Control of mammalian DNA methylation system components by protein arginine methylation.

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Thesis submitted towards the degree of Doctor of Philosophy

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Abstract

DNA methylation is essential for the survival and development of vertebrates. Methylated cytosine in the context of CpG-dinucleotides within the genome is recognized by proteins from the methyl-CpG DNA binding domain (MBD) family. When bound to methyl-CpG DNA, most MBD proteins can recruit histone deacetylase (HDAC) silencing complexes to the site of chromatin to remodel its structure, and this causes transcription repression. Loss of CpG-DNA methylation results in embryonic lethality in mice, but loss of methyl-CpG DNA recognizing MBD proteins produce a viable phenotype.

Our interest in MBD proteins arises from our discovery that a subset of them interact with RNA. Two MBD proteins (MBD2 and MeCP2) bind their RNA partners using a domain that contains arginine and glycine (RG) rich motifs. As proteins with RG rich motifs are often substrates of post-translational modifications catalyzed by Protein Arginine Methyltransferase (PRMT), I asked whether the two MBD proteins are methylated at their arginines, and the consequences of such modification.

By *in vitro* and *in vivo* labeling assays, I ascertained that MBD2 and MeCP2 are substrates of PRMT, and that PRMT1 and PRMT5 are responsible for catalyzing different forms of methylation on the MBD2 protein. The relationship between the two PRMTs with regard to MBD2 methylation was characterized. Subsequently, I identified the significance of MBD2 arginine methylation. Biochemically, methylated species of MBD2 protein have less affinity for the HDAC silencing complex and methyl-CpG DNA. In cells, the loss in repression activity of arginine methylated MBD2 was demonstrated.

As MBD2 mediated repression activity can be relieved by arginine methylation, I propose that this mechanism might possibly explains the discrepancy between the phenotypes of CpG-DNA methylation null and MBD null mice. This study provides the first evidence that PRMT participate in the DNA methylation system of chromatin control.

Acknowledgements

Somebody once told me "every PhD is a unique experience". After completion of this thesis, I couldn't find a better sentence to describe my extraordinary adventure during the past few years. The few years of my life which I learned a little more about science and so much more about myself. Therefore, I would like to take this opportunity to acknowledge everybody around me who had contributed to make this thesis a reality.

First, I am grateful to my supervisor, Sara Nakielny, for giving me the chance to work in Cancer Research UK, which in my opinion is definitely a world-class institute. My second supervisor Frank Uhlmann, who is always ready to help, and never run out of ingenious ideas and solutions. Caroline Hill, my third supervisor whose extensive expertise in many different areas had always spurred me on to search for a better way to improve the quality of my data. I would also like to thank Patrick Pollard for the encouragement given when I first started this work, that had instil my faith in this study, and Saori Kitao, for the fruitful discussions over so many weekends and late evenings in the lab during the past few years. Not to forget Ava Yeo and the whole of LRI administration team, whose supports are so often unnoticed unless one runs into trouble.

Getting to this stage is never an easy journey, especially if one tries to do it with a sober mind and an empty stomach for twenty-four seven. Therefore, I would like to thank my best mate, Gerald Hammond, Stephanie Bohnert and Jonathan Leslie for the countless sessions of consuming liquid bread in the Ship or the George, and Petros Takousis for the dinner sessions. Thank you for lending me a listening ear.

For Tamara Cavanna and Grant Otto, I would like to give both of you a huge applause for gallantly stepping in to proofread this thesis. As for members of the Nuclear Transport Lab, it was enjoyable to be in the "Big Brother House" with you guys and I wish you more Golden Years.

Finally, and most importantly, I would like to tell my parents, and my sister that how indebted, I am, to them for believing and endorsing my interest in science. To my fiancée, Scilla Chua (now my wife) who is always there waiting patiently for me, supporting me and making me believe in myself, I want you to know that you have played the most important role during these years. This thesis is dedicated to my grandmother who passed away peacefully in 2006.

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Tan, C. P., and S. Nakielny. 2006. Control of the DNA methylation system component MBD2 by protein arginine methylation. **Mol Cell Biol** 26:7224-35.

