeletal Proteins	1346343	Keratin, type II cytoskeletal 1	644	ARGGGRGSGFGRG
	22652113	Alpha 1 type XXII collagen	1626	ERGEKGTRGEKGERGL
	18375518	Alpha 1 type XI collagen isoform A preproprotein	1806	ARGVAGKPGPRGQRGPTGPRGSRGARGPT
	4502961	Alpha 1 type VII collagen precursor	2944	PRGERGEPGIRGEDGRPGQEGPRGLTGPPGSRGERG
	435476	Cytokeratin 9	623	GRGSRGG
	4803663	Ankyrin B	3925	RGNTNMVKLLLDRGG
	5902122	Spectrin, beta, non-erythrocytic 2	2390	PRGERQTRTRGP
	4557703	Keratin 2a	645	MSCQISCKSRGRGGGGGGFRGFSSGSAVVS
	13435369	Desmocollin 3 isoform Dsc3b preproprotein	839	NQTLESCRGAGHHHTLDSCRGGHTE
	19115954	Dynein, axonemal, heavy polypeptide 5	4624	-
	13376204	E- catherin binding protein E7	491	-
Enzymes	3915598	DNA-3-methyladenine glycosylase	298	EDEAAHSRGGRQTPRNRGMF
	15076827	Pentachlorophenol hydroxylase (Pcph)	428	VRGKLHQPEEVQRGSFYA
	118090	Peptidl-prolyl cis-trans isomerase B precursor	208	-
	224517	Kinase, phosphoglyserate	417	-
	26665879	CTAGE-2 / chromosome segration ATPase	754	RGGGRGSRGPGNPPDHQITKERG
	3121981	Probable ATP- dependent RNA helicase DDX10	875	-
Others	6841194	HSPC272	2011	TSRLSGNRGV QYTRLAVQRG
	7717446	PRED59	211	AAGGQTRGRRWDRGGPGGPC
	292059	MTHSP75	679	-
	11415038	Solute carrier family 22 member 3/ organic cation transporter 2	556	WNRTAPASRGPEPPERRGRC
	5042405	BC282485_1	477	LYKRGGRGSRGRGRPARPSP
	4503469	Early endosome antigen 1, 162kD	1410	-
	21553341	Synaptic nuclei expressed gene 2 nesprin 2	6885	-
	11877243	SSF1 / P2YII chimeric protein	794	-
	338490	52-kD SS-A / Ro autoantigen	475	-
	7513172	N-chimerin homolog F25965_3	903	YRAAPPAYGRGGELHRGSLY
	20539645	Similar to mKIAA0038 protein	228	AYSSFGSGRGSRGSAGGHGS
	14572619	Novel, protein, KIAA1769	2567	-
	6760015	Brain protein	344	PADDGAGPGGRGPRGRARGD
	3882199	KIAA0739 protein	1130	RTHGQKHRRRGRGKGASQGE
	10047331	KIAA1627 protein	468	RGGGRGGTSAGRGRERNRSNFRGERGGFRGGRGG
	3882183	KIAA0731 protein	1096	GGARASFRGRGRGRGRGRGRGRGGTRTHFD
	14017861	KIAA1822 protein	533	RGRPTAAPPAPTPRPARPTQQPGSRRGGGRRRG
	4204880	Heat shock protein	639	-
	11321605	Smcx homolog, X chromosome, XE169	1560	GSRARGRALERRRRRKVDRGGEGD
	29123596	SET binding factor 2	1849	-
	7512605	Hypothetical protein DKFZp434H244.1	845	RFELRRGSGGRGRFRGGDGV
	22044805	Hypothetical protein XP_065829	505	CLETAVLRGRRARGRFRGST
	18593376	Hypothetical protein XP_097805	161	AAASGARRHRGRGSIGGPFH
	27480502	Hypothetical protein XP_209301	261	PARGQHRTQPLRGRGWRGPL
	27486452	Hypothetical protein XP_211272	170	SGRTVRGSVSWKRPEQAAPETGRGPAVARG
	22044805	Hypothetical protein XP_065829	505	CLETAVLRGRRARGRFRGST
	27486452	Hypothetical protein XP_211272	170	TGRGPAVARG
	27479241	Hypothetical protein XP_212123	103	SERGQPTRGR
	18601673	Hypothetical protein XP_047499	232	SGARGGGRGARARRPGQRGR
	24432052	Hypothetical protein FLJ25348	696	GRGSIAPRGISAWQRPPRGRGR
	21389373	Hypothetical protein FLJ25359	506	-
	30156226	Similar to hypothetical protein FLJ35782	763	LRVARGLLATRVAGIRNRGNWRDG
	19913516	Unknown (protein for MGC:26598)	408	KRWGRRGRGL
	22477301	Unknown (protein for MGC:46140)	514	IEECRILRGPDGLSRGCAFV
	10435310	Unnamed protein product	193	TSVRDRRGDR HRGEGHVETE
	14336692	Unknown	321	PGGGAARGGP HLGGCGSAAR SGRGAGRGGA

Table 2.2 Proteins identified with the SYM11 sDMA-specific antibody.

2.5.1.2 ASYM24 and ASYM25

Three RNA helicases containing RG repeats were purified with ASYM25 (Table 2.4). One of these helicases, RH70, co-purifies with the U1 snRNP (Lee, 2002). In addition, several RNA binding proteins were identified including Sam68, the Src substrate in mitosis, which has been shown to function in various aspects of RNA metabolism (26). HnRNP Q2 protein was purified with ASYM24 (Table 2.3) suggesting that it contains aDMA. The RG sequences of hnRNP Q interact with SMN and may link SMN to the spliceosome (Mourelatos et al., 2001).

Function	Accession	Protein	a.a	ASYM24 epitope
RNA Processing	20450941	RR M containing RNA-binding protein	593	RGRGAAGNR
	15809568	HnRNP Q2	588	RGGPGSARGV RGARGGAQQQ RGRGVRGARG
	29733020	RRM similar to REKIN cDNA	425	GQVASSLFRGEHHSRGGTGR
DNA and Transcription	181488	Zinc finger DNA-binding protein	1902	YVYVRGRGRG
	12697318	PBX4 protein	330	PEKRGRGG
	1388162	Interleukin enhancer binding factor 2 (ILF 2)	609	TRARGRGR
Protein Translation	484950	Valine-tRNA ligase	1051	ALKERGLFRG
	20380061	Likely ortholog of mouse variant polyadenylation protein CSTF-64	616	RGMETCAMETRGMEARGMDARGLEMRG
	226021	Nuclear protein with sequence homology to translation initiation factor eIF-4A	594	RSRGRGGMK
	15029520	histidyl-tRNA synthetase	506	-
Receptor and Signaling	7446458	Protein kinase (EC 2.7.1.37) N beta	889	RGRGELASE
	4507009	Solute carrier family 25, member 14 isoform UCP5L	325	EGTRGLWRGV
	13632400	Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing beta	1634	TGRGRGMV
	631148	FLT3/FLK2 ligand (clone S109	245	RGESPARGCI AWTQRKLARG
	7768779	Transient receptor potential-related channel 7, a novel putative Ca2+ channel protein	1503	GLRGRGSL
Cytoskeletal	126363	Alpha-1 chain precursor (Laminin A chain)	3075	HRGKLPAGSD RGPRPLVAPC
	4502955	Alpha 5 type IV collagen isoform 1	1685	GLPGDRGPPG PPGIRGPPGP
	2119157	Collagen alpha 1(XIX) chain precursor	1142	PPGKEGQRGR RGKTGPPGKP
	18765746	Alpha 1 type XVIII collagen isoform 3 precursor; endostatin	1473	RGPRGFPGPP GGMRGIRGAD
	8953371	BA448E12.1 (collagen, type IV, alpha 6)	1116	GLKGARGDRG
	190394	Profilaggrin	1084	IRGHPGSRRG
	13518037	Matrilin 2 precursor	956	AEARERSRGR SISRGRHART
	27764561	TPA: keratin 1b	502	RGGGARGRSG
	27720593	Similar to cartilage intermediate layer protein	1069	QRASRGGLRR RGSMAPLRFS
	32140760	Collagen XXVII proalpha 1	1860	PPGPPGDRGP VGDRGDRGEP
	338440	Spectrin Rouen (beta-220-218) mutant coding sequence	2106	PTTASRGGRR DSRGGSSFPP
	135448	tubulin beta-1 chain	444	-
	4505037	Latent transforming growth factor beta binding protein 4 / LT BP-4	1587	RGPGGRGLLR
Enzyme	4503021	Carnitine palmitoyltransferase 1A	773	LRGRGPLM
	24429592	Chondroitin beta1,4 N-acetylgalactosaminyltransferase 2	542	EEFNRGRGLN
	30350206	AP20 region protein isoform B	170	KRGRGCNG
	27881700	Mitochondrial topoisomerase I	602	KIEPPGLFRG RGDHPKMGML
	13994294	AAA-ATPase TOB3	578	-
Other	2772564	ADP / ATP carrier protein	298	-
	4506457	reticulocalbin	317	-
	27462366	AG02	932	GKKRRGRRSKKERRRGRKEG
	7661890	Sorting nexin 17	470	SGSTSSPGRG RGEVRLELAF
	19263717	Similar to RIKEN cDNA 2610027L16 gene	536	RGRGAKGSG
	7209305	FLJ00002 protein	1513	LHRLRGHVAV RGLSKGFGLA
	18379346	VPS10 domain receptor protein SORCS 3	1222	AGERRGRGI
	19913532	Tubby like protein 2	520	GDGRGERGL
	15076827	Pcph proto-oncogene protein	428	VRGKLHQPEE VQRGSFYAFS
	4589624	KIAA0990 protein	802	ARGDARGAQL
	17474221	Similar to KIAA1595 protein	140	SLARGFSLLR MPKMPELRGK
	24899186	KIAA2011 protein	1091	RKTPRGKRGW
	4507285	Syntaxin 10	249	RGEVQKAVNT ARGLYQRWCE
	10092621	Oncostatin M precursor	252	FPSEETLRGL GRRGFLQTLN
	27436933	Orthodenticle 2 isoform b; homeobox protein OTX2	289	SSRGWQGRRG
	23397570	Hypothetical protein FLJ31579	304	LSERASRGSF HVSQAILTPR VKTIARGLVG
	27499658	Hypothetical protein XP_208775	364	EGRGRDRGRL
	29789407	Hypothetical protein LOC284996	400	PGGRGAAPWV ALVARGGCTF
	17484894	Hypothetical protein XP_066268	176	GRGPEPSGWE LRRGRCAFGK
	27734689	Hypothetical protein FLJ36112	283	ATLERGRGP
	7513003	Hypothetical protein KIAA0522	1560	VGPRPPRERG QLSRGASRSS
	30425424	Hypothetical protein FLJ40434	334	RGGPVGLLQGRGAAVAEADPLHHDEVRLLRAHGRG
	14150001	Hypothetical protein DKFZp434G118	939	SERGLHSPSQ RSHRGPSQRR
	27478389	Hypothetical protein XP_166555	166	YLRAAHGRGM ERGLLCVPRR
	8922609	Hypothetical protein FLJ10709	586	-
	30268243	Hypothetical protein	1679	FPVSQKRGTI ENERGKPLPS
	29744798	Similar to hypothetical protein FLJ33516	1982	YREGLVRGSF RGSFLDYYAA
	23270808	Similar to hypothetical protein FLJ31614	740	AITFQEFARG FLGSLRGGRR
	23272713	Unknown (protein for IMAGE:5722844)	557	DFTRGSRGRK
	22800407	Unknown (protein for MGC:15173)	399	SPRQPRGGRGGGACSAKKERGG
	7022583	Unnamed protein product	864	RGERGNDESA
	28070992	Unnamed protein product	124	RGPEFD ARPLPTRGCD

Table 2.3 Proteins identified with the ASYM24 aDMA-specific antibody.

Function	Accession	Protein	a.a	ASYM25
Pre-mRNA processing	5453840	RH70 RNA heliase	650	RGGGFGDRDRDRDRGGFGARGGG
	3915658	ATP-dependent RNA heliase A	1270	RGVSRGGFRGNSGGDYRGPSGGYRGSGGFGRG
	1041747	Sam 68	443	RGRGVPVRGRGAAPPPPPVPRGRGVGPPRGALVRG
Protein translation	35903	Ribosomal protein L7	248	-
	4506671	Ribosomal protein P2	115	-
	4503477	eEF1 beta 2	225	-
DNA transcription	29743861	Similar to widely-interspaced zinc finger motifs	957	VLRGGIPGPPLYPGRGRTAF
	13376064	Zinc finger protein 408	720	CGRAFRQRGNLRGHLRLHTG
Receptors and signaling	7656967	Cadherin EGF LAG seven-pass G-type receptor 1	3014	SVRRGFRGC
	21929089	Seven transmembrane helix receptor	1464	RGGQSSARGV
	30156191	Similar to MrgE G protein-coupled receptor	418	RVERGPQRPPPRGFPGLILL
	21594833	Platelet-derived growth factor receptor, beta polypeptide	1106	EQTVRCRGRG
	28422541	Phosphatidylserine receptor beta	441	PPRPGRGALVSGSGLRRGRS
	6760665	FLASH homolog RIP25	1982	EDSRRGRKDIRHSQFNRGTE
	22532415	Regulatory protein NOXO1-gamma / p47 phox	376	SGTGFRGGDDPAGEARGFPE
	14579311	Atypical PKC isotype-specific interacting protein long variant b	1273	RGRGCNESFR
Cytoskeletal proteins	446631	Collagen:SUBUNIT=alpha2:ISOTYPE=IX	618	RGGRGHPGMPGPPGIPGLPGRPGQAINGKDGDRGS
	115313	Collagen alpha 1(V) chain precursor	1838	RGQRGPTGPRGERGPRGITGKPGPKGNSGPPGERG
	13435369	Desmocollin 3 isoform Dsc3b preproprotein; desmocollin 4	839	SCRGAGHHHT LDSCRGGHTE
	18698322	Synemin	1251	ELRGRRGREG
DNA repair	4505339	Nibrin	754	-
	5031923	MRE11	708	GRGRGRRGGRGQNSASRGGSQRGRA
	5032017	Rad50	1318	-
Enzymes	181362	Cytochrome P-450 S-mephenytoin 4-hydroxylase	485	GRGIFPLAERANRGFGIVFS
	2209278	Oxytocinase splice variant 2	1011	EYEPRGSRLLVRGLGEHEME
	9966821	Lysosomal apyrase-like protein 1	604	HVRGRGDWV
	5305594	Ca2+-independent phospholipase A2 short isoform	752	AYMRGMYFRMKDEVFRGSRP
	7435520	Adenosylhomocysteinase (EC 3.3.1.1) DKFZp564A1523	797	HRGGSRRGKI
	15026974	Obscurin Rho GEF	6620	LGPRGPLGLFRPEPRGASPP
Others	29735143	Similar to KIAA1311 protein	949	RGRGRGRGGR
	18916736	KIAA1944 protein	657	RRGRGCTLQY
	3694661	Carrier protein-like; similar to Q01888	391	RGPAPCRAGPGARHLRPWPESPRPEPRGLPGPGRG
	22328128	Similar to Meningioma-expressed antigen 6/11 (MEA6) (MEA11)	811	GGEGRGSRGPGNPLDHQITKERGESSCERL
	11990772	BA534G20.4 (supervillin)	1084	RGRGAANDS
	1945155	MN1 probable tumor suppressor	1342	GRGRGRRK
	27499679	Similar to 2210403N09Rik protein	276	RGHSAGRGDE
	11359992	Hypothetical protein DKFZp434F117.1	834	RGRCDSRGNQ
	7657017	Hypothetical protein DJ328E19.C1.1	921	KGKKRRGRRSKKERRRGRKE
	20483460	Hypothetical protein XP_116897	258	LFRGKAGKPSQGRGMAVRLM
	29738552	Hypothetical protein XP_061446	344	RSELQARGLRRGNAGRRLEL
	30154584	Hypothetical protein XP_302601	114	QRGRSGSGNFGGGRGGGFSGNDN
	24432043	Hypothetical protein FLJ13511	668	RGRGGPESP
	22749001	Hypothetical protein FLJ30373	192	MGHEGRGQSGELGDLGARGP
	21732492	Hypothetical protein	1289	RGRPEISLDERGEGGHVHTS
	21732438	Hypothetical protein	723	RGRGCALQYQ
	13528825	Similar to hypothetical protein FLJ20003	316	EGRGRGRD
	21595426	Unknown (protein for MGC:40478)	412	KRTKDRGTMDDDDFRRGHPQ
	36575	Unnamed protein product	478	SQRGHSRGRN
	14336692	Unknown	321	SGRGAGRGGARGFSTVTRGH

Table 2.4 Proteins identified with the ASYM25 aDMA-specific antibody.

2.5.2 B) Protein translation

A protein complex that was affinity purified by SYM10, but not SYM11, was the cleavage and polyadenylation specificity factor (CPSF) complex including the 25, 68 and 100 kDa subunits (Table 2.1). The polyA binding proteins PABP1 and PABP2, as well as eIF-4G were purified (Kahvejian et al., 2001). CPSF6, a protein of 68kDa, has several RG repeats that are likely the epitope for SYM10 and the other components are likely co-associating proteins (Ruegsegger et al., 1998). The PABP has been shown to be asymmetrically dimethylated by CARM1 at arginine 455 and 460, which is not an epitope for SYM10 (Lee and Bedford, 2002b). Thus PABPs and eIF-4G are likely purified as co-associating proteins with the CPSF complex. SYM11 purified elongation factor 1 delta isoform which is a guanine nucleotide exchange factor (Table 2.2). ASYM24 and ASYM25 purified several ribosomal proteins and tRNA components (Table 2.3 and 2.4).

2.5.3 C) DNA transcription

The role of arginine methylation in transcriptional regulation is well established with histones being a major target of protein arginine methyltransferases (see introduction). As histones do not contain RG rich sequences, we did not expect to identify them and indeed histones were not identified. SYM11 purified transcriptional proteins involved in all aspects of transcription. Several histone modifying proteins including MYST histone acetyltransferase 2 (Table 2.1, (Utley and Cote, 2003)) and histone lysine methyltransferase DOT1L were identified (Table 2.2, (Feng et al., 2002)). Two proteins with methyl binding domains were identified (Table 2.2) including methyl-CpG binding protein 2 (MBD2) which plays a role in transcriptional control (Bhattacharya et al., 1999; Hendrich and Bird, 1998) and the transcription terminator factor I interacting protein 5. A protein involved in transcriptional elongation, DSIF p160, and a serine phosphatase TFIIF-associated CTD phosphatase I which is involved in the recycling of RNA polymerase II (Cho et al., 1999) were purified with

SYM11. Several transcription factors were also identified including the basic helix-loop-helix leucine zipper protein TFEB, p98 Rel homolog, interleukin enhancer binding factor 1 and 2 (ILF1, ILF2), Zn finger DNA binding proteins, homeodomain protein PBX4, the C/EBP induced protein and ZNP9. Interestingly, ILF2 was identified with SYM11 and ASYM24 suggesting that it contains both modifications. The amino acid sequence of TFEB has RG repeats that are likely candidates for SYM10 recognition. A chromosomal translocation leads to a promoter switching giving rise to an *AlphaTFEB* fusion gene which leads to renal cell carcinomas (Davis et al., 2003; Kuiper et al., 2003). The ZNF9, RING zinc finger protein 9, was identified by LC-MS/MS and the presence of RG repeats in its sequence suggest that it a direct epitope of SYM10. The function of ZNF9 is unknown but it is a nucleic acid binding protein with an AIR1 (arginine methyltransferase-interacting protein 1) domain (Inoue et al., 2000). The presence of CCTG repeats in intron 1 of *ZNF9* gene leads to myotonic dystrophy type 2 (Liquori et al., 2001). Thus arginine methylation may regulate the transcriptional initiation, elongation and termination.

2.5.4 D) Receptors and signaling

Six G-coupled receptors were identified with the DMA-specific antibodies. Both the alpha2CII-adrenergic receptor and the urotensin II receptor were purified by SYM10 (Table 2.1), alpha-2A adrenergic receptor was identified with SYM11 (Table 2.2), and three unclassified receptors with ASYM25 (Table 2.4). This analysis is the first indication that G-coupled receptors may contain dimethylated arginines and the presence of RG repeats suggest that the receptors were directly recognized by the antibodies. The alpha2 CII-adrenergic receptor is present in the axon terminals of neurons of spinal cord origin and may mediate nociceptive information (Olave and Maxwell, 2003). The urotensin II receptor has vasoactive properties and its increased expression in cardiomyocytes correlates with cardiac dysfunction (Douglas et al., 2002).

Other cell surface receptors that were identified include Roundabout 1 (Table 2.2) which is an axon guidance receptor that controls axon crossing of the central nervous system midline (Kidd et al., 1998). The platelet derived growth factor receptor beta was identified with ASYM25 and it contains RG repeats (Table 2.4). A phosphatidylserine receptor was identified with ASYM25. Phosphatidylserine receptor is required for recognition of the asymmetrical phosphatidylserine distribution of a dying cell (Fadok and PM., 2003). A potassium voltage-gated channel was identified with SYM10 (Table 2.1) and calcium channel was identified with ASYM24 (Table 2.3).

Intracellular proteins involved or associated with signaling proteins were also identified including the Crk-associated substrate p130cas, the breast cancer antiestrogen resistance 3(BCAR3), the STE20-like kinase from prostate (PSK), ataxia telangiectasia (ATM), protein kinase N beta, FLASH homolog RIP25, phosphatidylinositol-4-phosphate-3-kinase, and NOXO1. CAS is an SH3 domain docking protein that participates in FAK-dependent cell migration (Harte et al., 1996). BCAR3 contains a putative SH2 domain and a guanine nucleotide exchange activity and has been shown to associate with CAS (Riggins et al., 2003). The absence of RG repeats in CAS and BCAR3 indicates that they were likely co-purified. PSK contains several RG rich repeats and was identified with SYM11. PSK is known to activate mitogen-activated protein kinase pathways (c-Jun NH(2)-terminal kinase, p38, or extracellular signal-regulated kinase)(Mitsopoulos et al., 2003). ATM is a serine kinase that involved in DNA repair signal transduction, the absence of RG repeats indicates that it was co-purified (Savitsky et al., 1995a). Protein kinase N beta, is an unassigned kinase identified with ASYM24. The FLASH homolog RIP25 is known to be involved in signaling to the interleukin 2 gene expression and was identified with ASYM25. Phosphatidylinositol-4-phosphate-3-kinase C2 beta represents a class II PI3K and has been shown to be recruited to tyrosine kinase complexes (Wheeler and Domin, 2001). NOXO1 gamma is an RG

containing protein purified with ASYM24 (Table 2.3). NOXO1 was identified as a homolog of p47phox and has been shown to regulate superoxide production (Banfi et al., 2003).