Pichlmair, A., O. Schulz, **C. P. Tan**, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. **Science** 314:997-1001.

Abstracts

Tan, C. P., and Nakielny, S. 2004. Regulation of methyl-DNA binding domain (MBD) proteins by protein arginine methylation. FASEB Summer Research Conferences, Biological Methylation, August 2004, Vermont Academy Saxtons River, Vermont, USA.

Abbreviations

-/-	Homozygous deletion
5 Aza C	5-aza-cytidine
Adox	Adenosine, periodate oxidized or Adenosine-2′,3′-dialdehyde
Asym	Asymmetric (refers to arginine methylation)
BSA	Bovine serum albumin
CX	Cyclohexamide
CP	Chloramphenicol
DAPI	4,6-diamidino-2-methylindole
DBD	DNA binding domain
DMA	Dimethylarginine
DNMT	DNA methyltransferase
DTT	Dithiothreitol
ES cells	Embryonic stem cells
F/L	Full-length
HDAC	Histone deacetylase
HAT	Histone acetyltransferase
IP	Immuno-precipitation
k/MDa	Kilo Dalton, Mega Dalton
MBD	Methyl-CpG DNA binding domain
MBD proteins	Proteins
MBD2	Methyl-CpG DNA binding domain 2
MeCP2	Methyl-CpG DNA binding domain protein 2
Methyl-CpG	DNA in the context of CpG methylated on the cytosine
MMA	Monomethylarginine
Mta	5'-methyl-thioadenosine
MTA2	Metastasis associated protein 2
MTF	Mouse tail fibroblast
MW	Molecular weight markers
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethanesulfonyl fluoride
PRMT	Protein arginine methyltransferase
RbAp46/48	Retinoblastoma-binding protein 46/48
RG	Arginine and glycine
RLU	Relative luciferase unit
SAH	S-adenosylhomocysteine
SAM	*S*-(5′-Adenoysl)-L-methionine
SDS	Sodium dodecyl sulfate
Sym	Symmetric (refers to arginine methylation)
TRD	Transcription repression domain

Chapter 1 Introduction

1.1 Epigenetic control

Following the completion of Human Genome Project in 2003, scientists have the blueprint of every gene that constitutes a human being. However, this information is insufficient to decipher the mystery of how a single cell, upon fertilization, divides into different cells of specialized functions, expressing only a particular subset of genes in different space and time, and yet containing the same DNA sequence (191).

Changes in the phenotype of the cell/organism can be achieved without any alteration in the primary DNA sequence. This phenomenon is classified loosely under epigenetics, which was a term first introduced in the 1950s (155). To date, epigenetics has been used to describe the stable, heritable and yet reversible alterations in gene expression not involving mutations in the DNA sequence (92, 101).

Various epigenetic factors exist and most of them function interdependently to result in the phenotype of the cell. They include, DNA methylation, post-translation modification of core and variant histones, non-coding RNA and the overall structure of chromatin (147). While examples are aplenty, I focus on the epigenetic mechanisms in vertebrates that are related to this study.

1.2 CpG-DNA methylation

The symmetric methylation at the 5-position of the cytosine base of DNA in the context of CpG-dinucleotides is the most abundant covalent modification of the eukaryotic genome (79). In the human somatic cell, 70-80% of CpG-DNA are methylated (20, 61). Non-methylated CpG are generally located around transcription start site of genes (100, 192), particularly of those with tissue specific or house-keeping functions (8).

1.2.1 Heritability and reversibility of DNA methylation

When DNA sequences containing methylated CpG are integrated into the genome of cells, the epigenetic mark can be passed on for about 100 generations (202). The methyl-CpG landscape of the genome changes as totipotent embryonic stem cells

differentiate into tissue specific cells with specialized functions. Although this developmental process is considered irreversible as methyl-CpG patterns are stably inherited after differentiation, experimental manipulations have successfully reset the methyl-CpG pattern of terminally differentiated cells at specific promoters and render it more embryonic-like (51).