Several proteins implicated in apoptosis were also obtained including HtrA2, apoptosis inhibitor protein (IAP), and mortalin-2 (Vaux and Silke, 2003). These proteins are co-purified as they are devoid of RG repeats. The identification of the phosphatidylserine receptor with ASYM25 suggests that arginine methylation may play a role in cell death.

2.5.5 E) DNA repair

The MRE11/ Rad50/ NBS1 complex (Petrini, 2000) was purified by using ASYM25. The MRE11/ Rad50/ NBS1 complex localizes to radiation induced foci upon double-strand DNA breaks (Maser et al., 1997b). The presence of RG repeats in MRE11 suggests that MRE11 harbors an epitope for ASYM25 and that Rad50, NBS1 and ATM are co-purifying proteins with ASYM25 and SYM11.

2.5.6 F) Cytoskeleton

Many cytoskeletal proteins including keratin type II cytoskeletal 1, alpha 1 type VII, XI and XXII collagen precursors, spectrin, ankyrin, and dynein were purified (Tables 2.1-4). It is tempting to speculate that some of the components like keratin are contaminants but the presence of abundant RG repeats makes these proteins likely targets for the DMA-specific antibodies.

2.6 Discussion

In the present study we have identified over 200 proteins that contain RG rich repeats and are putative substrates of protein arginine methyltransferases. The identification and purification of proteins known to contain dimethylated arginines such as SmB/B', SmD1/D3, p80-coilin, EWS, TLS and Sam68 confirms our strategy. Most of these proteins are associated with RNA metabolism and contain RG-rich motifs. Gary and Clark (1998) searched for PRMT substrates by using databases with glycine arginine rich consensus sequences (GAR; (Gary and Clarke, 1998b)). The GAR consensus sequence was derived from the methylation sites of fibrillarin, nucleolin, hnRNP A1, basic fibroblast growth factor and MBP (Gary and Clarke, 1998b). Putative substrates identified included mammalian EWS, hTAFII68, hnRNPs, TLS and ribosomal S2. Thus the major category of putative substrates was RNA binding proteins. By using antibodies that are specific for sDMA or aDMA, we were able to minimize the amount of RNA binding proteins identified. Each antibody was designed with unique surrounding sequences and thus we were able to identify proteins that could not be predicted by using a GAR domain search.

The presence of Sm proteins and p80-coilin in SYM10 and SYM11 immunoprecipitations indicate the specificity of these antibodies for sDMA-containing proteins in cellular lysates. It has been reported that the abundant hnRNPs account for close to 65% of all arginine methylation that occurs in the cell (Liu and Dreyfuss, 1995). These proteins often contain long stretches of RG and RGG repeats which have been demonstrated to contain asymmetrically dimethylated arginines. The fact that the SYM10 and SYM11 immunoprecipitated only a few hnRNPs further demonstrate the specificity of the antibody for sDMA, and not aDMA or non-methylated RG rich containing proteins. We would have expected the ASYM24 and ASYM25 antibodies to recognize the spectrum of hnRNP proteins, the nucleolar proteins fibrillarin, nucleolin and ribosomal protein S2. In addition, we have not identified the cell death regulator aven (Chau et al., 2000) and 53BP1 (Iwabuchi

et al., 1994b), two proteins known to contain extensive RG motifs. This demonstrates that the methyl-antibodies that we generated recognize a subset of methylated proteins excluding the majority of hnRNPs. Actually, methylated peptides corresponding to hnRNP K arginine glycine repeats were not recognized by ASYM24 in an ELISA (Côté et al. 2003). In addition the mass spectrometry data did not identify the SMN and PRMT5 complexes in the SYM10 immunoprecipitations performed with HeLa S3 cells. SMN and PRMT5 was readily identified in SYM10 immunoprecipitations in T4 HeLa as shown previously (Boisvert et al., 2002). It has been shown that SMN complexes behave differently in certain HeLa cell types (Matera and Frey, 1998).

Thus, the proteins that were identified through the large-scale immunoprecipitation can only be present if 1) they contain DMA and are directly immunoprecipitated by the antibody, 2) they are present in a complex that contains DMA or 3) they are contaminating proteins that were non-specifically bound during the purification process. The presence of potential epitopes (RG repeats) suggest that most of the proteins were directly recognized by the methylarginine specific antibodies. It is possible that some of the cytoskeletal proteins are contaminants, especially keratin, but the presence of RG repeats in their sequences provides a reason for their selective purification. The identification of many proteins in the same complex such as the pre-mRNA complex and polyadenylation complex allows us to conclude that DMA is potentially involved in regulating the function of that complex.

The identification of proteins involved in pre-mRNA splicing and transcription was expected (McBride and Silver, 2001a). The identification of the G-coupled receptors, the polyadenylation complex and proteins involved in DNA repair and cell death was unexpected. The role of sDMA in snRNP assembly is known and has been proposed as a signal for targeting to the SMN complex (Friesen et al., 2001a). Treatment of cell extracts with SYM10 or with methylase inhibitors impairs pre-mRNA splicing (26). The

identification of several splicing factors including KSRP and the SR proteins suggest a new level of regulation of splicing by arginine methylation.

The role of arginine methylation in transcription has been known since the identification of CARM1 as a coactivator (Chen et al., 1999b). The regulation in most of those studies involves the histone arginine methylation. The identification of transcription factors including TFEB, ILF proteins, PBX as well as the CTD phosphatase, DSIF p160 transcription elongation factor and the transcription terminator interacting protein 5 will direct us towards new modes of transcription regulated by arginine methylation. The interplay between sDMA and transcription has been suggested (Fabbrizio et al., 2002), however, the substrates of PRMT5 were not identified. Finally, arginine methylation could also delineate the transition between promoter binding and transcription elongation, an example of which has been demonstrated for SPT5 (Kwak et al., 2003).

The presence of 6 G-coupled receptors in DMA-specific immunoprecipitations leads us to believe that these proteins are likely to be dimethylated on arginines. The presence of RG rich containing sequences further provide evidence that this will be the case. It will be interesting to determine whether the methylation interferes with the signaling or potentially with the recycling/ desensitization of the receptors.

The purification of the cleavage and polyadenylation specific complex by SYM10 demonstrates that a protein within the complex contains sDMA (Ruegsegger et al., 1998). The presence of RG repeats in CPSF6, the 68 kDa subunit, indicates that this protein is putatively methylated by type II PRMTs. The fact that SMN associates with sDMA containing proteins (Friesen et al., 2001a) and has been dubbed the 'master' assembler (Terns and Terns, 2001) suggest that the CPSF complex formation, localization and function may be regulated by SMN complex and/ or the methylosome.

The MRE11/ Rad50/ NBS1 complex (Petrini, 2000) was purified by using ASYM25. In addition, the ATM kinase was purified with SYM11. The MRE11/ Rad50/ NBS1 complex

localizes to radiation induced foci upon double-strand DNA breaks (Maser et al., 1997b) and the ATM kinase is involved in signaling DNA damage (Savitsky et al., 1995a). The presence of RG repeats in MRE11 suggests that MRE11 harbors an epitope for ASYM25 and that Rad50, NBS and ATM are co-purifying proteins. The purification of proteins involved in DNA repair indicates that arginine methylation will have a role in this cellular process. Arginine methylation may regulate the signaling of DNA damage, regulate protein-protein interactions or protein localization during DNA damage.

In conclusion, the identification of proteins containing DMA will allow us to shed some light on new roles for this post-translational modification. The methylation of every protein on the list of putative methylated proteins will have to be confirmed and their physiological roles demonstrated. The present study will facilitate the integration of arginine methylation in pre-mRNA splicing, protein translation, receptor signaling, transcription, DNA repair and the cytoskeleton.

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Chapter 3

Arginine methylation of MRE11 by PRMT1 is required for DNA damage checkpoint control.

3.1 Preface

A role for protein arginine methylation in DNA damage checkpoint control and DNA repair was up to now largely unknown. Our previous study aimed at discovering new substrates of arginine methyltransferases identified the MRN DNA repair complex using an antibody recognizing asymmetrically dimethylated arginines. This chapter describes the identification and characterization of the methyltransferase responsible for the methylation of MRE11, the protein from the MRN complex that we found to be arginine methylated and recognized by our methylarginine specific antibodies. Moreover, we analyzed the role of arginine methylation in the regulation of MRE11 biochemical and signalling functions during the cellular response to DNA damage.

3.2 Abstract

The role of protein arginine methylation in the DNA damage checkpoint response and DNA repair is largely unknown. Herein we show that the MRE11 checkpoint protein is arginine methylated by PRMT1. Mutation of the arginines within MRE11 severely impaired the exonuclease activity of MRE11, but did not influence its ability to form complexes with RAD50 and NBS1. Cells containing hypomethylated MRE11 displayed intra-S-phase DNA damage checkpoint defects that were significantly rescued with the MRE11-RAD50-NBS1 complex. Our results suggest that arginine methylation regulates the activity of MRE11-RAD50-NBS1 complex during the intra-S-phase DNA damage checkpoint response.

3.3 Introduction

Protein arginine methylation is a post-translational modification that results in symmetrical or asymmetrical dimethylarginines (sDMA, aDMA)(Gary and Clarke, 1998b). In humans, protein arginine methyltransferases (PRMT) represent a family of 8 known enzymes that utilize S-adenosyl methionine (AdoMet) as a methyl donor (McBride and Silver, 2001b). The substrates of these PRMT include glycine arginine rich (GAR) nucleic acid binding proteins (Gary and Clarke, 1998b). Arginine methylation has been shown to regulate nuclear export (Yu et al., 2004), nuclear import (Yun and Fu, 2000a), protein-protein interactions (Bedford et al., 2000b), ribosome biogenesis (Bachand and Silver, 2004b; Swiercz et al., 2004), pre-mRNA splicing (Boisvert et al., 2002; Friesen et al., 2001a), transcriptional elongation (Kwak et al., 2003) and transcription (Chen et al., 1999b; Cuthbert et al., 2004a; Rezai-Zadeh et al., 2003; Wang et al., 2001a; Wang et al., 2004a). However, it is not known whether arginine regulates cell cycle checkpoints and the DNA damage response.

DNA double-strand breaks (DSB) are a common form of damage that occurs due to external factors such as ionizing radiation or exposure to certain chemicals. Failure to properly repair the damaged DNA results in genetic instability, which can ultimately lead to cancer. Genetic defects underlying chromosomal instability include ataxia-telangiectasia (A-T), A-T like disorders (A-TLD) and Nijmegen breakage syndrome (NBS) (Shiloh, 2003b). Patients with any one of these disorders exhibit hypersensitivity to ionizing radiation, immunodeficiency and an increased predisposition to the development of malignancies. These phenotypic outcomes indicate that ATM, MRE11 and NBS1, the gene products deficient in A-T, A-TLD and NBS respectively, play an important role in maintaining genomic integrity (Carney et al., 1998; Savitsky et al., 1995b; Stewart et al., 1999; Varon et al., 1998). A biochemical link between DSB repair and mammalian cellular responses to DNA damage was uncovered by the observation that NBS1 functions in a complex with the

highly conserved DSB repair proteins MRE11 and RAD50 (Carney et al., 1998). The MRE11/RAD50/NBS1 (MRN) complex is known to be involved in the DNA damage response (Carson et al., 2003b; Lee and Paull, 2004; Stewart et al., 1999; Theunissen et al., 2003b; Uziel et al., 2003; van den Bosch et al., 2003). Upon exposure to ionizing radiation, the MRN complex becomes rapidly associated with the DNA DSBs at radiation-induced nuclear foci and remains at these sites until the damage is repaired (Lisby et al., 2004b; Nelms et al., 1998). MRE11 is a conserved protein with an N-terminal nuclease domain (Paull and Gellert, 1998) as well as a C-terminal DNA binding region (de Jager et al., 2001c; Hopfner et al., 2002) and a GAR domain of unknown function.

Herein, we report that MRE11 contains aDMA within its GAR domain and is a substrate of PRMT1. We show that the GAR domain regulates the exonuclease activity of MRE11. Cells treated with methylase inhibitors, cells treated with PRMT1 siRNA and PRMT1$^{-/-}$ cells displayed DNA damage induced intra-S-phase checkpoint defects. This phenotype was significantly rescued by the addition of purified MRE11-RAD50-NBS1 complex. Our findings identify a novel role for protein arginine methylation in the regulation of the DNA damage response.

3.4 Materials and Methods

3.4.1 Antibodies

The peptides used to generate rabbit antibodies against MRE11 and methylated 587 MRE11, were as follows: MRE11 (amino acids 597 to 621) (KSTRQQPSRNVTTKNYSEVIEVDES) and Arg587 (KGQNSASR*GGSQRGR) where the arginine marked with an asterisk is aDMA. Antibodies against PRMT1 and Sam68, SYM10 were from Upstate Biotechnology and anti-NBS1 and -RAD50 were from Novus Biologicals (Littleton, CO).

3.4.2 DNA Constructs

The full length human MRE11 was amplified by PCR from HeLa cells cDNA and cloned in pFAST-Bac1. The arginine to alanine MRE11 mutations was generated by ligating double-stranded oligonucleotides with the following sequence (5'-GGA GCA GGC GCA GGA GCA GGT GCA GCA GGT GGA GCA GGG CAA AAT TCA GCA TCG GCA GGA GGG TCT CAA GCA GGA GCA-3') and the arginine to lysine MRE11 mutation was generated by ligating (5'-GG AAA AGG CAA AGG AAA AGG TAA GAA AGG TGG AAA AGG GCA AAA TTC AGC ATC GAA AGG AGG GTC TCA AAA AGG AAA G-3') into a SmaI site created by inverse PCR using the following oligonucleotides (5'-TCC CCC GGG GTT GGT TGC TGC TGA GAT GCT ATC-3') and (5'-TCC CCC GGG GAC ACT GGT CTG GAG ACT TCT ACC-3').

3.4.3 Mass Spectrometry

Endogenous MRE11 was immunopurified from 5 x 10^8 HeLa cells by using 1 mg of our rabbit anti-MRE11 antibody coupled to 1 g of protein A-Sepharose (Sigma-Aldrich). After extensive washings with lysis buffer and 1x phosphate-buffered saline (PBS), the bound proteins were eluted with 250 μM of the MRE11 peptide in 1x PBS. Eluted proteins were resolved by SDS-PAGE and revealed by Coomassie Blue staining. The apparent bands were

excised, in-gel digested with trypsin, and analyzed by MALDI-TOF analysis on a Voyager DE-STR mass spectrometer (U. of Calgary, Alberta).

3.4.4 Methylation Assays

GST- MRE11 (554-680; from M. Bedford) was incubated with GST-PRMT1, -PRMT3, -PRMT4 or with immunoprecipitated PRMT5 with 0.55 μCi of [methyl-^{3}H] AdoMet in the presence of 25 mM Tris-HCl at pH 7.5 for 1 h at 37°C in a final volume of 30 μl. Reactions were stopped by adding 20 μl of 2x SDS-PAGE sample buffer, followed by heating at 100°C for 10 min. Samples were loaded on 9% SDS-polyacrylamide gels and stained with Coomassie Blue. The destained gels were soaked in EN^{3}HANCE (PerkinElmer Life Sciences) according to manufacturer instructions and visualized by fluorography. The *in vivo* methylation assay was performed by metabolically labeling the cells with L-[methyl-^{3}H]methionine directly in methionine-free medium for 3 h in the presence of cycloheximide and chloramphenicol, as described previously (Boisvert et al., 2002). L-[^{35}S]methionine was also used as a control under the same conditions to exclude incorporation during protein synthesis (not shown). The cell lysates were immunoprecipitated and the ^{3}H-labeled proteins were visualized by fluorography after SDS-PAGE.

3.4.5 Purification of MRE11 from insect cells and exonuclease assays

Recombinant MRE11-His$_6$, MRE11R/A-His$_6$ or MRE11R/K-His$_6$ proteins were produced from baculovirus-infected Sf9 cells, using the Bac-to-Bac expression system (Invitrogen). 500 ml spinner flasks of Sf9 cells (1 x 10^6 per ml) infected with either MRE11, MRE11R/A or MRE11R/K baculoviruses (M.O.I. ~ 10) for 2 days at 27°C as described (Masson et al., 2001; Paull and Gellert, 1998). Reactions contained 100 nM DNA in MOPS buffer (25 mM MOPS pH 7.0, 60 mM KCl, 0.2 % Tween-20, 2 mM DTT and 5 mM $MnCl_2$). After 5 min at 37°C, the indicated amount of MRE11, MRE11R/A or MRE11R/K was added and

incubation was continued for 1h. Reaction products were analyzed by 10% Urea-PAGE using followed by autoradiography.

3.4.6 Intra-S-phase checkpoint assays and protein transduction

HeLa cells treated for 24h with 750 μM MTA or PRMT1-downregulated with siRNA or pure proteins introduced with Chariot™ were labeled with 20 nCi/ml [^{14}C] thymidine for 24 h, washed and incubated for an additional 24 h. The cells were DNA damaged with 50 μM etoposide for 1h, and labeled with 2.5 μCi/ml [^{3}H]-thymidine for 45 min. Inhibition of DNA synthesis was measured as described (Falck et al., 2002). Protein transduction was performed according to the manufacturer protocol using 100 ng of MRN complex or MRE11 R/A purified from insect cells or control immunoglobulin G and 6 μl of Chariot™ (ActiveMotif).

3.5 Results and Discussion

3.5.1 A novel aDMA-specific antibody recognizes MRE11

To study the role of methylated arginines and find new proteins containing this modification, an aDMA-specific antibody, named ASYM25, was raised using the GAR peptide N-KFRGGGRGGGRGGFGGRGGRGG-C where all the arginines were aDMA. Purified ASYM25 recognized the immunizing aDMA containing GAR peptide and not the un- or sDMA-methylated GAR peptides (Suppl. Figure 3.1A). To identify proteins specifically immunoprecipitated by ASYM25, a large-scale immunoprecipitation was performed with HeLa cells followed by MALDI-TOF mass spectrometry identification of the trypsin digested proteins. A representative immunoprecipitation using ASYM25 on total cell lysates labeled with ^{35}S-methionine revealed many proteins that are specifically immunoprecipitated by the antibody (Suppl. Figure 3.1B). A protein with an apparent molecular mass of 85 kDa was identified as MRE11 (Boisvert et al., 2003). MRE11 contains multiple repeated arginine and glycine residues between amino acids 566 to 600 constituting a GAR domain. The function of the MRE11 GAR domain and its post-translational regulation are unknown. To confirm that MRE11 can be recognized by ASYM25, HeLa cell extracts were immunoprecipitated with control, ASYM25, and SYM10 antibodies, the latter is an sDMA-specific antibody (Boisvert et al., 2003). MRE11 was detected in ASYM25, but not SYM10 immunoprecipitations, as visualized by immunoblotting with anti-MRE11 antibodies (Figure 3.1A). Moreover, immunoprecipitated MRE11 was directly recognized by ASYM25, and not by SYM10 (Figure 3.1B). These findings confirm that MRE11 contains aDMA and is the component of the MRN complex that is directly recognized by ASYM25.

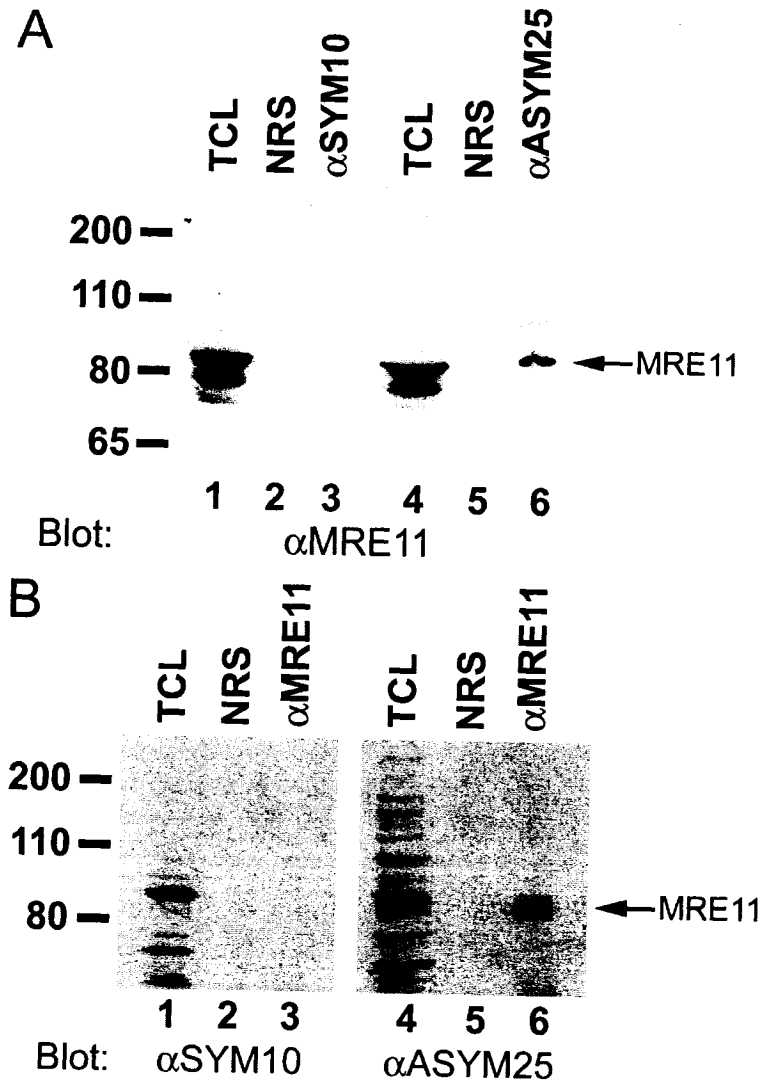
The presence of methylated arginines in MRE11 was also analyzed by mass spectrometry. We raised an MRE11 antibody to purify sufficient amounts of MRE11 from HeLa cells. The immunopurified MRE11 was separated by SDS-PAGE, visualized with Coomassie Blue, excised as a distinct band of ~85 kDa, digested with trypsin, and analyzed

by MALDI-TOF mass spectrometry. The mass of 28 tryptic peptides matched MRE11 predicted peptides (Suppl. Figure 3.2 and Suppl. Table 3.1). Peptides had additional mass corresponding to the presence of methyl groups and based on the predicted versus observed tryptic peptide masses, it was calculated that 9 arginines from MRE11 residues 566 to 600 were dimethylated (Suppl. Table 3.1). Collectively, our data show that MRE11 contains aDMAs within its GAR domain residing between residues 566 to 600.