Using monoclonal antibodies against methyl-CpG and performing bisulphite sequencing of the genome, it is able to study how CpG methylation changes throughout the life cycle of an organism (152, 175). In mice, the genome wide demethylation of methyl-CpG DNA can occur twice. This demethylation occurs first in germ cell development when methyl-CpG of the haploid genome is demethylated and then remethylated at maturation (187). The second occurrence is after fertilization, when the paternal genome is rapidly demethylated and the maternal genome becomes passively demethylated after a few cell divisions (152). Methyl-CpG gradually reappears in the embryonic genome after the fifth cell division and increases until the end of blastula stage at which the amount of methyl-CpG remains stable (200).

The fact that paternal DNA in the mouse zygote undergoes active demethylation without DNA replication had spurred researchers on in the search for CpG-DNA demethylating enzymes. Although a strong candidate protein was reported as the possible enzyme (MBD2, see Chapter 1.4.2)(19), the report has yet been reproduced by a different laboratory to date (40).

1.2.2 DNA methyltransferases

While methyl-CpG DNA demethylating enzymes are not identified, advances have been made on enzymes that catalyze the methylation of CpG-DNA; these enzymes are named DNA methyltransferase (DNMT). The first mammalian DNMT was cloned in 1988 (17) and five DNMT with conserved catalytic domain have been characterized, they are: DNMT1, 2, 3a, 3b and 3L (18) (Figure 1-1).

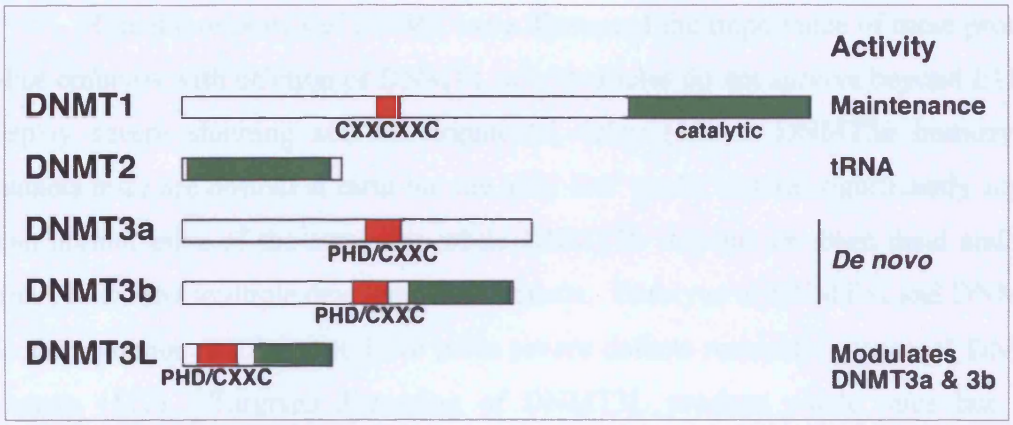

Figure 1-1 The DNMT protein family.

Five DNMT protein shares the conserved catalytic domain (Green). The PHD/CXXC (plant homeodomain-like) zinc finger domain (Red) and the activities of each DNMT are indicated. DNMT3L does not contain catalytic activity but is a co-factor of the DNMT3a and DNMT3b.

Even before their discovery, predicted activities of DNMT were categorized into maintenance or *de novo* DNA methylation (93, 190). Now, DNMT1 is regarded as the maintenance methyltransferase as it methylates cytosines on the newly synthesized DNA strand to duplicate the CpG-methylation pattern of the parental DNA strand in proliferating somatic cells (79, 136). Although proteolytically cleaved DNMT1 also displayed strong *de novo* DNA methyltransferase activity (18), absence of the enzyme in cells does not affect the ability of the genome to derive methyl-CpG from non-methylated double stranded DNA by *de novo* methylation (132).

In the search for genes encoding *de novo* methyltransferases in cells, DNMT3a and DNMT3b were identified (173). Cells lacking both enzymes fail to demonstrate *de novo* methylation activity against retroviral reporter DNA that is integrated into the genome (172). In embryos, the CpG-methylation ability of DNMT3a and DNMT3b depends on their interaction with a regulatory factor, DNMT3L (28). This protein shares a homology with DNMT3a and DNMT3b in the PHD zinc finger domain (1) but lacks conserved critical residues in the methyltransferase motif and has no DNA-methylation activity both *in vitro* and *in vivo* (84, 214) (Figure 1-1). Similar to DNMT3L, DNMT2 proteins does not to show any DNA methylation activity (174) and is recently identified to be a tRNA methyltransferase (80).