Figure 3.1 MRE11 contains asymmetrically dimethylated arginines.

(*A*) Antibodies recognizing sDMA (SYM10) or aDMA (ASYM25) were used to perform immunoprecipitations from HeLa cell extracts, and after washing, bound proteins were analyzed by Western blotting to detect MRE11. The total cell lysate (TCL) shows 10% of the extract used in each binding assay. The migration of MRE11 is indicated on the right. The molecular mass markers are shown on the left in kDa. Normal rabbit serum (NRS) was used as a negative control.

(*B*) MRE11 was immunoprecipitated from HeLa cell extracts, and after washing, bound proteins were analyzed by immunoblotting using SYM10 and ASYM25. Many methylated proteins are visualized in the TCL and the migration of MRE11 is shown with an arrow.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NRS
αMRE11
TCL
NRS
αMRE11
200
110
80
MRE11
1 2 3 4 5 6
Blot:
αSYM10
αASYM25

3.5.2 MRE11 is arginine methylated by PRMT1

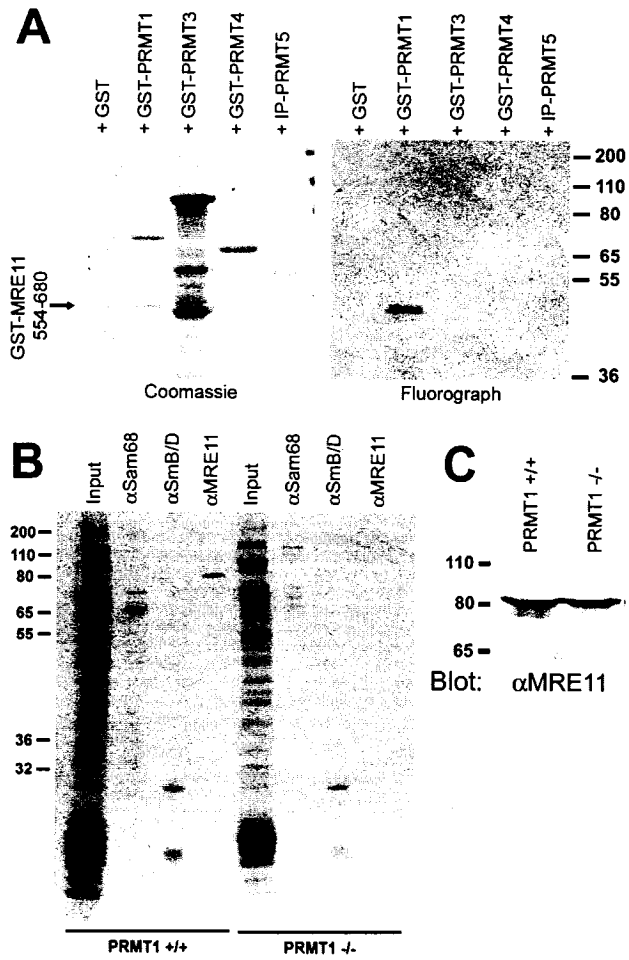
To identify the PRMT that methylates MRE11, an *in vitro* methylation assay was performed using [^{3}H]-AdoMet. A recombinant glutathione-S-transferase (GST) MRE11 fusion protein containing the amino acids 554 to 680 of MRE11 encompassing the GAR domain was methylated by PRMT1, but not by PRMT3, PRMT4 and PRMT5 (Figure 3.2A). All the methyltransferases were active when incubated with known substrates (not shown). Active PRMT5 was immunoprecipitated from HeLa cells using an anti-PRMT5 antibody as described (Boisvert et al., 2002). PRMT1 is required for embryogenesis as PRMT1$^{-/-}$ mice are early embryonic lethal, however, embryonic stem (ES) cells devoid of PRMT1 are viable (Pawlak et al., 2000). To determine whether PRMT1 was the physiological enzyme methylating MRE11, the methylation of endogenous MRE11 was compared between PRMT1$^{+/+}$ and PRMT1$^{-/-}$ ES cell extracts. The cells were metabolically labelled 24 h after transfection with L-[*methyl*-^{3}H]-methionine for 3 h in the presence cycloheximide and chloramphenicol to ensure incorporation into methyl groups and not during protein synthesis as described (Boisvert et al., 2002). Immunoprecipitations in ES cells were performed with the indicated antibody and the labelled methylated protein separated by SDS-PAGE and visualized by fluorography. MRE11 was methylated in PRMT1$^{+/+}$ ES cells, but not in PRMT1$^{-/-}$ ES cells (Figure 3.2B, lanes 4 and 8). Sam68, a known PRMT1 substrate (Cote et al., 2003), was employed as a positive control and its methylation was lost in PRMT1$^{-/-}$ ES cells (lanes 2 and 6). SmB/D, known substrates of PRMT5 (lanes 3 and 7), remained methylated in PRMT1$^{-/-}$ cells as expected. Although the methylation was generally lower in PRMT1$^{-/-}$ cells (lanes 1 and 5), MRE11 protein expression remained equivalent between the ES cell lines (Figure 3.2C). Taken together, our results demonstrate that MRE11 is arginine methylated within the GAR domain by PRMT1.

Figure 3.2 Arginine methylation of MRE11 by PRMT1.

(*A*) Recombinant GST-PRMTs or PRMT5 immunoprecipitated from HeLa cells were incubated with GST-MRE11 (554-680) in the presence of [^{3}H]-AdoMet as the methyl donor. Proteins were separated by SDS-PAGE and visualized by Coomassie staining (left) and the [^{3}H]-labeled proteins visualized by fluorography (right).

(*B*) PRMT1$^{+/+}$ and PRMT1$^{-/-}$ were metabolically labeled with [methyl-^{3}H]-*L*-methionine, and cell lysates were immunoprecipitated with Sam68, Sm (Ana128; Cappel) or MRE11 antibodies. The *in vivo* methylated proteins were visualized by fluorography after SDS-PAGE.

(*C*) Equivalent amount of TCL from normal ES cells or ES cells PRMT1$^{-/-}$ were immunoblotted with anti-MRE11 antibodies.


A
+ GST
+ GST-PRMT1
+ GST-PRMT3
+ GST-PRMT4
+ IP-PRMT5
GST-MRE11 554-680
Coomassie
Fluorograph
200
110
80
65
55
36
B
Input
αSam68
αSmB/D
αMRE11
200
110
80
65
55
36
32
PRMT1 +/+
PRMT1 -/-
C
PRMT1 +/+
PRMT1 -/-
110
80
65
Blot: αMRE11

3.5.3 Arginines within the GAR domain are necessary for MRE11 exonuclease activity

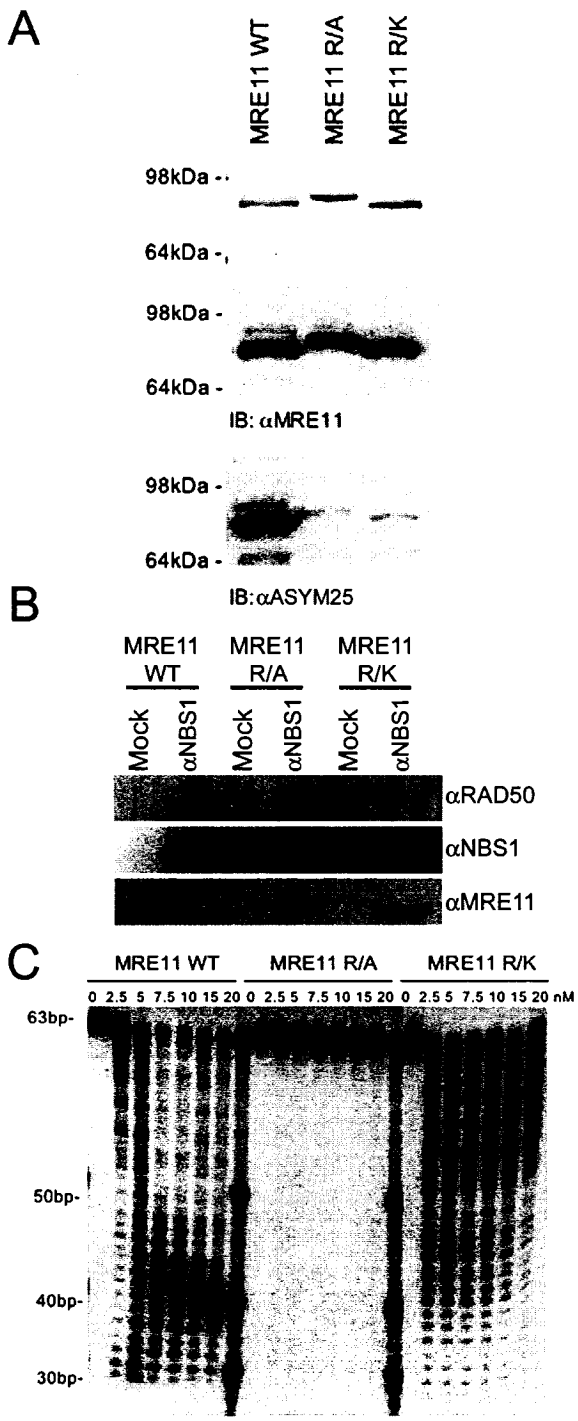
The 3'-5' exonuclease activity is one of the most important biochemical features of MRE11(Paull and Gellert, 1998). To investigate whether the GAR domain affected the enzymatic activity of MRE11, two mutant MRE11 proteins were generated where the arginines between residues 570-594 were replaced with alanines (R/A) or lysines (R/K). MRE11 wild-type, R/A and R/K baculoviruses were generated and the corresponding proteins were purified to homogeneity from insect cells, a species known to contain PRMTs (Boulanger et al., 2004). Purified human MRE11 was methylated as analyzed by mass spectrometry (not shown) and was recognized by ASYM25, unlike the R/A and R/K mutant proteins, which were not significantly methylated (Figure 3.3A). All MRE11 proteins associated with RAD50 and NBS1, as observed in anti-NBS1 immunoprecipitates, demonstrating that the GAR domain does not influence the formation of the MRN complex (Figure 3.3B). When compared with wild-type, the exonuclease activity of MRE11 on double-stranded DNA was absent for the R/A mutant. These data demonstrate that either the charge or the methyl groups on the arginines are required (Figure 3.3C, MRE11 R/A). Amino acid substitution of arginines to lysines to maintain the charge severely impaired the exonuclease activity, demonstrating that the arginines or methylarginines are necessary for optimal MRE11 activity (Figure 3.3C, MRE11 R/K). At 2.5nM the R/K appears more active, but it lacks the processivity to cleave the DNA into smaller fragments unlike the wild-type MRE11 (Figure 3.3C). Hypomethylated MRE11 could not be obtained and assayed for exonuclease activity because insect cells could not be grown in presence of sufficient amount of methyltranferase inhibitors (data not shown). Our findings suggest that the MRE11 GAR domain regulates the exonuclease activity, an enzymatic activity important for the checkpoint response (Uziel et al., 2003).

Figure 3.3 The MRE11 GAR domain regulate the exonuclease activity.

(*A*) HIS-tagged human MRE11 wild-type, R/A and R/K baculoviruses were generated and the corresponding proteins were purified to homogeneity from insect cells. Purified MRE11, MRE11R/A and MRE11R/K were visualized by Coomassie Blue (top panel), and immunoblotted with MRE11 (middle panel) and ASYM25 (lower panel).

(*B*) To determine whether the methylated arginines are necessary for the formation of the MRN complex, cells infected with NBS1, RAD50 and either MRE11, MRE11R/A or MRE11R/K baculoviruses were lysed and immunoprecipitated with beads alone or anti-NBS1 antibodies. The presence of RAD50 and MRE11 as well as NBS1 was visualized by immunoblotting. Their identities are indicated on the right.

(*C*) Nuclease assays were performed with the indicated amount of each of MRE11 WT, MRE11R/A or MRE11R/K, in 5 mM $MnCl_2$ on a [^{32}P]-5'-labeled at one end 63-bp dsDNA substrate. Reactions were incubated for 60 min at 37°C before separation on a 10% denaturing polyacrylamide gel and visualized by autoradiography.


A
MRE11 WT
MRE11 R/A
MRE11 R/K
98kDa -
64kDa -
98kDa -
64kDa -
IB: αMRE11
98kDa -
64kDa -
IB: αASYM25
B
MRE11 WT
MRE11 R/A
MRE11 R/K
Mock
αNBS1
Mock
αNBS1
Mock
αNBS1
αRAD50
αNBS1
αMRE11
C
MRE11 WT
MRE11 R/A
MRE11 R/K
0 2.5 5 7.5 10 15 20 0 2.5 5 7.5 10 15 20 0 2.5 5 7.5 10 15 20 nM
63bp-
50bp-
40bp-
30bp-

3.5.4 Inhibition of MRE11 methylation results in S phase checkpoint defects.

A-TLD cells are known to display intra-S-phase checkpoint defects and fail to suppress DNA synthesis in response to DNA damage (Stewart et al., 1999). To examine whether cells pre-treated with the methyl-thio-adenosine (MTA) methylase inhibitor exhibited a similar phenotype, we examined the ability of the cells to progress through S-phase following DNA damage. Indeed, ~70% of HeLa cells and the human primary fibroblasts CRL2097 (ATCC, not shown) pre-treated with MTA progressed through the S-phase like A-TLD derived cells following DNA damage (Figure 3.4A). In contrast, ~40% mock-treated HeLa cells progressed through S-phase following DNA damage (Figure 3.4A). Since MTA has a broad specificity, we down-regulated the expression of PRMT1 using siRNA generated by pBS/U6(Rezai-Zadeh et al., 2003). The PRMT1 expression was down-regulated by ~70% in HeLa cells and the methylation of MRE11 Arg587 was severely impaired in these cells, as detected by immunoblotting with αArg587 (Figure 3.4B), an anti-aDMA 587 site-specific MRE11 antibody that we generated (Suppl. Figure 3.1C). The hypomethylation of MRE11 was also observed with ASYM25 (not shown). The PRMT1 siRNA-treated cells displayed a phenotype similar to MTA and A-TLD cells treated with etoposide, a topoisomerase II inhibitor (Figure 3.4A). Since PRMT1 methylates many substrates, we purified and introduced the MRN complex into the cells by protein transduction with Chariot^{TM}. The MRN complex containing methylated MRE11 to a large extent rescued the PRMT1 siRNA phenotype and this effect was dose-dependent (Figure 3.4C and Suppl. Figure 3.3A). The introduction of equal amounts of purified MRE11 R/A alone (Figure 3.4C) or control immunoglobulin G (Suppl. Figure 3.3B) did not rescue the intra-S-phase defect in PRMT1 siRNA treated cells. These findings demonstrate that arginine methylation of MRE11 by PRMT1 is required for the intra-S-phase DNA damage checkpoint. To determine the effect of DNA damage on cell cycle profiles, PRMT1$^{-/-}$ ES cells were exposed to a low dose of etoposide (500 nM). The ability to repair the damaged DNA

and progress through the cell cycle was assessed 16 h after damage. Approximately 24% of the wild-type ES cells remained in the S-phase and 70% were in the G_2/M phase, while 46% of the PRMT1$^{-/-}$ cells remained accumulated in S-phase and 37% progressed to the G_2/M phase following DNA damage (Figure 3.4D). These findings suggest that PRMT1$^{-/-}$ ES cells progress slower through the S-phase than PRMT1$^{+/+}$ cells following DNA damage.

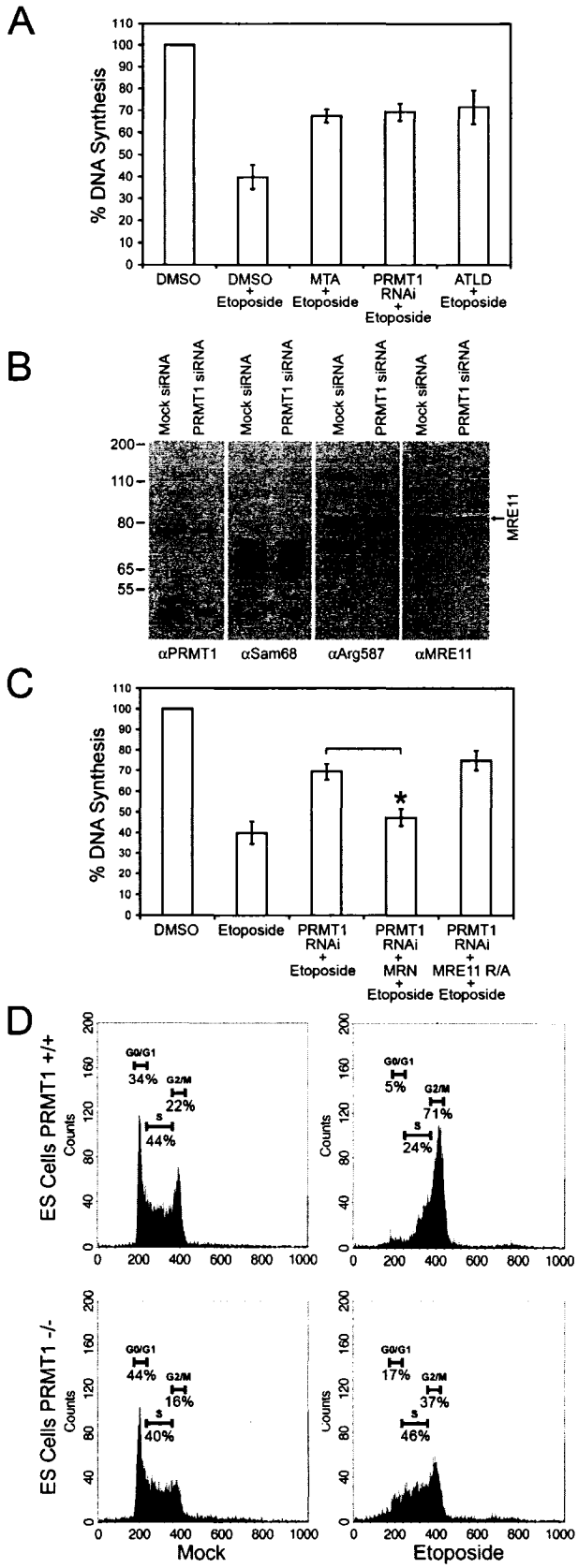
Figure 3.4 Arginine methylation of MRE11 regulates the intra-S-phase checkpoint.

(*A*) DNA synthesis following DNA damage in HeLa cells pre-treated with the methylase inhibitor MTA, in cells treated with PRMT1-siRNA or in A-TLD cells. DNA synthesis was assessed 1 h after 50 μM etoposide treatment. The results represent an average of six independent experiments.

(*B*) Cell extracts from mock- and PRMT1-siRNA transfected HeLa cells were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with PRMT1, Sam68, Arg587 and MRE11 antibodies as indicated.

(*C*) DNA synthesis following DNA damage in PRMT1-siRNA cells protein transfected with 100 ng of the MRE11 complex (MRN) or MRE11 R/A alone using Chariot^{TM}. DNA synthesis was assessed 1 h after 50 μM etoposide treatment. The results represent an average of six independent experiments. The asterisk represents a significant difference between PRMT1 RNAi and PRMT1 RNAi + MRN ($P < 0.01$).

(*D*) S-phase progression was followed using flow cytometry of PRMT1$^{+/+}$ ES cells (top panels) or PRMT1$^{-/-}$ ES cells (lower panels) 16 h following either mock treatment (left panels) or DNA damage induced with a low-dose of 500 nM etoposide (right panels). The percentage of cells in the G_0/G_1, S or G_2/M phases of the cell cycle is indicated.


A
% DNA Synthesis
DMSO
DMSO + Etoposide
MTA + Etoposide
PRMT1 RNAi + Etoposide
ATLD + Etoposide
B
Mock siRNA
PRMT1 siRNA
MRE11
αPRMT1
αSam68
αArg587
αMRE11
C
% DNA Synthesis
DMSO
Etoposide
PRMT1 RNAi + Etoposide
PRMT1 RNAi + MRN + Etoposide
PRMT1 RNAi + MRE11 R/A + Etoposide
D
ES Cells PRMT1 +/+
ES Cells PRMT1 -/-
Counts
G0/G1
S
G2/M
34%
22%
44%
5%
71%
24%
44%
16%
40%
17%
37%
46%
Mock
Etoposide

3.6 Discussion

In this study, we provide evidence that MRE11 is arginine methylated by PRMT1. First, we showed that the aDMA-specific ASYM25 antibody and Arg587 MRE11 site-specific antibody directly recognized MRE11 by immunoblotting. It was also shown that MRE11 incorporated [^{3}H]-methyl groups in a known *in vivo* methylation assay and this incorporation was absent in PRMT1$^{-/-}$ cells. It was also shown that the MRE11 GAR domain was methylated by PRMT1 *in vitro*. Lastly, we identified by MALDI-TOF, peaks that corresponded to the mass of methylated MRE11 peptides and most of the peptide peaks were less abundant or totally absent in the MTA-treated cells (data not shown). The identification by mass spectrometry of multiple methylated peptides corresponding to MRE11 and the low abundance of unmethylated peptides within the GAR domain suggests that MRE11 exists predominantly in the methylated form. By using the methyl-specific antibodies Arg587 and ASYM25, we observed no difference in MRE11 methylation or global methylation following genotoxic treatments (not shown). Similarly, the methylation of histone H3 lysine 79, involved in the recruitment of 53BP1 to sites of DNA damage, is not regulated with DNA damage (Huyen et al., 2004a). Cells harboring mutations in ATM, MRE11 or NBS1 (Carney et al., 1998; Savitsky et al., 1995b; Stewart et al., 1999; Varon et al., 1998) or cells down-regulated in the protein MDC1 (Goldberg et al., 2003; Lou et al., 2003; Stewart et al., 2003) display intra-S-phase checkpoint defects. Our study suggests a role for arginine methylation and PRMT1 in this process. Cells pre-treated with methyltransferase inhibitors displayed an intra-S-phase defect following DNA damage, demonstrating a requirement for methylation in this response. As methyltransferase inhibitors have a broad specificity, other type of methylation events cannot be ruled out solely based on this observation. The PRMT1 siRNA-treated cells displayed an intra-S-phase checkpoint defect similar to MTA-treated cells, narrowing down the observed phenotype to the inhibition of PRMT1 activity. Moreover, PRMT1$^{-/-}$ ES cells displayed a slower progression through S-phase following DNA damage.

Based on these experiments, it is likely that several methylated proteins contribute to this intra-S-phase checkpoint defect. However, we have shown that re-introduction of the purified arginine methylated baculovirus produced MRN complex into PRMT1 siRNA-treated cells significantly rescued the phenotype. These findings demonstrate that the MRN complex can alleviate the intra-S-phase checkpoint defect observed in these cells. Collectively, our results suggest that the arginine methylation may regulate the MRE11 exonuclease activity during the intra-S-phase checkpoint response. In summary, the results presented herein suggest that arginine methylation, a poorly characterized post-translational modification, plays a crucial role in regulating the DNA damage response.

3.7 Acknowledgments

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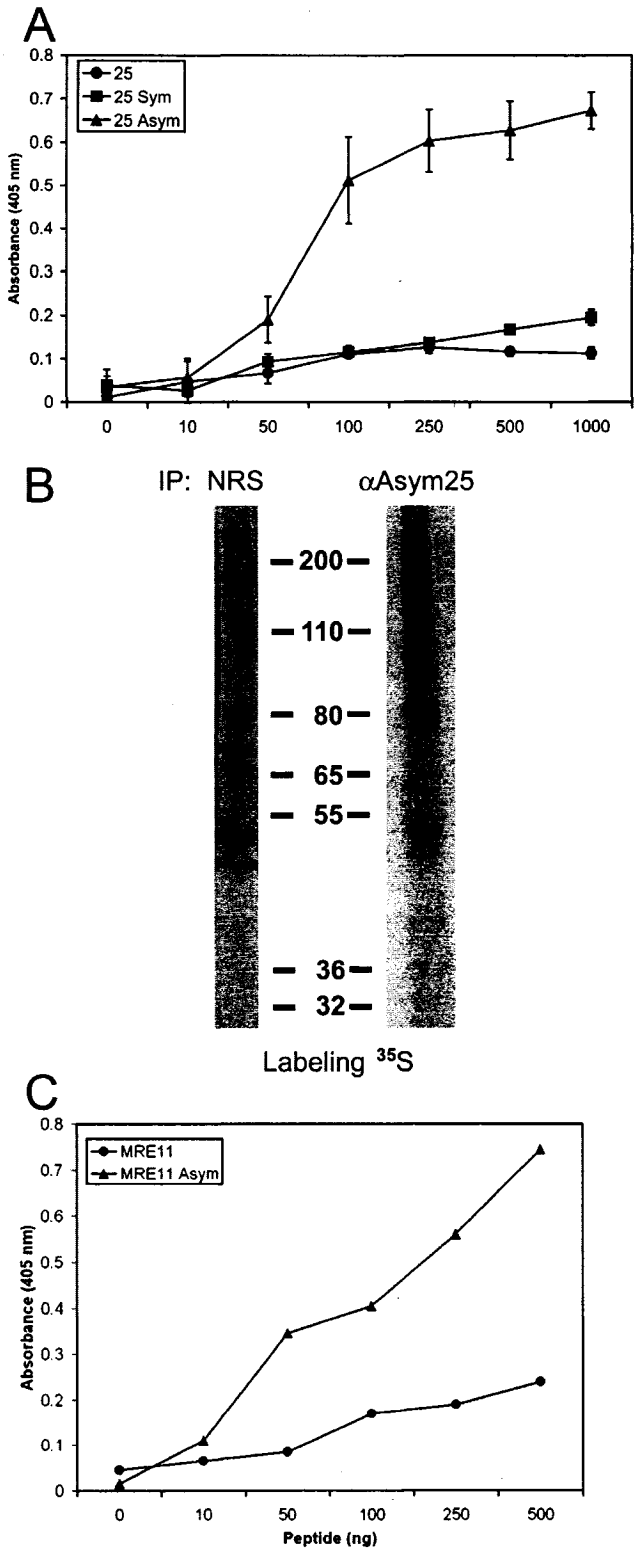
Supplemental Figure 3.1 Characterization of dimethylarginine specific antibodies.

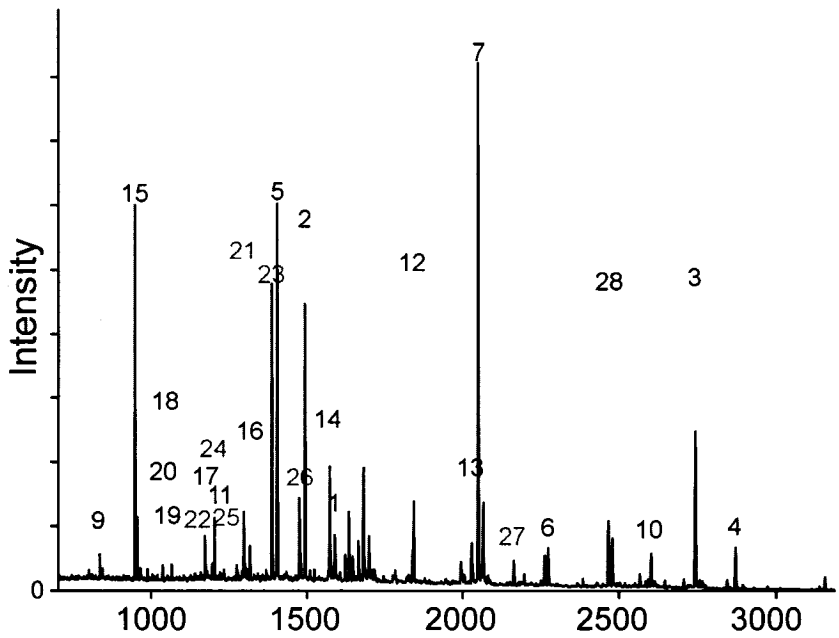
(*A*) An ELISA demonstrates the specificity of the ASYM25 antibody for the aDMA immunizing peptide (triangles, 25 Asym), but not the corresponding unmethylated (circles, 25) or sDMA-containing peptides (squares, 25 Sym). The ordinate shows the absorbance at 405 nM and the abscissa shows the peptide concentration in (ng). (*B*) ^{35}S-methionine metabolically labeled HeLa cell extracts were immunoprecipitated with the aDMA-specific ASYM25 antibody or control normal rabbit serum (NRS). The bound proteins were separated by SDS-PAGE and visualized by fluorography. The migration of the molecular mass markers is shown in kDa.

(*C*) An ELISA demonstrates the specificity of the MRE11 site-specific antibody αArg587. This antibody was raised with a peptide containing arginine 587 asymmetrically dimethylated. αArg587 recognizes the aDMA immunogenic MRE11 peptide (triangles, MRE11 Asym) but only weakly recognized the non-methylated peptide at higher concentrations (circles, MRE11).


A
25
25 Sym
25 Asym
Absorbance (405 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
50
100
250
500
1000
B
IP: NRS
αAsym25
200
110
80
65
55
36
32
Labeling 35S
C
MRE11
MRE11 Asym
Absorbance (405 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
50
100
250
500
Peptide (ng)

Supplemental Figure 3.2 Mass spectrometry analysis of human MRE11 purified from HeLa cells.

MRE11 immunopurified from HeLa cells was separated by SDS-PAGE, visualized with Coomassie Blue, excised as a distinct band the size of MRE11, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry. A representative spectrum is shown with the relative intensity as the ordinate and the peptide mass (daltons) on the abscissa. The numbered peaks correspond to the peptides in Table 3.1.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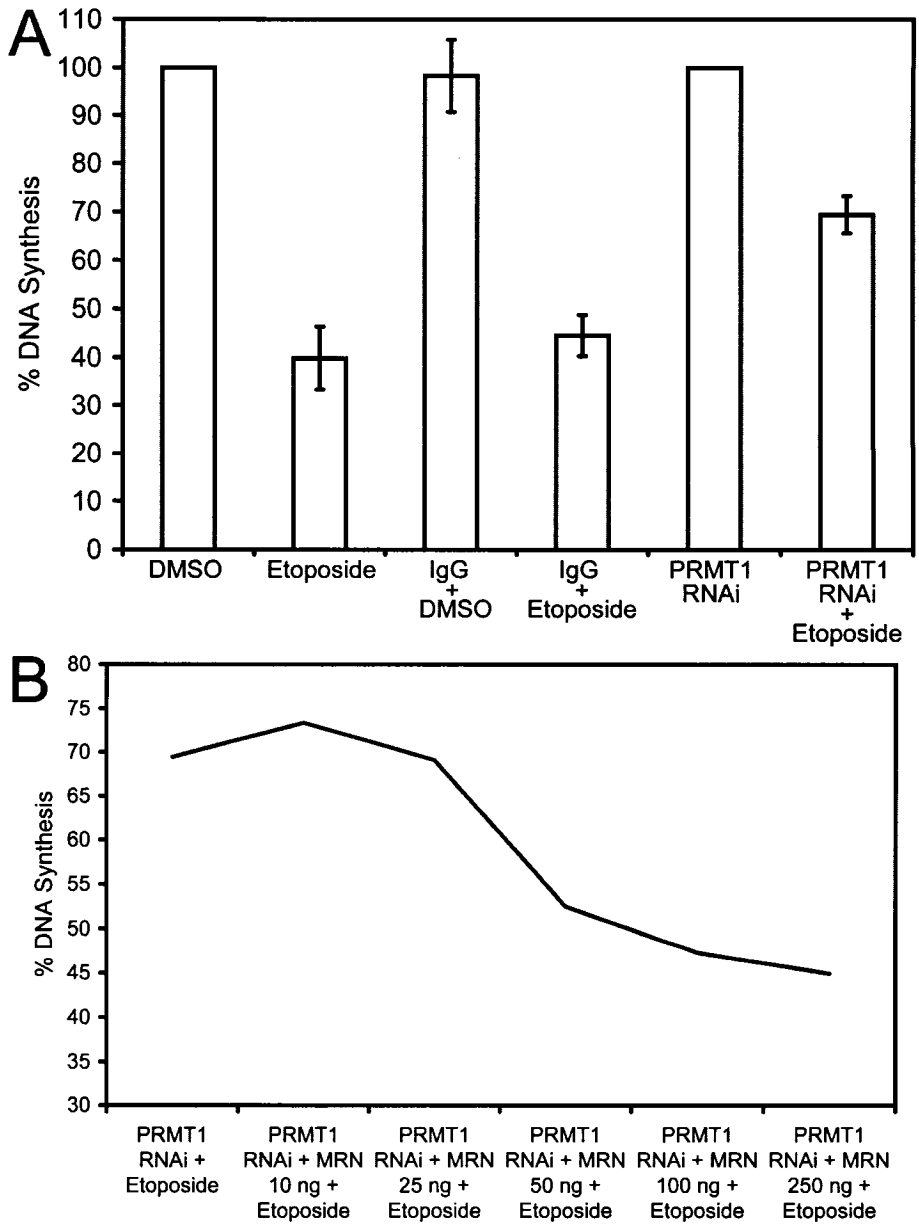
0
1000
1500
2000
2500
3000
15
5
2
21
23
12
7
28
3
18
16
14
24
20
17
26
13
11
1
9
19
22
25
27
6
10
4

Supplemental Figure 3.3 Arginine methylation of MRE11 regulates the intra-S-phase checkpoint.

(*A*) DNA damage-resistant DNA synthesis in HeLa cells expressing PRMT1-siRNA and protein transfected with increasing amount (0-250 ng) of the MRE11 complex (MRN) using Chariot™. DNA synthesis was assessed 1 hr after mock (DMSO) or 50 μM etoposide treatment.

(*B*) DNA damage-resistant DNA synthesis in HeLa cells mock treated (DMSO and etoposide) or transfected with 100 ng of mouse control immunoglobulin (IgG + DMSO and IgG + etoposide) or HeLa expressing a PRMT1 siRNA (PRMT1 RNAi or PRMT1 RNAi + Etoposide). DNA synthesis was assessed 1 hr after mock (DMSO) or 50 μM etoposide treatment as described in Materials and Methods.


A
% DNA Synthesis
110
100
90
80
70
60
50
40
30
20
10
0
DMSO
Etoposide
IgG + DMSO
IgG + Etoposide
PRMT1 RNAi
PRMT1 RNAi + Etoposide
B
% DNA Synthesis
80
75
70
65
60
55
50
45
40
35
30
PRMT1 RNAi + Etoposide
PRMT1 RNAi + MRN 10 ng + Etoposide
PRMT1 RNAi + MRN 25 ng + Etoposide
PRMT1 RNAi + MRN 50 ng + Etoposide
PRMT1 RNAi + MRN 100 ng + Etoposide
PRMT1 RNAi + MRN 250 ng + Etoposide

A

Peptide	Mass Observed	Mass Expected	Residues	Sequence	Modifications
1	1586.851	1586.861	15 - 28	(K)ILVATDIHLGFMEK(D)	
2	1492.752	1492.764	33 - 45	(R)GNDTFVTLDEILR(L)	
3	2741.387	2741.389	46 - 69	(R)LAQENEVDFILLGGDLFHENKPSR(K)	
4	2869.464	2869.484	46 - 70	(R)LAQENEVDFILLGGDLFHENKPSRK(T)	
5	1403.738	1403.753	176 - 188	(K)IALYGLGSIPDER(L)	
6	2273.157	2273.5	203 - 220	(R)PKEDENSWFNLFVIHQNR(S)	
7	2047.959	2047.963	205 - 220	(K)EDENSWFNLFVIHQNR(S)	
8	694.399	694.436	283 - 288	(K)HVGLLR(I)	
9	835.482	835.515	299 - 305	(K)IPLHTVR(Q)	
10	2601.238	2601.2448	306 - 327	(R)QFFMEDIVLANHPDIFNPDNPK(V)	
11	1233.569	1233.579	340 - 349	(K)IEEMLENAER(E)	
12	1841.937	1841.955	365 - 380	(R)LRVDYSGGFEPFSVLR(F)	
13	2028.095	2028.095	365 - 380	(K)ILVATDIHLGFMEKDAVR(G)	
14	1572.767	1572.769	367 - 380	(R)VDYSGGFEPFSVLR(F)	
15	947.493	947.51	394 - 400	(K)DIIHFFR(H)	
16	1315.745	1315.758	417 - 428	(K)LITKPSEGTTLR(V)	
17	1172.644	1172.664	443 - 452	(K)NVQLSLLTER(G)	
18	1065.541	1065.569	476 - 483	(K)YQLEKTQR(F)	
19	955.569	955.509	569 - 576	(K)GRGRGRGR(R)	6 Met
20	955.569	955.509	573 - 580	(R)GRGRRGGR(G)	6 Met
21	1296.684	1296.694	566 - 576	(K)TNKGRGRGRGR(R)	6 Met
22	1168.644	1168.631	571 - 580	(R)GRGRGRRGGR(G)	6 Met
23	1386.664	1386.711	575 - 587	(R)GRRGGRGQNSASR(G)	2 Met
24	1202.611	1202.219	577 - 587	(R)RGGRGQNSASR(G)	4 Met
25	1233.369	1233.24	581 - 592	(R)GQNSASRGGSQ(R)	2 Met
26	1475.777	1475.701	581 - 594	(R)GQNSASRGGSQRG(R)	4 Met
27	2163.042	2163.286	581 - 600	(R)GQNSASRGGSQRGRAFKSTR(Q)	4 Met
28	2477.995	2477.989	683 - 704	(K)GVDFESSEDDDDDPFMNTSSLR(R)	

B

565 **611**

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    *  *  *  **   *        *      *  *
KTNKGRGRGRGRRGGRGQNSASRGGSQRGRAFKSTRQQPSRNVTTKN
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Supplemental Table 3.1 Mass spectrometric analysis of MRE11

Chapter 4

Methylation of MRE11 regulates its nuclear compartmentalization

4.1 Preface

Following the characterization of the role of arginine methylation on MRE11 functions, we decided to extend our study to look into the effect of arginine methylation on the cellular localization of MRE11. MRE11 is well-known to be a very dynamic protein, localizing in several nuclear compartment prior to its localization to sites of DNA damage where it will eventually achieve its primary function. This chapter describes the characterization of the role of methylation in regulating MRE11 cellular localization, nuclear structures interactions and recruitment to sites of DNA damage.

4.2 Abstract

The cellular response to DNA damage includes the orderly recruitment of many protein complexes to DNA lesions. The MRE11-RAD50-NBS1 (MRN) complex is well known to localize early to sites of DNA damage, but the post-translational modifications required to mobilize it to DNA damage sites are poorly understood. Recently, we have shown that MRE11 is arginine methylated in a C-terminal glycine-arginine rich (GAR) domain by protein arginine methyltransferase 1 (PRMT1). Arginine methylation is required for the exonuclease activity of MRE11 and the intra-S phase DNA damage response. Herein, we report that cells treated with methylase inhibitors failed to re-localize MRE11 from PML nuclear bodies to sites of DNA damage and formed few γ-H2AX foci. We also demonstrate that PRMT1 is a component of PML nuclear bodies where it co-localizes with MRE11. Using cellular fractionation, we demonstrate that methylated MRE11 is predominantly associated with nuclear structures and that MRE11 methylated arginines were required for this association. These results suggest that MRE11 methylation regulates its association with nuclear structures such as PML nuclear bodies and sites of DNA damage.

4.3 Introduction

DNA double-strand breaks (DSB) occur naturally when a replication fork collapses, or exogenously when cells are exposed to ionizing radiation or certain chemicals. The initial recruitment of proteins involved in DNA damage signalling and repair to sites of DNA double-strand breaks (DSB) represents the earliest response mechanism that is triggered by DNA damage (Lisby et al., 2004b). The ensuing response to DNA damage includes the accumulation of signalling–repair complexes into nuclear foci in the vicinity of the DSBs (Rouse and Jackson, 2002b). Following the initial recognition of DNA lesions, histone H2AX becomes rapidly phosphorylated in the chromatin surrounding a DSB (Rogakou et al., 1999). Although H2AX deficiency is not essential for cellular survival, H2AX is required for the accumulation of numerous essential proteins into irradiation induced foci (IRIF) (Celeste et al., 2003c; Celeste et al., 2002). The MRE11/ RAD50/ NBS1 (MRN) complex is one of the early DNA repair complex that is recruited at DNA DSBs (Maser et al., 1997b; Nelms et al., 1998). Mutations in the NBS1 and MRE11 genes lead to genomic instability disorders: the Nijmegen breakage syndrome (NBS) (Carney et al., 1998; Varon et al., 1998) and ataxia-telangiectasia (A-T)-like disease (A-TLD) (Stewart et al., 1999), respectively. MRE11 is a conserved protein with an N-terminal nuclease domain (Paull and Gellert, 1998) as well as a C-terminal DNA binding region (de Jager et al., 2001a; Hopfner et al., 2002) and a glycine-arginine rich (GAR) domain regulating both MRE11 exonuclease activity and its ability to signal DNA damage (Boisvert et al., 2005c). An intact MRE11 C-terminus, deleted in A-TLD1/2 cells, is also required to assemble signalling proteins at sites of DNA damage (Costanzo et al., 2004b; Petrini and Theunissen, 2004). The MRN complex localizes in discrete nuclear foci at DNA DSBs and the MRN complex is involved in DNA repair, DNA damage and checkpoint signalling (Carson et al., 2003b; Lee and Paull, 2004; Theunissen et al., 2003b; Uziel et al., 2003).

Protein arginine methylation is a post-translational modification that results in the mono- and dimethylation of the guanidino nitrogen atoms of arginine (Boisvert et al., 2005b; Gary and Clarke, 1998b; McBride and Silver, 2001b). Arginines can be dimethylated either in a symmetrical or asymmetrical manner (sDMA, aDMA). In humans, protein arginine methyltransferases (PRMT) represent a family of 8 known methyltransferases that utilize S-adenosyl methionine as a methyl donor and are divided into type I and type II enzymes. There are 5 known type I enzymes that catalyze the formation of aDMA including PRMT1 (Lin et al., 1996a), PRMT2 (Scott et al., 1998b), PRMT3 (Tang et al., 1998c), PRMT4 (CARM1) (Chen et al., 1999b) and PRMT6 (Frankel et al., 2002). Type II enzymes catalyze the formation of sDMA and include PRMT5 (Branscombe et al., 2001; Pollack et al., 1999b) and PRMT7 (Lee et al., 2004; Miranda et al., 2004b). Protein arginine methylation has been shown to regulate RNA metabolism, protein-protein interaction, protein localization and transcription (Boisvert et al., 2005b; Gary and Clarke, 1998b; McBride and Silver, 2001b).

We have previously demonstrated that MRE11 is arginine methylated by PRMT1, and that methylation regulates both MRE11 exonuclease activity and its ability to signal DNA damage (Boisvert et al., 2005c). We now report that MRE11 interacts and colocalizes with PRMT1 in PML nuclear bodies. The sites of arginine methylation within MRE11 were replaced with alanines and it was observed that MRE11 R/A had an increased mobility by fluorescence recovery after photobleaching analysis and was loosely associated with nuclear structures unlike wild-type MRE11. Antibodies that recognize arginine methylated MRE11 demonstrated that methylated MRE11 was associated with nuclear structures. Cells treated with methylase inhibitors prevented the recruitment of MRE11 to sites of DNA damage and inhibited phosphorylation of histone H2AX in response to DNA damage. Our findings

identify a new role for protein arginine methylation in recruitment of DNA repair proteins to sites of DNA damage.

4.4 Materials and Methods

4.4.1 Antibodies

The sequence of the peptides used to raise antibodies against methylated MRE11 (MeMRE11) and non-methylated MRE11 (UnMRE11) was KGRGR GRGRR GGRGQ NSASR GGSQR GRA where all arginine residues are aDMA or arginines, respectively. The antibodies were affinity purified over the antigenic peptide coupled to Affigel beads (Bio-Rad). PRMT1, ASYM25 and SYM10 antibodies were described previously (Côté et al., 2003). γ-H2AX was from Upstate Biotechnology, PML (PG-M3) and PRMT1 (N-19) were from Santa Cruz Biotechnology, MRE11 and NBS1 antibodies were from Novus Biologicals (Littleton, CO). ELISAs were performed as described previously (Côté et al., 2003).