Genetic removals of DNMT have illustrated the importance of these proteins. Mice embryos with deletion of DNMT1 in both alleles do not survive beyond E11 and display severe stunning and developmental delay (138). DNMT3a homozygous mutants mice are normal at birth but die after few weeks and are significantly smaller than normal mice of the same age while DNMT3b mutants are born dead and their embryos exhibit multiple developmental defects. Embryos of DNMT3a and DNMT3b double mutation are found to have more severe defects resembling those of DNMT1 mutants (172). Targeted disruption of DNMT3L produce viable mice but adult homozygous males suffer from hypogonadism and homozygous females produce ova that cannot survive longer than 9.5 days (28).

Human diseases also arise from aberrant expression of DNMT genes. Reduced levels of DNMT1 in T-cells cause the autoimmune disease SLE (*Systemic lupus erythematosus*) and mutations of the DNMT3b gene result in the autosomal recessive disease ICF (*Immunodeficiency, centromeric instability and facial anomalies*) syndrome. In both instances, the cells affected display global or partial genome hypomethylation (191). Abnormal *de novo* methylation of CpG-DNA in the genome can be observed during establishment of primary cells in culture (101) and has been widely correlated to oncogenesis as many tumor suppressor genes are reported to be methylated in cancer cells.

Apart from disease, normal methylation of CpG-DNA in the mammalian genome is known to be essential for imprinting (137), transposon silencing (225), X chromosome dosage compensation (13) and chromosomal stability (60). The role of CpG-DNA methylation from the evolutionary standpoint has been argued to either serve as a host defense strategy against parasitic self-replicating DNA sequences (235) or to prevent the expression of irrelevant genes that would otherwise interfere with the agenda of the cell (21). Whichever theory is correct, they both still support the fact that methyl-CpG DNA mediates these phenomena by means of chromatin silencing.

1.3 Chromatin silencing mediated by DNA methylation

Methylation of CpG-DNA was correlated with transcription repression since the 1970s when methyl-CpG was found to be enriched in constitutive heterochromatin

(159). Studies of differential methylation of β-globulin genes from different chicken cell types revealed that DNA hypomethylation correlated with increased gene activity (186). The strongest clue provided then was observed in cells treated with 5-aza-cytidine; a potent DNA methylation inhibitor that triggered the reactivation of previously silenced genes in these mouse embryonic cells (104).

1.3.1 Factors repelled by methyl-CpG

The methyl-CpG mark on the genome can be interpreted by binding factors that are either repelled or attracted by it to regulate chromatin activity (115). CTCF protein is an example of a DNA binding factor that is sensitive to methyl-CpG. Genomic imprinting program of cells have instructed the expression of *H19* gene only from the maternally inherited chromosome and *Igf2* gene from the paternal. This specificity is caused by CpG-methylation of the paternally inherited chromosome at the particular locus while the same position on the maternal chromosome remains unmodified (Figure 1-2). Only the non-methylated locus of the maternal chromosome is able to recruit the CTCF protein that acts as an insulator to prevent the maternal *Igf2* promoter from gaining access to the transcriptional enhancers that are shared with the *H19* promoter. On the CpG-methylated paternal locus, CTCF is not present and the *Igf2* promoter interacts with the enhancers for transcription and thereby shutting down the expression of *H19* gene (134).

Direct occlusion of transcription machinery from reconstituted chromatin can also be observed when a single CpG base within the upstream control elements of the ribosomal RNA gene is methylated (199). Binding of transcription activators to promoters of neurofibromatosis gene *in vitro* are also inhibited by methylated CpG DNA (149).