4.4.2 Cell culture

The primary human foreskin fibroblasts CRL2097, HeLa and SK-N-SH cells are from the ATCC and were cultured in DMEM supplemented with 10% calf bovine serum, 2% L-glutamine, 1.2% sodium pyruvate and 1.2% Pen/Strep. DNA transfection and immunoprecipitations have been described elsewhere (Côté et al., 2003).

4.4.3 DNA Constructs

SUMO1 was amplified by PCR from HeLa cells cDNA and cloned into the *EcoR*I site of pEGFP (Clontech). The full length MRE11 and MRE11 R/A were described previously (Boisvert et al., 2005c) and they were subcloned into the Kpn I site of pEYFP-C1 (Clontech).

4.4.4 Immunofluorescence, in situ fractionation and FRAP

To visualize MRE11, PRMT1 and sites of DNA damage, cells were subjected to an extraction protocol prior to fixation, according to a previously reported procedure (Mirzoeva and Petrini, 2001b; Moore and Haber, 1996). Briefly, cells were washed once with PBS,

incubated for 5 min on ice in cytoskeleton buffer (10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) pH 6.8, 100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$, 1 mM EGTA and 0.5% Triton X-100) followed by a 5 min incubation on ice in cytoskeleton stripping buffer (10 mM Tris-HCL pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1% Tween 40 and 0.5% sodium deoxycholate). Cells were then washed 3 times with PBS, fix in 1% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 10 min, blocked in 10% goat serum and labeled for immunofluorescence with primary antibodies against various proteins and the appropriate Alexa488- (green, Molecular Probes) and Alexa546- (red, Molecular Probes) conjugated secondary antibodies. Cells were mounted in Immuno-Mount (Thermo Shandon, Pittsburgh, PA) containing DAPI (Sigma-Aldrich) at 1 μg/ml. Images were collected with an Olympus immunofluorescence microscope. For FRAP analysis, YFP-MRE11 or R/A transfected cells were maintained at 37 °C and analyzed for FRAP as described (Kruhlak et al., 2000). The average recovery values for 10 cells were plotted.

4.4.5 Cellular Fractionation and Nuclear Structures Isolation

Nuclei were prepared essentially as described by (Cockerill and Garrard, 1986). In brief, cells were washed in phosphate-buffered saline and subjected to hypotonic lysis in RSB buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride). Cells were incubated on ice for 10 min, homogenized with a Dounce homogenizer, and centrifuged at 750 x g for 10 min. The supernatant corresponding to the cytoplasm-containing fraction was designated fraction number 1. The nuclei recovered in the pellet were washed twice in RSB buffer. The subsequent steps allowing subnuclear fractionation and nuclear structures isolation were performed essentially as described by (He et al., 1990). The washed nuclei were freed of the chromatin by digestion with 20 units of RNase-free DNase-1 (Promega, Madison, WI) per 1 $x10^6$ cells at 30 °C for 50 min in digestion buffer (10 mM Pipes, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% (v/v)

Triton X-100, 1.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A). The digested nuclei were then extracted by the addition of ammonium sulfate from a 1 M stock to a final concentration of 0.25 M. The 750 x g supernatant containing the digested chromatin was designated fraction number 2. The pellet corresponds to nuclear structures containing ribonucleoprotein complexes (Nickerson et al., 1992). This nuclear fraction was further fractionated after resuspension of the pellet in digestion buffer and extraction by the addition of NaCl to a final concentration of 2 M from a 5M stock in digestion buffer. The supernatant obtained after a 750 x g centrifugation was designated fraction number 3. The pellet was resuspended in digestion buffer and incubated for 1 h at room temperature with or without RNase A (Sigma-Aldrich) at 100 μg/ml and RNase T1 (MBI Fermentas, Canada) at 40 units/ml. The fractions were then centrifuged at 750 x g. The supernatant and pellet treated with the RNases were designated fraction numbers 4 and 5, respectively. All steps used for cell fractionation and nuclear structures isolation were performed at 4 °C unless otherwise specified.

4.5 Results

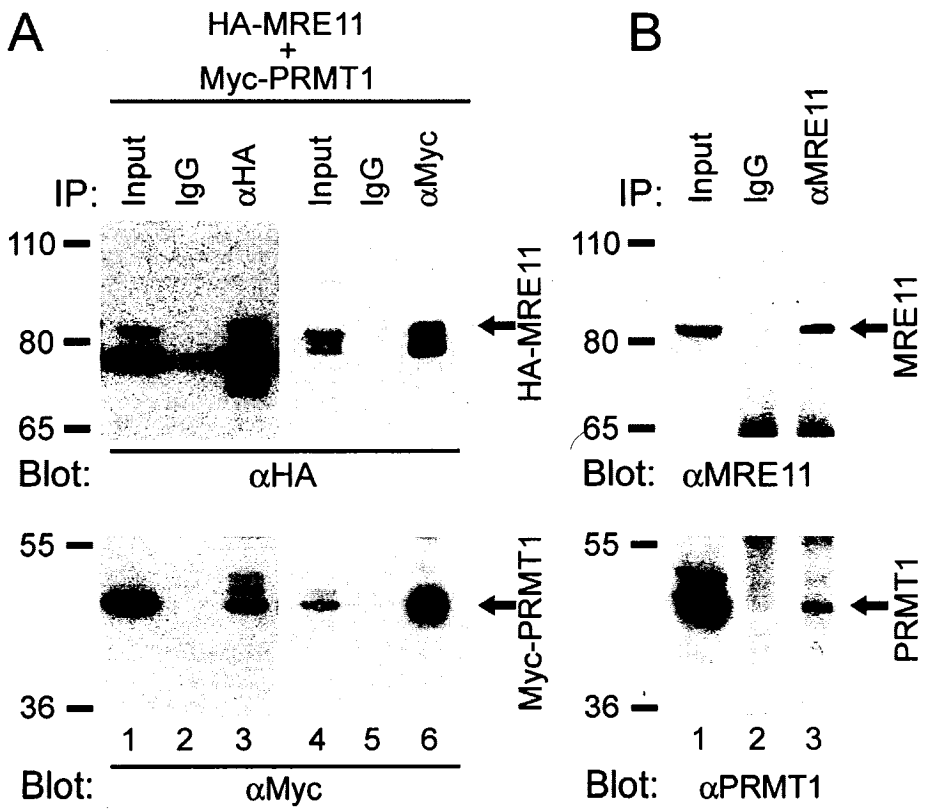
4.5.1 In vivo interaction between MRE11 and PRMT1.

The PRMT1 arginine methyltransferase often associate with its substrates and to determine whether MRE11 and PRMT1 interact, HeLa cells were co-transfected with expression vectors encoding hemagglutinin (HA)-tagged MRE11 and myc-tagged PRMT1. Myc-PRMT1 was readily observed in anti-HA immunoprecipitates, demonstrating co-immunoprecipitation (Figure 4.1A, lane 3 bottom panel). The top part of the same gel was immunoblotted with HA antibodies to visualize the immunoprecipitated HA-MRE11 (Figure 4.1A, lane 3 top panel). The converse was also observed as HA-MRE11 was detected in Myc immunoprecipitates (Figure 4.1A, lanes 4-6, top panel). The lower part of the gel was immunoblotted with Myc antibodies to visualize Myc-PRMT1 (Figure 4.1A, lane 4-6 bottom panel). These data show that over-expressed PRMT1 and MRE11 associate in transfected cells. We next examined whether or not endogenous PRMT1 and MRE11 interact. Untransfected HeLa cell extracts were immunoprecipitated with either control immunoglobulin G (IgG) or anti-MRE11 antibodies. Anti- MRE11 immunoprecipitates, but not control immunoprecipitates contained PRMT1, as detected by immunoblotting with anti-PRMT1 antibodies (Figure 4.1B, lanes 1-3, lower panel). The upper part of the same gel was immunoblotted with MRE11 antibodies, confirming the presence of MRE11 (Figure 4.1B). These data demonstrate that PRMT1 and MRE11 interact in vivo in human cells.

Figure 4.1 *In vivo* interaction between MRE11 and PRMT1.

(A) Co-immunoprecipitations assays in HeLa cells with HA-MRE11 and Myc-PRMT1 were performed. The immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotted with anti-HA and anti-Myc antibodies as indicated (lane 1-6).

(B) HeLa cell extracts were immunoprecipitated with anti-MRE11 or normal rabbit serum (NRS) antibodies (lane 1-3). The immunoprecipitated proteins were analyzed by SDS-PAGE and Western blotting with anti-MRE11 and PRMT1 antibodies (lane 1-3).


A
HA-MRE11
+
Myc-PRMT1
IP:
Input
IgG
αHA
Input
IgG
αMyc
110
80
65
Blot:
αHA
HA-MRE11
55
36
Myc-PRMT1
1 2 3 4 5 6
Blot:
αMyc
B
IP:
Input
IgG
αMRE11
110
80
65
MRE11
Blot:
αMRE11
55
36
PRMT1
1 2 3
Blot:
αPRMT1

4.5.2 PRMT1 localizes within PML nuclear bodies with MRE11

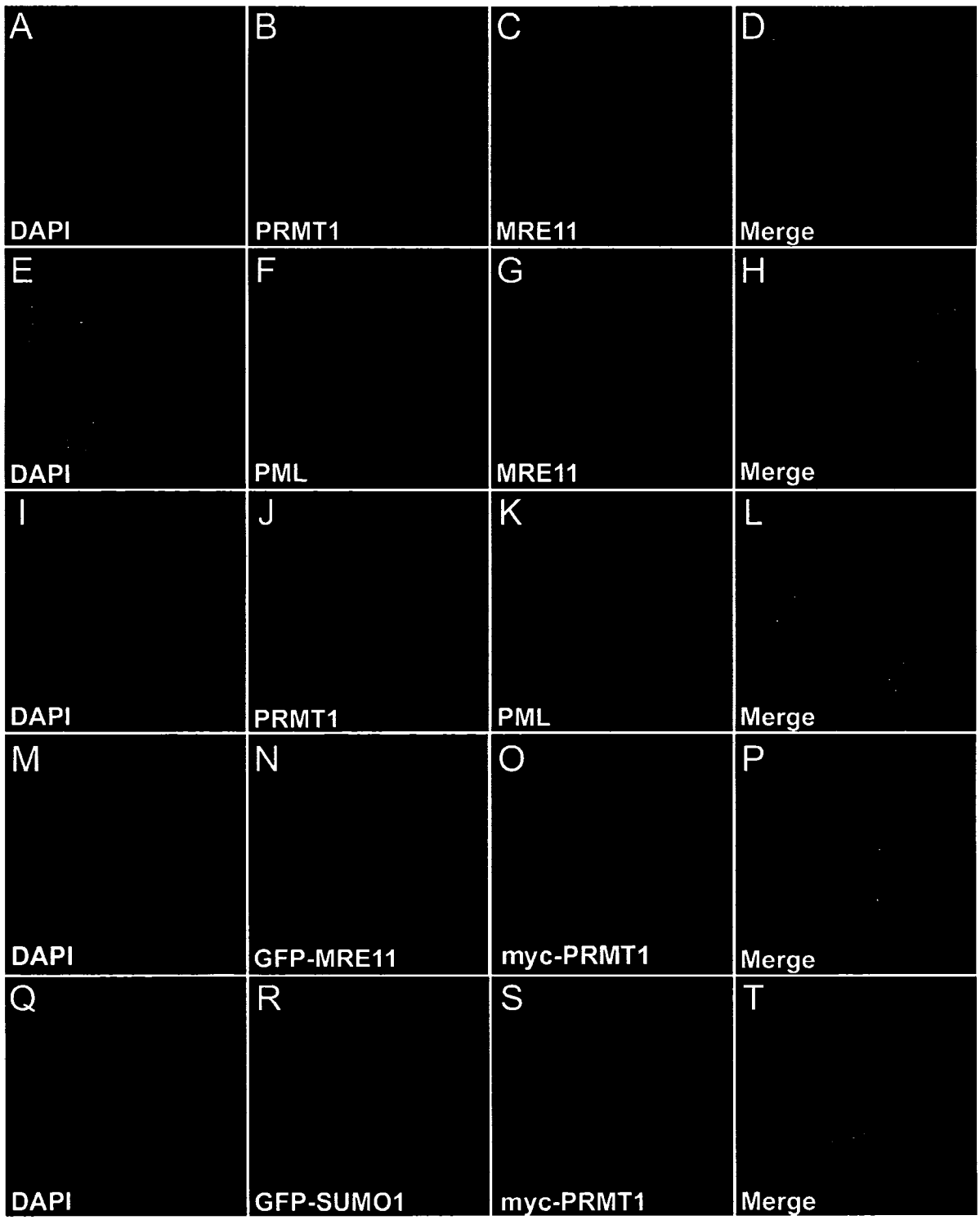
PRMT1 is a ubiquitiously expressed and localized within the cytoplasm and the nucleus (Tang et al., 1998c). MRE11 resides within the nucleus and a fraction of MRE11 is associated with the nuclear structure within PML nuclear bodies (Lombard and Guarente, 2000; Mirzoeva and Petrini, 2001b). This PML nuclear body localization of MRE11 is visualized by using a detergent extraction step before the immunostaining which reduces the signal contributed by diffuse nucleoplasmic MRE11 and reveals the PML nuclear bodies. To examine whether endogenous PRMT1 was also present in PML nuclear bodies, CRL2097 human normal diploid fibroblasts were detergent extracted and the localization of endogenous PRMT1 was visualized by indirect immunofluorescence. Indeed PRMT1 localized within discrete nuclear foci (Figure 4.2B) that co-localized with MRE11 (Figure 4.2C and D). In addition, PRMT1 also stained in cytoplasmic and peripheral foci that may represent focal adhesion contacts (Figure 4.2B). To confirm the localization of MRE11 in PML nuclear bodies, we performed co-immunostaining using anti-PML (Figure 4.2F) and anti-MRE11 (Figure 4.2G) antibodies. Indeed, MRE11 was found in PML nuclear bodies in CRL2097 human primary fibroblasts (Figure 4.2H). To confirm that PRMT1 was also present in PML nuclear bodies, co-immunostaining was performed with anti-PRMT1 (Figure 4.2J) and anti-PML (Figure 4.2K) antibodies. Endogenous PRMT1 co-localized with PML (Figure 4.2L), confirming the presence of PRMT1 in PML nuclear bodies.

The localization of PRMT1 within PML nuclear bodies was also confirmed using transfected PRMT1. Because fibroblasts are nearly impossible to transfect with acceptable efficiency, SK-N-SH cells were cotransfected with GFP-MRE11 along with an expression vector encoding myc-epitope tagged PRMT1. The cells were labeled for immunofluorescence using an anti-myc antibody. GFP-MRE11 was visible in nuclear foci and indeed myc-PRMT1 colocalized with MRE11 within the nuclear foci that resembled

PML nuclear bodies (Figure 4.2M-P). To further confirm the authenticity of the PML nuclear bodies, we co-transfected HeLa cells with myc-PRMT1 and GFP-SUMO1 expression plasmids. GFP-SUMO-1, a PML nuclear body marker (Boddy et al., 1996b), colocalized with myc-PRMT1 within PML nuclear bodies (Figure 4.2Q-T). Our results suggest that PML nuclear bodies may represent a nuclear site for the arginine methylation of MRE11 or its sequestration.

Figure 4.2 PRMT1 co-localizes with MRE11 in PML nuclear bodies.

(A-H) Human normal fibroblasts CRL2097 were labeled for immunofluorescence using anti-MRE11 (panel C and G), anti-PRMT1 (panel B and J) and anti-PML (panel F) antibodies. Nuclei were stained with DAPI (A, E, I) and the merges are shown in panels (D, H, L). (M-P) SK-N-SH neuroblastoma cells transfected with GFP-MRE11 and Myc-PRMT1 or GFP-SUMO1 and Myc-PRMT1 (Q-T) were labeled for immunofluorescence using a Myc antibody (9E10). Nuclei were stained with DAPI (M, Q) and the merges are shown in panels (P, T).


A
DAPI
B
PRMT1
C
MRE11
D
Merge
E
DAPI
F
PML
G
MRE11
H
Merge
I
DAPI
J
PRMT1
K
PML
L
Merge
M
DAPI
N
GFP-MRE11
O
myc-PRMT1
P
Merge
Q
DAPI
R
GFP-SUMO1
S
myc-PRMT1
T
Merge

4.5.3 Arginine methylated MRE11 associates with nuclear structures

To determine whether arginine methylated MRE11 was present in PML nuclear bodies, we generated antibodies that recognize fully methylated MRE11 (MeMRE11) and unmethylated MRE11 (UnMRE11). The specificity of each antibody was confirmed by ELISA (Figure 4.3A, 4.3B). The antibodies were examined for their ability to detect unmethylated bacterial produced GST-MRE11 amino acid 554 to 680 or methylated baculovirus produced hMRE11 by immunoblotting. GST-MRE11 was recognized by UnMRE11, and slightly recognized by MeMRE11 (Figure 4.3C). Baculovirus hMRE11 was recognized by a general aDMA-specific antibody ASYM25 and by MeMRE11 (Figure 4.3C), confirming their specificity. We utilized these antibodies to detect MRE11 by immunofluorescence and we did not detect any MRE11 specific staining (Suppl. Figure 4.1) consistent with these antibodies detecting additional proteins besides MRE11 (data not shown). We next proceeded to cell fractionation followed by immunoprecipitation studies to identify the fractions that contain unmethylated and methylated MRE11.

The cytoplasm was separated from the nuclei of HeLa cells by hypotonic lysis. The washed nuclei were lysed and freed of the chromatin using Triton X-100 and digestion with DNaseI. The digested nuclei were extracted by the addition of ammonium sulfate to a final concentration of 0.25 M. The supernatant containing the digested chromatin was designated the nucleoplasm. The proteins weakly associated with the insoluble nuclear fraction were washed with a 2M NaCl solution (2M extraction). The pellet was then incubated with RNase A and T1 to elute RNA bound proteins. The supernatant and pellet were designated ribonucleoproteins (RNPs) and nuclear structure, respectively. Aliquots of each fraction were immunoblotted with anti-Actin, anti-Sam68 and MRE11. Actin was detected only in the cytoplasmic fraction and the majority of the Sam68 RNA binding protein was observed in the nucleoplasm as expected (Figure 4.3D). MRE11 was detected in both the nucleoplasm and the fraction that corresponds to nuclear structures (Figure 4.3D). To identify where

methylated and unmethylated MRE11 reside, each fraction was divided equally and immunoprecipitated with either control, UnMRE11 and MeMRE11 antibodies. The bound proteins were separated by SDS-PAGE and immunoblotted with an MRE11 antibody that detects both forms. Our data revealed that the nucleoplasm contained equal amounts of methylated and unmethylated MRE11 (Figure 4.3E, lanes 5-8). In addition, the unmethylated MRE11 was detected in the 2M extraction fraction (lanes 9-12), whereas the methylated MRE11 was predominantly detected in the nuclear structure fraction (Figure 4.3E, lanes 17-20). These data suggest that arginine methylation of MRE11 regulates the association with nuclear structures.

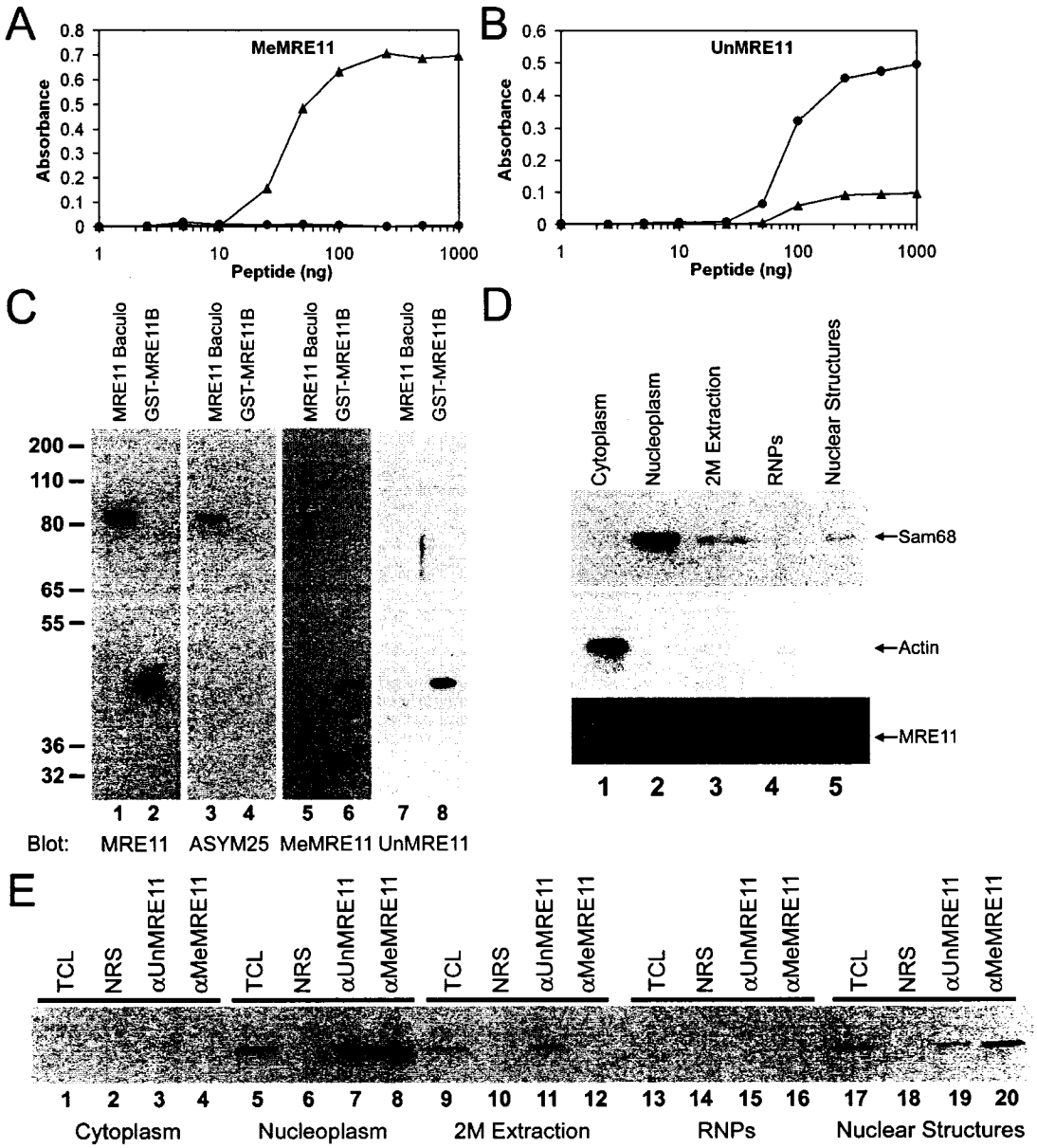
Figure 4.3 Arginine methylation of MRE11 influences its nuclear localization.

(A, B) The ELISA demonstrates the specificity of the methyl-specific (MeMRE11) or the non-methyl-specific (UnMRE11) anti-MRE11 antibodies using a methylated MRE11 peptide (triangles) and an unmethylated peptide (circles).

(C) Baculovirus human methylated MRE11 and unmethylated GST-MRE11 residues 554-680 were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(D) Fractionated HeLa cells were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-actin, -Sam68 and -MRE11 antibodies

(E) Fractionated HeLa cell lysates were immunoprecipitated with NRS, UnMRE11 and MeMRE11 antibody. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with MRE11 antibodies.


A
MeMRE11
Absorbance
Peptide (ng)
B
UnMRE11
Absorbance
Peptide (ng)
C
MRE11 Baculo
GST-MRE11B
200
110
80
65
55
36
32
1 2 3 4 5 6 7 8
Blot: MRE11 ASYM25 MeMRE11 UnMRE11
D
Cytoplasm
Nucleoplasm
2M Extraction
RNPs
Nuclear Structures
←Sam68
←Actin
←MRE11
1 2 3 4 5
E
TCL
NRS
αUnMRE11
αMeMRE11
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Cytoplasm
Nucleoplasm
2M Extraction
RNPs
Nuclear Structures

4.5.4 The MRE11 GAR domain is required for association with nuclear structures

MRE11 is arginine methylated within its glycine arginine rich (GAR) domain (Boisvert et al., 2005c). To investigate whether the arginines within the GAR domain affected MRE11 cellular localization, wild-type MRE11 and a mutant that contains the arginines within the GAR domain substituted for alanines (MRE11 R/A) were expressed in HeLa cells as YFP-fusion proteins. We first examined the ability of the YFP-MRE11 fusion protein to move in the presence or absence of DNA damage (10 Gy) by fluorescence recovery after photobleaching (FRAP) analysis. The recovery of the fluorescence in a photo-bleached area was determined and the normalized intensity of recovery of 10 different cells was plotted against time. The recovery of YFP-MRE11 occurred in seconds (Figure 4.4A) and as previously observed with another MRN protein, NBS1 (Lukas et al., 2003b), and was independent of γ-irradiation (Figure 4.4A). To address whether the arginines or methylarginines within the MRE11 GAR domain were involved in MRE11 mobility, FRAP analysis was performed with the fusion protein. Indeed, the kinetics of recovery of the YFP-MRE11 R/A was rapid and appeared to consist of a single kinetic population (Figure 4.4B). In contrast, the wild-type YFP-MRE11 after photo-bleaching had a significantly slower recovery and was found in both fast and slow recovering populations (Figure 4.4B). These findings are indicative of an association of wild-type MRE11 with a nuclear structure and this association is absent or reduced with MRE11 R/A. These data suggest that the MRE11 GAR domain is required for association with nuclear structures consistent with our fractionation studies of Figure 4.3.

The cellular localization of the YFP-MRE11 fusion protein was examined by immunoblotting after fractionation. Both YFP-MRE11 fusion proteins and endogenous MRE11 were observed in both the nucleus and the cytoplasm (Figure 4.4C, YFP-MRE11 and MRE11). Transfected MRE11 is known to localize in the cytoplasm as well as the nucleus

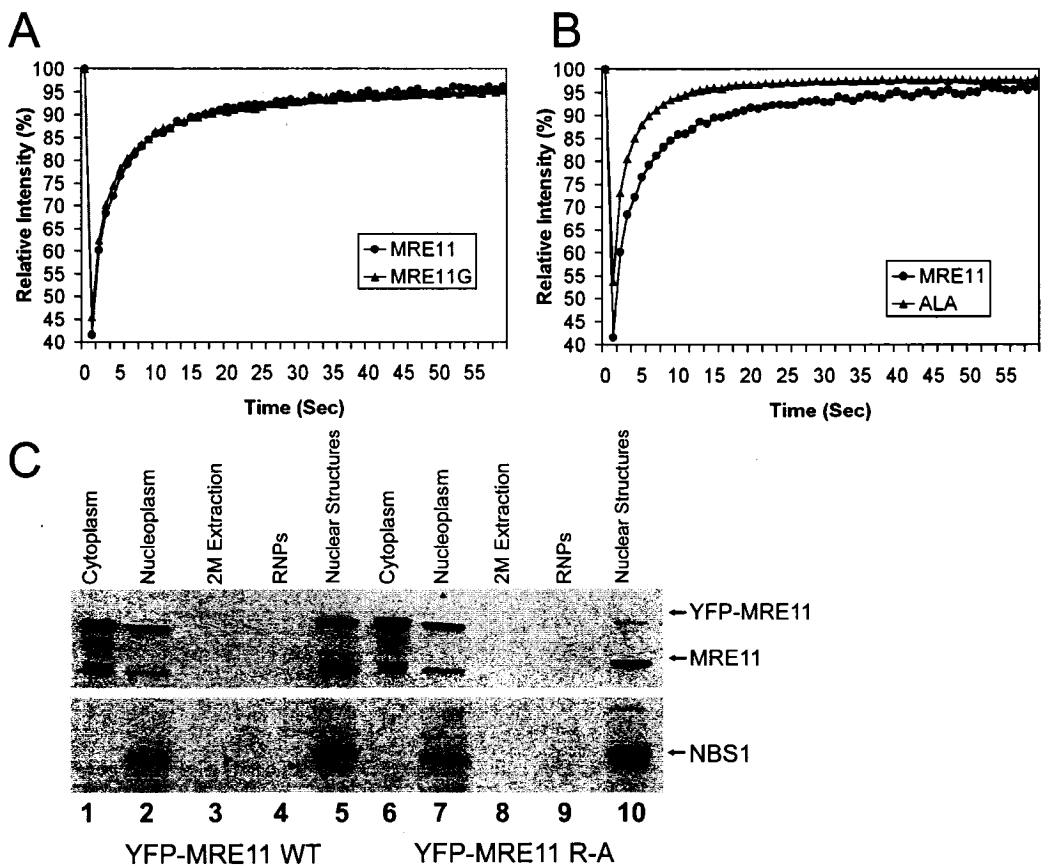
due to limiting NBS1 (Carney et al., 1998). However, as expected endogenous MRE11 and YFP-MRE11 were strongly associated with nuclear structures (Figure 4.4C). In contrast, YFP-MRE11 R/A behaved differently, as it was weakly associated with nuclear structures (Figure 4.4C, compare lanes 5 and 10). Endogenous NBS1 was only present in nucleoplasm and within nuclear structures (Figure 4.4C), as expected for this protein (Lukas et al., 2003b). Collectively, these data identify a role for the MRE11 GAR domain in protein localization and mobility.

Figure 4.4 Arginines in the MRE11 C-terminus regulate nuclear structure association.

(A) FRAP analysis of YFP-MRE11 in HeLa cells in the absence (MRE11) or presence of γ-irradiation (MRE11G).

(B) FRAP analysis of YFP-MRE11 (MRE11) and -MRE11R/A (ALA).

(C) Cellular fractionation of HeLa cells transfected with YFP-MRE11 (lane 1-5) or YFP-MRE11 R/A (lane 6-10). Proteins were visualized by immunoblotting with anti-MRE11 and anti-NBS1 antibodies.


A
Relative Intensity (%)
Time (Sec)
MRE11
MRE11G
B
Relative Intensity (%)
Time (Sec)
MRE11
ALA
C
Cytoplasm
Nucleoplasm
2M Extraction
RNPs
Nuclear Structures
Cytoplasm
Nucleoplasm
2M Extraction
RNPs
Nuclear Structures
YFP-MRE11
MRE11
NBS1
1 2 3 4 5 6 7 8 9 10
YFP-MRE11 WT
YFP-MRE11 R-A

4.5.5 Methylase inhibitors prevent the recruitment of MRE11 to sites of DNA damage

To address whether arginine methylation affected the relocalization of MRE11 following DNA damage, CRL2097 human primary fibroblasts were mock treated (Figure 4.5A) or treated with a moderate (Figure 4.5B) or high (Figure 4.5C) dose of methylase inhibitors MTA (5-deoxy-5 -methylthioadenosine) and Adox (adenosine-2',3'-dialdehyde) for 24 h, followed by DNA damage introduced by 1h treatment of 50 μM of the topoisomerase II inhibitor, etoposide. The phosphorylation of serine 139 of the histone variant H2AX (γ-H2AX) is induced rapidly following DNA damage and antibodies against γ-H2AX serve as a marker of DNA damage foci (Rogakou et al., 1999). The phosphorylation of H2AX is not required for the initial recruitment of DNA damage complexes, but rather is necessary for association with chromatin regions distal to the break and the initiation of the DNA damage response (Celeste et al., 2003c). Mock-treated cells contained MRE11 in PML nuclear bodies with few γ-H2AX foci, as visualized by indirect immunofluorescence with anti-MRE11 and anti-γ-H2AX antibodies (Figure 4.5A, top row). Etoposide treatment resulted in the formation of DNA damage induced foci that contained both MRE11 and γ-H2AX, (Figure 4.5A, bottom row) but not PRMT1 (data not shown). Cells pretreated with a moderate dose of methylase inhibitors also contained MRE11 within PML nuclear bodies and few γ-H2AX foci (Figure 4.5B, top row), suggesting that methylase inhibitors do not disrupt the integrity of PML nuclear bodies. Etoposide treatment stimulated the appearance of γ-H2AX foci, but interestingly only a subset of these foci contained MRE11 (Figure 4.5B, bottom row). The MRE11 nuclear foci that did not colocalize with γ-H2AX foci appeared to be PML nuclear bodies, suggesting that the relocalization of MRE11 to sites of DNA damage is partially impaired at this methylase inhibitor concentration. Cells treated with the higher concentration of methylase inhibitors contained only weakly visible γ-H2AX foci and MRE11 remained localized in PML nuclear bodies (Figure 4.5C). Visualization of a larger field confirmed that

γ-H2AX foci were greatly diminished in cells treated with methylase inhibitors (Figure 4.6H) compared to mock-treated cells (Figure 4.6D). These findings demonstrate that methylation is required to localize MRE11 to sites of DNA damage and for the appearance of γ-H2AX foci.

Figure 4.5 Methylase inhibitors prevent the localization of MRE11 at sites of DNA damage.

The human primary fibroblasts CRL2097 were mock treated with DMSO (A), pre-treated for 24 hr with the methylase inhibitors MTA at 750 μM and Adox at 250 μM (B) or pre-treated for 24 hr with the methylase inhibitors MTA at 1000 μM and Adox at 500 μM (C) Cells were then treated with etoposide at 50 μM (A, B and C, second row) for 1 hour, allowed to recover for another hour, fixed and labeled for immunofluorescence with anti-MRE11 and anti-γ-H2AX antibodies and the nuclei stained with DAPI.

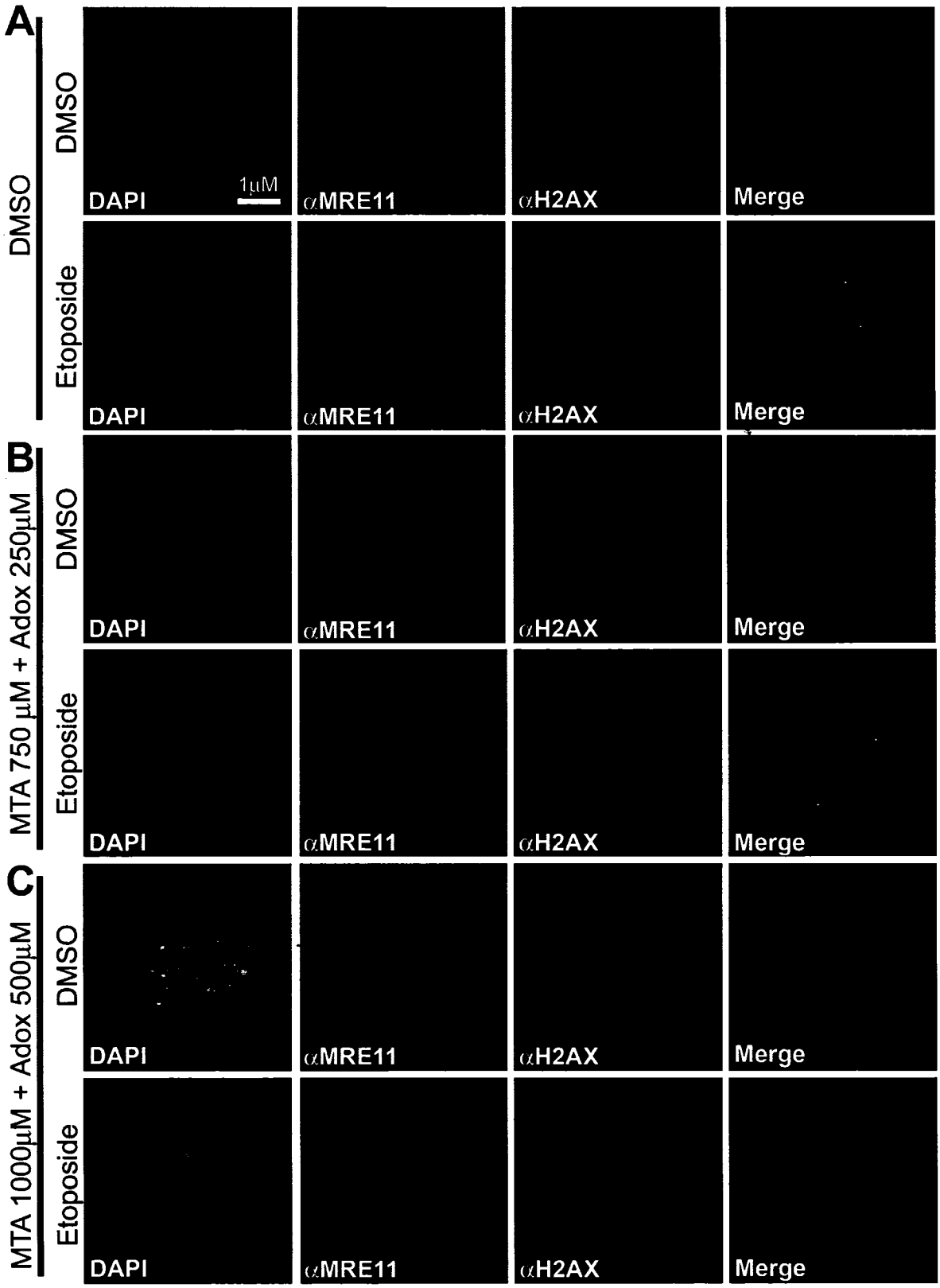
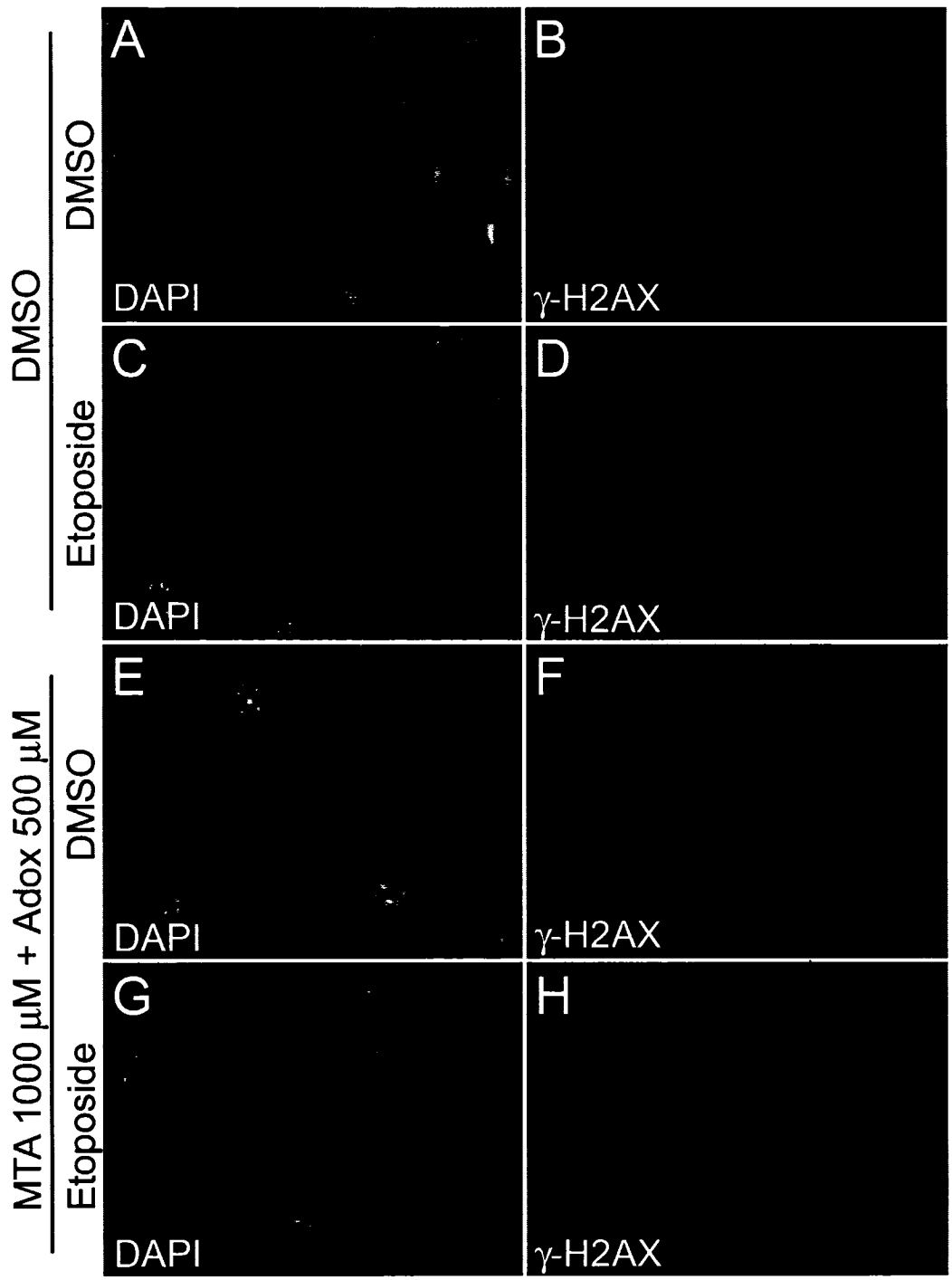

A
DMSO
DMSO
Etoposide
B
MTA 750 µM + Adox 250µM
DMSO
Etoposide
C
MTA 1000µM + Adox 500µM
DMSO
Etoposide
1µM
DAPI
αMRE11
αH2AX
Merge

Figure 4.6 Inhibition of methylation inhibits H2AX phosphorylation following DNA damage.

The human primary fibroblasts CRL2097 were mock treated with DMSO (A-D) or pre-treated for 24 hrs with the methyltransferase inhibitors MTA at 1000 μM and Adox at 500 μM (E-H) Cells were then treated with etoposide at 50 μM (C, D, G and H) for 1 hr, allowed to recover for another hour, fixed and labeled for immunofluorescence with the anti-γ-H2AX (Upstate) antibody and the nuclei stained with DAPI.


DMSO
DMSO
Etoposide
MTA 1000 µM + Adox 500 µM
DMSO
Etoposide
A
DAPI
B
γ-H2AX
C
DAPI
D
γ-H2AX
E
DAPI
F
γ-H2AX
G
DAPI
H
γ-H2AX

4.6 Discussion

In the present study, we establish a new role for protein methylation in the DNA damage response. Inhibition of methylation prevented the recruitment of the double-strand repair protein MRE11 to sites of DNA damage and prevented the formation of γ-H2AX foci. MRE11 associated *in vivo* with the arginine methyltransferase PRMT1 and the two proteins co-localized within PML nuclear bodies in normal diploid fibroblasts. Using our newly generated MRE11 methylarginine-specific antibodies, we show that arginine methylated MRE11 is preferentially associated with nuclear structures and that amino acid substitutions replacing the arginines within the MRE11 glycine arginine rich (GAR) domain prevented association with nuclear structures. These findings suggest that arginine methylation of the MRE11 GAR domain is required for its association with nuclear structures including sites of DNA damage.

We showed previously that MRE11 contains aDMA by using mass spectrometry and aDMA-specific antibodies. We also demonstrated that MRE11 is a substrate of PRMT1 in vitro and in vivo (Boisvert et al., 2005c). Now we report that MRE11 and PRMT1 associate and co-localize in PML nuclear bodies. PRMT1 was shown previously to reside in both the cytoplasm and the nucleus (Tang et al., 1998c). By reducing the nucleoplasmic signal using a detergent extraction step prior to immunostaining, we observed endogenous PRMT1 in cytoplasmic foci that may represent focal adhesion contacts and we identified PRMT1 in PML nuclear bodies. Biochemical experiments and *in situ* fractionation show that many DNA repair proteins are part of a dynamic nuclear structure complex that requires PML (Bischof et al., 2001; Mirzoeva and Petrini, 2001b) and dispersal of PML nuclear bodies following DNA damage may facilitate the enhanced release of DNA repair proteins in order to respond adequately to extensive DNA damage (Conlan et al., 2004). PML nuclear bodies are known to be modified following DNA damage and are recruited to sites of DNA breaks, along with the MRN complex, p53 and other proteins involved in DNA repair (Carbone et

al., 2002b). The function of PML nuclear bodies remains undefined, but the presence of a number of protein modification activities including acetylation (LaMorte et al., 1998b), phosphorylation (Hofmann et al., 2002a), SUMOylation (Boddy et al., 1996b) and now arginine methylation, suggests that many proteins localize to PML nuclear bodies to acquire nuclear post-translational modifications. The fact that the majority of the MRN complex was present in the nucleoplasm and only a fraction was localized to PML nuclear bodies, suggests that MRE11 may transit through the PML nuclear bodies to receive methyl groups. This model would suggest that PML nuclear bodies represent a nuclear structure where the maturation of MRE11 takes place. Thus, arginine methylation of MRE11 within PML nuclear bodies could possibly prepare the MRN complex for further activation through phosphorylation and recruitment to sites of DNA damage.

The cellular response to DNA damage includes the initial recognition and recruitment of several protein complexes in an orderly fashion (Lisby et al., 2004b). The localization of the MRN complex to sites of DNA damage is very rapid (Lukas et al., 2003b) and is required for activation of the ensuing intra-S checkpoint (Carson et al., 2003b). We noticed that replacing the arginines with alanines within the MRE11 GAR domain accelerated the movement of MRE11. Since these amino acid substitutions do not affect the MRN complex this suggests that the GAR domain is required to 'anchor' MRE11 to nuclear structures. One of the roles of MRN complex at sites of DNA damage is to activate the ATM kinase (Carson et al., 2003b; Lee and Paull, 2004; Uziel et al., 2003). ATM phosphorylates known effectors including H2AX, p53, Chk2 and other substrates and this initiates the DNA damage response (Shiloh, 2003b). The absence of MRE11 at sites of DNA damage coincides with few γ-H2AX foci with methylase inhibitor treatment is consistent with the requirement for the MRN complex at sites of DNA damage prior to the phosphorylation of H2AX.

We have shown previously that the exonuclease activity of MRE11 is abrogated without arginine methylation and that cells display intra-S phase defects with methylase inhibitors or

in cells treated with PRMT1 siRNA (Boisvert et al., 2005c). Our observation that arginine methylation may impair the ability of MRE11 to localize to DNA damaged sites suggests that the intra-S phase defects in the absence of methylation may be due to both the abnormal exonuclease activity and the inability of the MRE11 to localize to DNA damaged sites. Since we showed that intra-S phase defects occurred in PRMT1 siRNA treated cells, this confirms the role of arginine methylation within this process. Recently, the methylation of lysine 79 was shown to be required for recruitment of 53BP1 to sites of DNA damage (Huyen et al., 2004a). Thus, it demonstrates that methylation is a modification that is essential for the recruitment of several DNA repair complexes to sites of DNA damage.

Until recently, methylation of arginines and lysines was presented as an irreversible modification (Bannister et al., 2002; Lukong and Richard, 2004b). However, recent evidences identifying enzymes capable of removing methyl groups on those residues suggested that methylation of lysine and arginine might by a more dynamic process then first anticipated (Cuthbert et al., 2004a; Shi et al., 2004; Wang et al., 2004a). This raises the possibility that the methylation of MRE11 might not be a constitutive and irreversible modification, but that its arginine methylation might be a reversible, regulated process that can switch the active state of the protein to an inactive state. Thus, the role of arginine methylation in DNA repair might not solely be in the assembly of an active MRE11 complex, but also in the dynamic regulation of both the enzymatic activity of MRE11 as well as its ability to signal DNA damage.

In conclusion, we provide evidence that PRMT1 is localized within PML nuclear bodies and that methylation may regulate the intranuclear trafficking of proteins. More specifically, we show that arginine methylation is required for the mobility of MRE11 and its association with sites of DNA damage. There data suggest that protein methylation regulates protein localization and recruitment during the DNA damage response.

4.7 Acknowledgements

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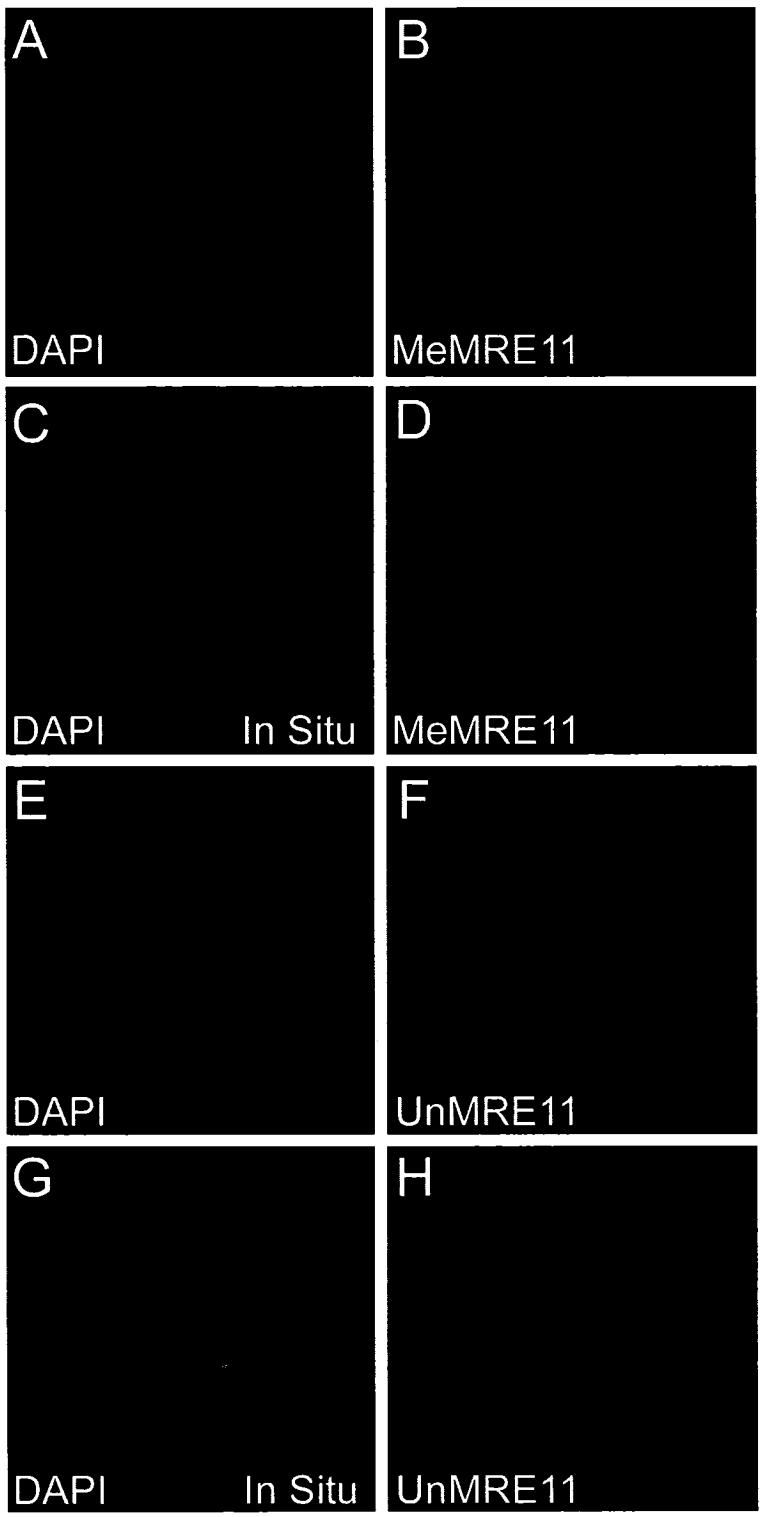
Supplemental Figure 4.1 Immunofluorescence using MeMRE11 and UnMRE11 antibodies.

The human primary fibroblasts CRL2097 were untreated (A, B, E and F) or detergent extracted (C, D, G and H), fixed and labeled for immunofluorescence using the anti-MeMRE11 antibody (A-D) or the anti-UnMRE11 antibody (E-H) and the nuclei stained with DAPI.


A
DAPI
B
MeMRE11
C
DAPI
In Situ
D
MeMRE11
E
DAPI
F
UnMRE11
G
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In Situ
H
UnMRE11

Chapter 5

General discussion

5.1 Arginine methylation: more abundant then previously thought.

The identification of over 200 new proteins using a proteomic approach has provided new insights into possible new cellular functions for arginine methylation. However, the antibodies used in our study were all biased towards recognizing methylated residues when found in clusters in a glycine arginine rich environment (GAR domain), allowing possibly the identification of mostly PRMT1, PRMT3 and PRMT5 targets which preferentially methylate arginines in such domains (Gary and Clarke, 1998a). We used the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) to search for short, nearly exact matches with RGRGRG as a query, a sequence that is almost certain to be a target for methylation and identified 1770 GAR domains in 956 different human proteins in the REFSEQ non-redundant database using no more than one mismatch as criteria. The proteins were classified by function according to the Gene Ontology Consortium (GO_Molecular Function) and ranked by functional significance based on co-occurrence of sets of genes with the same function (P Values) (Table 5.1). Consistent with the fact that RNA binding proteins are the most represented group of arginine methylated proteins (Gary and Clarke, 1998a), the first nine most significant functional categories of potentially arginine methylated proteins as predicted by the presence of a GAR domain (Table 5.1) are all related to some aspect of RNA metabolism. Interestingly, many of the new functions for arginine methylation that we have proposed in our proteomic study are also very significantly represented in that analysis, such as transcription, signal transduction, cytoskeleton and DNA repair. In addition, there are several protein functions that are unexpectedly present and that arginine methylation has not

been involved or described as playing a role, such as general cellular metabolism, cell growth, cell cycle and cell adhesion. This analysis suggests that our proteomic study has only identified a fraction of the proteins containing the epitope that was used for their generation (Chapter 2). Moreover, it has been shown that PRMT4 and PRMT6 prefer arginines not necessarily found in GAR domains or not necessarily found in repeats of methylated arginines (Boulanger et al., 2005; Lee and Bedford, 2002a). Our antibodies have a lower affinity for peptides containing single methylated arginines (Boisvert et al., 2002), suggesting that our proteomic study mostly identified proteins containing several clusters of methylated arginines and rarely proteins containing single methylated arginines, or methylated arginines not found in repeats. Thus, there is likely a far wider spectrum of proteins with methylated arginine beyond the scope of our proteomics studies. Therefore there may be well over 1000 cellular substrates for arginine methyltransferases, a number far greater than predicted for a rare post-translational modification like arginine methylation.

Most of the arginine methylated proteins characterized to date are substrates for PRMT1, leaving some of the methyltransferase with not a single known protein substrate to this day (see Table 1.2 in Chapter 1). Although it has been shown that PRMT1 is responsible for 54% of cellular methylated arginines (Pawlak et al., 2000), it was also shown that most of the proteins methylated by PRMT1 are very abundant RNA binding proteins containing repeats of RGG motifs, which account for a large proportion of the cellular methylated arginines. Thus, it would not be surprising to identify many less abundant protein substrates for the other methyltransferases. It is interesting to note, however, that there are only 9 known arginine methyltransferases, and that few examples of their regulation and specificities have been demonstrated. Thus, perhaps arginine methylation is a constitutive post-translational modification and therefore is not a post-translational modification that regulates the function of proteins that harbors potential methylation sites, but may rather play a role in the maturation process of protein complexes. Future studies aimed at identifying and

characterizing substrates for all methyltransferases and will be of crucial importance to our understanding of the role of arginine methylation.

Function	Number	P Value
RNA PROCESSING	36	1.10614E-16
RNA METABOLISM	40	2.97753E-16
MRNA PROCESSING	20	2.88137E-10
MRNA METABOLISM	21	2.95762E-10
NUCLEOTIDE AND NUCLEIC ACID METABOLISM	108	1.63155E-08
RNA SPLICING	15	2.89812E-07
RNA SPLICING, VIA TRANSESTERIFICATION REACTIONS	12	2.32085E-06
NUCLEAR MRNA SPLICING, VIA SPLICEOSOME	12	2.32085E-06
RNA SPLICING, VIA TRANSESTERIFICATION REACTIONS	12	2.32085E-06
METABOLISM	194	8.36914E-05
CELL GROWTH AND/OR MAINTENANCE	112	8.83416E-05
CELL PROLIFERATION	44	0.000102557
CELL ORGANIZATION AND BIOGENESIS	33	0.000124876
DNA UNWINDING	4	0.000284918
CELLULAR PHYSIOLOGICAL PROCESS	122	0.00038247
REGULATION OF BIOLOGICAL PROCESS	85	0.000771218
CELLULAR PROCESS	174	0.001366095
CELL CYCLE	30	0.002306228
TRANSCRIPTION	62	0.002764993
RAS PROTEIN SIGNAL TRANSDUCTION	5	0.002793053
CHROMOSOME ORGANIZATION AND BIOGENESIS	13	0.003199359
NUCLEAR ORGANIZATION AND BIOGENESIS	13	0.003673329
CHROMATIN REMODELING	5	0.006189059
REGULATION OF NUCLEOTIDE AND NUCLEIC ACID METABOLISM	58	0.006480705
POSITIVE REGULATION OF TRANSCRIPTION, DNA-DEPENDENT	5	0.006828787
REGULATION OF TRANSCRIPTION	57	0.007897897
DNA TOPOLOGICAL CHANGE	3	0.008294787
TRANSCRIPTION, DNA-DEPENDENT	57	0.009172042
REGULATION OF PHYSIOLOGICAL PROCESS	62	0.009685634
REGULATION OF METABOLISM	60	0.010063051
REGULATION OF TRANSCRIPTION, DNA-DEPENDENT	55	0.010444734
REGULATION OF CELL CYCLE	17	0.010669822
WNT RECEPTOR SIGNALING PATHWAY	7	0.011236972
NUCLEOCYTOPLASMIC TRANSPORT	7	0.013693363
CELL-MATRIX ADHESION	6	0.019189204
DEVELOPMENT	51	0.019768157
REGULATION OF CELL PROLIFERATION	12	0.022853929
CELLULAR MORPHOGENESIS	9	0.023189129
POSITIVE REGULATION OF TRANSCRIPTION	5	0.028061504
PROTEIN COMPLEX ASSEMBLY	7	0.029408745
REGULATION OF CELLULAR PROCESS	26	0.031080915
POSITIVE REGULATION OF NUCLEOTIDE AND NUCLEIC ACID METABOLISM	5	0.033163903
CELL CYCLE ARREST	5	0.033163903
RIBOSOME BIOGENESIS	4	0.040178993
RIBOSOME BIOGENESIS AND ASSEMBLY	4	0.051946022
SPLICEOSOME ASSEMBLY	3	0.057447343
CELL COMMUNICATION	89	0.071076464
MRNA-NUCLEUS EXPORT	3	0.074566445
MRNA TRANSPORT	3	0.074566445
CHOLESTEROL BIOSYNTHESIS	3	0.080592092
POSITIVE REGULATION OF METABOLISM	5	0.083003734
NEUROPEPTIDE SIGNALING PATHWAY	6	0.089140534
DNA-DEPENDENT DNA REPLICATION	5	0.092069245
REGULATION OF GTPASE ACTIVITY	2	0.098852154
POSITIVE REGULATION OF GENE EXPRESSION, EPIGENETIC	2	0.098852154

Table 5.1 Function of predicted arginine methylated proteins.

5.2 Defining a role for arginine methylation in DNA repair.

A function for arginine methylation in DNA repair was postulated following the observation of the interaction between PRMT1 and BTG2/TIS21/PC3 (Berthet et al., 2002). BTG1 and BTG2 are proteins thought to play an important role in G2/M control following genotoxic treatment (Rouault et al., 1996). These proteins are upregulated in a p53 dependent manner following DNA damage and, relevant to this argument, have been shown to activate PRMT1 activity *in vitro* (Lin et al., 1996b). This induction of BTG1 and BTG2 after genotoxic treatment was correlated with a modulation of protein methylation (Cortes et al., 2000). However, no evidence for the importance of the functions of BTG1 and BTG2 in regulating the cellular response to DNA damage has been demonstrated so far. Another methyltransferase, PRMT7, was originally found in a genetic screen for proteins conferring resistance to a topoisomerase II inhibitor (Gros et al., 2003). PRMT7 has been shown to methylate histone H4, myelin basic protein (MBP) and SmD3 *in vitro* (Lee et al., 2005b). However, no *in vivo* substrates for PRMT7 have been confirmed so far and it remains to be shown why PRMT7 is implicated in cell resistance to DNA damage. The cold-inducible RNA-binding protein (CIRP or A18 hnRNP) was originally found in mammalian cells as a protein that is overexpressed upon a temperature downshift (Nishiyama et al., 1997). CIRP is a protein shuttling between the nucleus and the cytoplasm, and is thought to be involved in mRNA transport (Aoki et al., 2002; Matsumoto et al., 2000). Overexpression of CIRP increases mRNAs stability and consequently enhances translation in a dose-dependent manner (Yang and Carrier, 2001). CIRP has also been shown to be induced and translocated from the nuclei to the cytoplasm after exposure to UV radiation (Yang and Carrier, 2001). Forty-six mRNA transcripts bound by CIRP were identified, most of which are stress- or

UV-responsive genes such as the replication protein A (RPA2) (Yang and Carrier, 2001). Since arginine methylation by overexpressed PRMT1 has been shown to cause cytoplasmic accumulation of CIRP (Aoki et al., 2002), arginine methylation may perhaps play a role in regulating translation of many DNA repair proteins. 53BP1 was first identified in a yeast two-hybrid screen for proteins interacting with the tumor suppressor p53 (Iwabuchi et al., 1994a). In response to DNA damage, 53BP1 becomes hyperphosphorylated by ATM and forms discrete nuclear foci that co-localizes with MRE11 (Anderson et al., 2001; Rappold et al., 2001; Schultz et al., 2000; Xia et al., 2001). 53BP1 is also required for at least a subset of ataxia telangiectasia-mutated (ATM)-dependent phosphorylation events at sites of DNA breaks (DiTullio et al., 2002). 53BP1 contains a BRCT domain that consists of approximately 95 amino acid residues and occurs as a tandem repeat at the carboxyl terminus of numerous proteins including BRCA1. This domain has been characterized as a phosphopeptide-binding module (Manke et al., 2003). Interestingly, 53BP1 also contains two tandem tudor domain that bound to histone H3 methylated on Lys 79 *in vitro* (Huyen et al., 2004b; Sanders et al., 2004). The tudor domain of another protein, SMN, has been proposed as a methylarginine binding domain (Buhler et al., 1999; Selenko et al., 2001), suggesting a possible role for 53BP1 tudor domain in binding methylated arginines. Interestingly, the tudor domains of 53BP1 are preceded by a glycine-arginine rich region that is most likely a target of methyltransferases.

Although these observations point to a possible role for arginine methylation in DNA repair, no PRMT substrates involved in DNA repair have been identified and how arginine methylation regulates any of the DNA repair mechanisms is unknown. This body of work is the first to implicate arginine methylation in cell cycle checkpoint control and DNA repair.

We have uncovered one specific example by which methylation is required for regulating DNA repair mechanisms, namely the recruitment of MRE11 to sites of DNA damage and the regulation of its exonuclease activity. It is likely that additional mechanisms where arginine methylation regulates proteins involved in the DNA damage response will be found. For example, if arginine methylation is a targeting signal for the MRE11 complex to sites of DNA damage, a methyl-dependent binding protein or complex will be required and these proteins will likely associate with the methylated GAR domain of MRE11. Altogether, our results presented here show that arginine methylation plays a crucial role in regulating the DNA damage response and DNA repair.

5.3 Regulation of MRE11 DNA repair functions through arginine methylation.

5.3.1 Arginine methylation and MRE11 exonuclease activity.

The 3'-5' exonuclease activity is one of the most important biochemical features of MRE11 (Paull and Gellert, 1998) and is thought to be a necessary step in the resolution of double-stranded break by creating protruding ends that can invade the sister chromatid during homologous recombination (Hopfner et al., 2002). Our results demonstrate that a mutant MRE11 protein where the arginine residues between the amino acids 570-594 were replaced with alanines was totally impaired in its exonuclease activity compared to wild-type MRE11. These data demonstrate that either the charge or the methyl groups on the arginines are required for the normal enzymatic activity of MRE11. Amino acid substitution of arginines to lysines to maintain the charge also severely impaired the exonuclease activity as well, demonstrating that the arginines or methylarginines are necessary for optimal MRE11 activity (Chapter 3, Figure 3.3C). However, the residual activity of the R/K mutant is

insufficient to cleave the DNA into smaller fragments like the wild-type MRE11. Our findings suggest that the MRE11 GAR domain regulates the exonuclease activity, an enzymatic activity important for the checkpoint response (Uziel et al., 2003). These results demonstrate for the first time a role for the MRE11 C-terminal GAR domain in the regulation of its exonuclease activity. Moreover, these results provide the first example of the implication of methylated arginine in regulating the enzymatic activity of a protein.

5.3.2 Arginine methylation and MRE11 localization to PML nuclear bodies

Biochemical experiments and *in situ* fractionation show that many DNA repair proteins are part of a dynamic nuclear structure complex that requires PML (Bischof et al., 2001; Mirzoeva and Petrini, 2001a). Dispersal of PML nuclear bodies following DNA damage may facilitate the enhanced release of DNA repair proteins to adequately response to extensive DNA damage (Conlan et al., 2004). PML nuclear bodies are known to be modified following DNA damage and are recruited to sites of DNA breaks, along with the MRN complex, p53 and other proteins involved in DNA repair (Carbone et al., 2002a). The function of PML nuclear bodies remains undefined, but the presence of a number of protein modification activities including acetylation (LaMorte et al., 1998a), phosphorylation (Hofmann et al., 2002b), SUMOylation (Boddy et al., 1996a) and now arginine methylation (Chapter 4), suggests that many proteins localize to PML nuclear bodies to acquire nuclear post-translational modifications. By immunofluorescence and nuclear fractionation, the majority of the MRE11 complex was observed in the nucleoplasm and only a fraction is localized to PML nuclear bodies, indicating that PML nuclear bodies do not represent a reservoir for the MRE11 complex prior to its localization to sites of DNA repair in the presence of DNA damage (Mirzoeva and Petrini, 2001a). Instead, PML nuclear bodies may represent a transient localization of MRE11. The presence of PRMT1 in PML nuclear bodies indicates that this nuclear structure might be the site of arginine methylation of MRE11. Thus, arginine

methylation of MRE11 within PML nuclear bodies could possibly prepare the MRN complex for further activation through phosphorylation and recruitment to sites of DNA damage. The presence of PRMT1 in PML nuclear bodies has implications not only for DNA repair, but also for other processes that occur at PML nuclear bodies. Many histones modifying activities have been associated with PML nuclear bodies (Boisvert et al., 2000; LaMorte et al., 1998a; Lin et al., 1998), suggesting that arginine methylation of histones or other proteins might also be increased at PML nuclear bodies. There are many viral proteins that localize to PML nuclear bodies following infection during viral cycle (Moller and Schmitz, 2003). Interestingly, many of those proteins have either been found to be arginine methylated, or contains RG repeats that are usual consensus sequence for arginine methylation (Gary and Clarke, 1998a; Mears and Rice, 1996). Thus, the presence of PRMT1 in PML nuclear bodies may partially explain the localization of viral proteins to PML nuclear bodies. Our data not only suggest that MRE11 localization to PML nuclear bodies might be a maturation step required for its activation, but also provide interesting insights into the regulation and localization of arginine methyltransferases.

5.3.3 Methylation regulates MRE11 recruitment to sites of DNA damage.

The cellular response to DNA damage includes the initial recognition and recruitment of several protein complexes in an orderly fashion (Lisby et al., 2004a). The localization of the MRN complex to sites of DNA damage is very rapid (Lukas et al., 2003a) and is required for activation of the ensuing intra-S checkpoint (Carson et al., 2003a). One of the roles of MRN complex at sites of DNA damage is to activate the ATM kinase (Carson et al., 2003a; Lee and Paull, 2004; Uziel et al., 2003). ATM phosphorylates known effectors including H2AX, p53, Chk2 and other substrates and this initiates the DNA damage response (Shiloh, 2003c). We noticed that inhibition of methylation resulted in an impaired recruitment of MRE11 to

sites of DNA damage (Chapter 4, Figure 4.5). The absence of MRE11 at sites of DNA damage coincides with few γ-H2AX foci with methyltransferase inhibitor treatment is consistent with the requirement for the MRN complex at sites of DNA damage prior to the phosphorylation of H2AX (Lee and Paull, 2004). However, methyltransferase inhibitors are not specific to arginine methylation and certainly not limited to MRE11 methylation. So, we cannot rule out solely on this observation that MRE11 arginine methylation is required for its recruitment to sites of DNA damage and H2AX phosphorylation. For example, it was recently demonstrated that histone H3 methylation on lysine 79 is required for recruitment of 53BP1 to sites of DNA damage (Huyen et al., 2004b). Methylation of lysine 79 was not increased in response to DNA damage, and it was suggested that changes in higher-order chromatin structure could allow exposure of a new binding site for 53BP1. Inhibition of methylation could potentially affect the recruitment of 53BP1, thus hindering the normal cellular response to DNA damage. However, H2AX phosphorylation happened normally in 53BP1 deficient mice (Fernandez-Capetillo et al., 2002), placing 53BP1 downstream of H2AX in the response to DNA damage. Nonetheless, our results demonstrate for the first time that methylation is necessary for MRE11 recruitment to sites of DNA damage and for a proper cellular response to DNA damage.

Previous work from our laboratory demonstrated that the presence of dimethylarginines in proline-rich ligands inhibits binding to Src homology 3 (SH3) domains *in vitro*, but not to WW domain, indicating that arginine methylation can regulate specific protein-protein interactions (Bedford et al., 2000a). Arginine methylation of Sm proteins by PRMT5 has been shown to enhance its interaction with SMN (Friesen et al., 2001b), promoting the assembly of the Sm proteins into spliceosomal snRNPs. The regulation of the interaction between SMN and dimethylarginine-modified proteins was shown to be mediated by the

Tudor domain of SMN (Selenko et al., 2001). Our data demonstrate that the interaction between MRE11 and the other members of the MRN complex, RAD50 and NBS1, as well as the assembly of the MRN complex, is not dependent on arginine methylation, since mutation of the methylated arginines to either lysine or alanine did not disrupt complex formation (Chapter 3, Figure 3.3). We noticed that replacing the arginines with alanines within the MRE11 GAR domain resulted in faster mobility of MRE11 within the nucleus. Since the MRN complex is still properly formed with the amino acid substitutions, this suggests that the GAR domain is required to 'anchor' MRE11 to nuclear structures. Thus, arginine methylation of MRE11 may regulate its interaction with other proteins involved in the recruitment of the MRN complex to sites of DNA damage through the GAR domain or proteins involved in maintaining a higher order nuclear structures, as suggested by the inability of the MRN complex to relocalize to sites of DNA damage in the presence of methyltransferase inhibitors. It will be interesting to identify proteins that could bind to the methylated arginines of MRE11 and regulate the recruitment of MRE11 to sites of DNA damage.

5.4 Regulation of methylation

We have demonstrated that MRE11 contains aDMA within a GAR domain and is a substrate of PRMT1. The identification by mass spectrometry of multiple methylated peptides corresponding to MRE11 and the low abundance of unmethylated peptides suggests that MRE11 exists predominantly in the methylated form. By using the MRE11 methyl-specific antibodies Arg587 and ASYM25, we observed no difference in MRE11 methylation or global methylation following genotoxic treatments such as γ-irradiation, etoposide, adriamycin and mitomycin C (Chapter 3, 4 and data not shown). Similarly, recruitment of 53BP1 to damaged DNA through methylation of lysine 79 of histone H3 was not increased in

response to DNA damage, and it was suggested that changes in higher-order chromatin structure could expose a new binding site for 53BP1 (Huyen et al., 2004b). So far, it was observed that methylation of arginines or lysines is essential for a proper cellular response to DNA damage, but it does not represent a DNA-damage-induced signal.

Until recently, methylation of arginines and lysines was reported as an irreversible modification (Bannister et al., 2002; Lukong and Richard, 2004a). However, recent studies indicate the presence of enzymes capable of removing methyl groups from arginines and lysines suggested that methylation may in fact be a more dynamic process than first anticipated (Cuthbert et al., 2004b; Shi et al., 2004; Wang et al., 2004b). This raises the possibility that the methylation of MRE11 is reversible and not constitutive. It is therefore possible that methylation regulates the equilibrium between the active and the inactive states of MRE11. Thus, the role of arginine methylation in DNA repair might not solely be in the assembly of an active MRE11 complex, but also in the dynamic regulation of both the enzymatic activity of MRE11 as well as its ability to signal DNA damage.

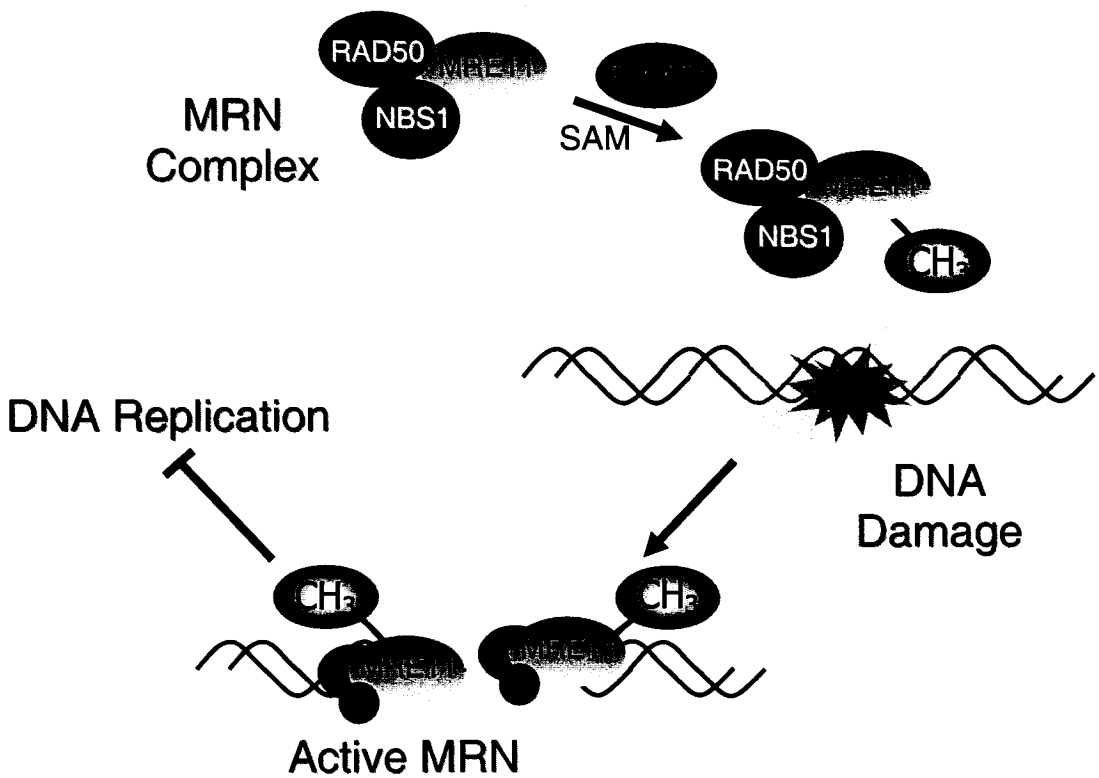
5.5 A model for the role of arginine methylation in regulating MRE11.

We have demonstrated that MRE11 is arginine methylated by PRMT1. The colocalization of PRMT1 and MRE11 in PML nuclear bodies with MRE11 and the fact that methylation appears to be ubiquitous and is not required for the MRN complex assembly would suggest that arginine methylation of MRE11 occurs prior to the presence of DNA damage. However, methylation of MRE11 is required for the MRN complex activities following DNA damage, but is not for nuclear import or MRN complex assembly. Nevertheless, our observation that inhibition of arginine methylation or mutating MRE11 methylated arginines results in impaired exonuclease activity, failure to recruit MRE11 to

sites of DNA damage and inhibition of downstream signaling events that contribute to cell cycle arrest rather attests the importance of methylation in the function of the MRN complex in DNA repair, as illustrated in Figure 5.1.

Figure 5.1. A model for the regulation of MRE11 through methylation.

Methylation of MRE11 by PRMT1 is required for recruitment of MRE11 at sites of DNA damage. This active MRN complex can then repair DNA and signal the presence of DNA lesions to the DNA replication machinery and cell cycle checkpoints.


RAD50
MRE11
NBS1
MRN
Complex
SAM
RAD50
MRE11
NBS1
CHa
DNA Replication
DNA
Damage
CHa
CHa
MRE11
MRE11
Active MRN

5.6 Future directions in the role of arginine methylation of MRE11.

Although we have now identified a role for arginine methylation in modulating the DNA repair response through MRE11, many aspects of the role of arginine methylation in DNA damage still have to be addressed. First of all, the biochemical mechanism by which MRE11 methylated arginines contribute to the regulation of its enzymatic activity need further investigation. It has been reported that the activity of N-terminal nuclease domain alone of MRE11 in *Pyrococcus Furiosus* is equivalent to the full length protein (Hopfner et al., 2000a), indicating that the C-terminal that includes the methylated arginines does not contribute to the nuclease activity *per se*, but rather plays a regulatory role.

At the protein level, we have demonstrated that arginine methylation does not prevent formation of the MRN complex. However, it would be interesting to investigate the methylarginine-dependent interaction of the MRN complex with other components of the DNA damage response, and to determine how the methylated arginine signals DNA damage and the downstream cascades. The only known methylarginine-binding module is the tudor domain of SMN (Selenko et al., 2001). It is interesting to note that 53BP1, another protein involved in the early recognition of DNA breaks also contain a tudor domain, perhaps interacting with methylated MRE11. Nonetheless, finding a protein module whose interaction with MRE11 is methylarginine-dependent would certainly provide clues on the role of arginine methylation in the regulation of MRE11 activity and another example of a methylarginine binding domain.

At the cellular level, it will be crucial to elucidate the mechanism regulating MRE11 localization to different nuclear structures. We have found that a subset of MRE11 co-localizes with PRMT1 in PML nuclear bodies in primary fibroblasts, perhaps confirming a

role for PML nuclear bodies as a nuclear depot (Negorev and Maul, 2001). We have also demonstrated that inhibition prevents the recruitment of MRE11 to sites of DNA damage. It will be important to investigate the mechanism regulating MRE11 nuclear localization. Perhaps the methylated arginines contribute directly to the recognition of DNA breaks, or indirectly through interaction with a protein that can directly recognizes DNA lesions.

Finally, at the genetic level, it would be particularly interesting to determine the role of MRE11 methylated arginines in a whole organism. For example, generation of knock-in mice expressing mutated versions of MRE11 that can no longer be methylated could lead us to a better understanding of the physiological roles of that region. The DNA damage signaling and DNA replication checkpoints defects that we have described suggest that such a mice could recapitulate the ATLD phenotype, similar to what is found in human patients.

5.7 Concluding Remarks

The field of arginine methylation has been rapidly evolving in the last years, particularly following the cloning of the first methyltransferase, PRMT1, in 1996. A growing number of arginine methylated proteins have since been reported, implicating arginine methylation in a multitude of cellular functions. A complete identification of the substrates for each methyltransferase and characterization of their respective consensus target site will be essential for understanding and identification of new roles for arginine methylation. In the future, characterization of the roles of arginine methylation in these pathways will surely reveal many different aspects of cellular functions regulated by arginine methylation and should underscore the importance of this post-translational modification. More interestingly, it will be necessary to determine how arginine methyltransferase activity is regulated in the cell, and whether deregulation is associated with distinct cellular conditions, such as differentiation, cell cycle progression, viral infection, transformation or other type of diseases.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The present work has focused on the identification and the functional characterization of the role of arginine methylation in DNA repair. The exonuclease MRE11 was identified as an aDMA containing protein, and the role of arginine methylation on its function has been addressed. These studies have been published in peer-reviewed journals. The major contributions of this work to original knowledge are summarized below:

1 A proteomic study identifying over 200 previously unknown arginine methylated proteins.

2 The identification and characterization of the PRMT1 methylation sites in the DNA repair protein MRE11.

3 The characterization of the role of arginine methylation in regulating MRE11 exonuclease activity and DNA damage signaling properties.

4 The identification of PRMT1 as a component of PML nuclear bodies, where it co-localizes with MRE11.

5 The finding that arginine methylation regulates MRE11 recruitment to sites of DNA damage and its association with nuclear structures.

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Appendix

The appendix contains the following items:

- Permit for radioactivity granted to the Lady Davis Institute
- Permission to the student to use radioactivity
- Acknowledgement that the student followed the required course on radioactivity
- Permission to the student for the use of biohazard materials
- Copyright waiver from publishers allowing partial or complete reproduction of published material:
    - Chapter 1: Science STKE
    - Chapter 2: Molecular & Cellular Proteomics
    - Chapter 3: Genes & Development
    - Chapter 4: Cell Cycle

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April 13, 2005

To whom it may concern,

This letter confirms that François Michel Boisvert attended, and successfully passed, a Radiation Safety course at the Lady Davis Institute for Medical Research (LDI). On October 23, 2001 he received authorization to use radioisotopes and radiation-emitting devices governed by the license issued to the LDI by the Atomic Control Board. Therefore, he was authorized to use radioisotopes during the entire course of his doctoral research in the laboratory of Dr. Stéphane Richard.

Sincerely,

Andrew Mouland, Ph.D.
Radiation Safety Officer,
Lady Davis Institute for Medical Research

AM/mr

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Canadian Nuclear Safety Commission | Commission canadienne de sûreté nucléaire

04094-7-07.0

NUCLEAR SUBSTANCES AND RADIATION DEVICES LICENCE | PERMIS PORTANT SUR LES SUBSTANCES NUCLÉAIRES ET LES APPAREILS À RAYONNEMENT | Licence Number / Numéro de permis

I) LICENSEE

Pursuant to section 24(2) of the Nuclear Safety and Control Act, this licence is issued to:

Hôpital Général Juif/
Jewish General Hospital
Sir Mortimer B. Davis
3755 Côte Ste-Catherine Road
Montréal, QC
H3T 1E2
Canada

hereinafter «the licensee».

II) PERIOD

This licence is valid from: August 1 2002 to July 31 2007.

III) LICENSED ACTIVITIES

This licence authorizes the licensee to possess, transfer, import, export, use and store the nuclear substances and the prescribed equipment listed in section IV) of this licence.

This licence is issued for: laboratory studies: 10 or more laboratories where radioisotopes are used or handled (836)

IV) NUCLEAR SUBSTANCES AND PRESCRIBED EQUIPMENT

ITEM	NUCLEAR SUBSTANCE	UNSEALED SOURCE MAXIMUM QUANTITY	SEALED SOURCE MAXIMUM QUANTITY	EQUIPMENT MAKE AND MODEL
1	Carbon 14	400 MBq	n/a	n/a
2	Calcium 45	40 MBq	n/a	n/a
3	Cerium 141	100 MBq	n/a	n/a
4	Chromium 51	100 MBq	n/a	n/a
5	Iron 55	100 MBq	n/a	n/a
6	Iron 59	1 GBq	n/a	n/a
7	Hydrogen 3	2 GBq	n/a	n/a
8	Iodine 125	1 GBq	n/a	n/a
9	Phosphorus 32	5 GBq	n/a	n/a
10	Phosphorus 33	100 MBq	n/a	n/a
11	Sulfur 35	3 GBq	n/a	n/a
12	Scandium 46	100 MBq	n/a	n/a
13	Strontium 85	20 MBq	n/a	n/a
14	Cesium 137	n/a	40 kBq	n/a
15	Cesium 137	n/a	1480 kBq	Beckman LS (series)
16	Radium 226	n/a	370 kBq	PerkinElmer Wallac 1200 series LS Counters
17	Europium 152	n/a	740 kBq	PerkinElmer Wallac 1400 series LS Counters

The total quantity of an unsealed nuclear substance in possession shall not exceed the corresponding listed unsealed source maximum quantity. The total quantity of nuclear substance per sealed source shall not exceed its corresponding listed sealed source maximum quantity. Sealed sources shall only be used in the corresponding listed equipment.

V) LOCATION(S) OF LICENSED ACTIVITIES

used or stored at:

Lady Davis Institute for Medical Research
3755 Côte Ste-Catherine Road
Montréal, QC

VI) CONDITIONS

1. Prohibition of Human Use
This licence does not authorize the use of nuclear substances in or on human beings.
(2696-0)

2. Area Classification
The licensee shall classify each room, area or enclosure where more than one exemption quantity of an unsealed nuclear substance is used at a single time as:

(a) basic-level if the quantity does not exceed 5 ALI,
(b) intermediate-level if the quantity used does not exceed 50 ALI,
(c) high-level if the quantity does not exceed 500 ALI,
(d) containment-level if the quantity exceeds 500 ALI; or
(e) special purpose if approved in writing by the Commission or a person authorized by the Commission.

Except for the basic-level classification, the licensee shall not use unsealed nuclear substances in these rooms, areas or enclosures without written approval of the Commission or a person authorized by the Commission.
(2108-1)

3. Laboratory Lists
The licensee shall maintain a list of all areas, rooms and enclosures in which more than one exemption quantity of a nuclear substance is used or stored.
(2569-1)

4. Laboratory Procedures
The licensee shall post and keep posted, in a readily visible location in areas, rooms or enclosures where nuclear substances are handled, a radioisotope safety poster approved by the Commission or a person authorized by the Commission, which corresponds to the classification of the area, room or enclosure.
(2570-1)

5. Thyroid Monitoring
Every person who
(a) uses at a single time a quantity of volatile iodine-125 or iodine-131 exceeding;
(i) 5 MBq in an open room;
(ii) 50 MBq in a fume hood;
(iii) 500 MBq in a glove box;
(iv) any other quantity in other containment approved in writing by the Commission or a person authorized by the Commission; or
(b) is involved in a spill of greater than 5 MBq of volatile iodine-125 or iodine-131;
(c) or on whom iodine-125 or iodine-131 external contamination is detected; and shall, undergo thyroid screening within five days following the exposure to iodine-125 or iodine-131.
(2046-7)

6. Thyroid Screening
Screening for internal iodine-125 and iodine-131 shall be performed using:
(a) a direct measurement of the thyroid with an instrument that can detect 1 kBq of iodine-125 or iodine-131; or
(b) a bioassay procedure approved by the Commission or a person authorized by the Commission.
(2600-1)

7. Thyroid Bioassay
If thyroid screening detects more than 10 kBq of iodine-125 or iodine-131 in the thyroid, the licensee shall immediately make a preliminary report to the Commission or a person authorized by the Commission and have bioassay performed within 24 hours by a person licensed by the Commission to provide internal dosimetry.

(2601-4)

8. Extremity Dosimetry
The licensee shall ensure that any person who handles a container which contains more than 50 MBq of phosphorus 32, strontium 89, yttrium 90, samarium 153 or rhenium 186 wears a ring dosimeter. The dosimeters must be supplied and read by a dosimetry service licensed by the Commission.
(2578-0)

9. Contamination Criteria
The licensee shall ensure that for nuclear substances listed in the licence application guide table titled "Classification of Radionuclides";
(a) non-fixed contamination in all areas, rooms or enclosures where unsealed nuclear substances are used or stored does not exceed:
(i) 3 becquerels per square centimetre for all Class A radionuclides;
(ii) 30 becquerels per square centimetre for all Class B radionuclides; or
(iii) 300 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres; and
(b) non-fixed contamination in all other areas does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; or
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres.
(2642-2)

10. Decommissioning
The licensee shall ensure that prior to decommissioning any area, room or enclosure where the licensed activity has been conducted;
(a) the non-fixed contamination for nuclear substances listed in the licence application guide table titled "Classification of Radionuclides" does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; and
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres;
(b) the release of any area, room or enclosure containing fixed contamination, is approved in writing by the Commission or person authorized by the Commission;
(c) all nuclear substances and radiation devices have been transferred in accordance with the conditions of this licence; and
(d) all radiation warning signs have been removed or defaced.
(2571-2)

11. Storage
The licensee shall:
(a) ensure that when in storage radioactive nuclear substances or radiation devices are accessible only to persons authorized by the licensee;
(b) ensure that the dose rate at any occupied location outside the storage area, room or enclosure resulting from the substances or devices in storage does not exceed 2.5 microSv/h; and
(c) have measures in place to ensure that the dose limits in the Radiation Protection Regulations are not exceeded as a result of the substances or devices in storage.
(2575-0)

12. Disposal (Laboratories)
When disposing of unsealed nuclear substances to municipal garbage or sewer systems, the licensee shall ensure that the following limits are not exceeded:

COLUMN 1	COLUMN 2(a)	COLUMN 3(b)

Declaration By Radiation Users

The purchase and use of radioisotopes and of radiation-emitting devices in the Lady Davis Institute is governed by the license issued to the LDI by the Atomic Control Board. It is a condition of this license that every person designated as a Radiation User shall read and understand the relevant sections of the Radiation Safety Manual.

Department or Laboratory

Responsible User RICHARD JIS BOISVERT. ROOM 402.

Radiation Safety Monitors Dr. Robyn Schecter
Dr. Moulay Alaoui-Jamali

I hereby declare that I have read the sections of the Radiation Safety Manual relevant to my work, as listed against my name, and that I understand the meaning and implications of these sections. I further declare that, before signing my name, I have been given adequate opportunity to discuss and clarify the Radiation Safety Manual with my Radiation Safety Monitors in the field of radiation safety.

Signature

Date 23/10/2007

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