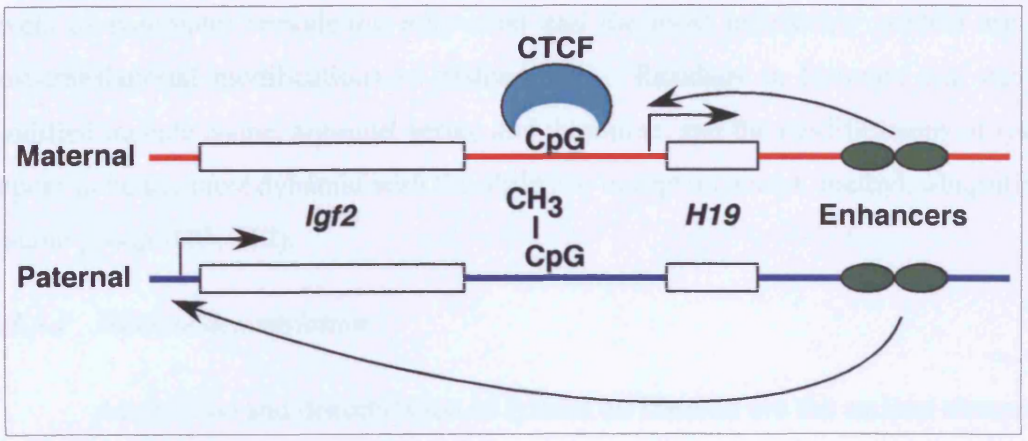

Figure 1-2 Regulation of gene expression by imprinting.

Differential methylation of paternal (Blue) and maternal (Red) locus result in the ability of CTCF protein to control the accessibility of enhancers (Green) to interact with promoters of imprinted genes (White boxes).

1.3.2 Factors attracted by methyl-CpG

Among the protein factors that are attracted to methyl-CpG DNA, the proteins from methyl-CpG DNA binding domain (MBD) family are the most thoroughly characterized. MBD proteins are the major research interest for this study and will be discussed in the following sections.

Insights gained from biochemical and mechanistic properties of the MBD had lead to the discovery of other transcription repressors, notably the Kaiso. First identified as an interacting partner of the human p120 catenin, Kaiso is recognized as a transcriptional repressor after its ability to bind methyl-CpG DNA, and repress the expression of reporter plasmids in cells (183). Mouse Kaiso protein is also capable of binding specific non-methylated DNA sequences (55) but this interaction has not been reported to mediate repression. Genetic removal of Kaiso in mice yields healthy phenotypes (184) but inhibiting the expression of this protein in frog embryos resulted in severe developmental defects and apoptosis (195). In HeLa cell, Kaiso interacts with components of the chromatin remodeling complex at the promoter of specific genes to mediate transcription repression (236).

1.3.3 Chromatin remodeling implicated with methyl-CpG

Evidence gathered from repressive factors attracted to methyl-CpG DNA point towards changes in chromatin structure during the silencing process. Various

levels of chromatin remodeling may exist and the most intensively studied are the post-translational modifications of histone tails. Residues in histones that can be modified include lysine, arginine, serine and threonine, and the modifications of lysine appear to be the most dynamic with the ability to accept an acetyl, methyl, ubiquitin or a sumo group (103, 212).

1.3.3.1 Histone deacetylation

Acetylation and deacetylation of lysines on histones are the earliest chromatin modifications linked with transcriptional control and this modification is considered a central switch regulating the interconversion between the permissive and repressive chromatin structure (223).

Early experiments using episomal chromosome, bearing methyl-CpGs in cells showed that addition of sodium butyrate, the histone deacetylase inhibitor, relieves methyl-CpG dependent transcriptional repression on the chromosome (96). Acetylation of the lysine residue on the N-terminal tail of the histone results in neutralization of the positive charge and this leads to weaker affinity of the histone to the negatively charged backbone of the DNA, thereby increasing the flexibility of the nucleosome structure. The presence of the acetyl group on the lysine residue and the increased chromatin flexibility facilitates the interaction of transcription factors and the outcome is positive transcription (109) (Figure 1-3).

Acetylation of lysine residue in the histone tail is carried out by histone acetyltransferases (HAT) and the antagonistic deacetylation reaction is carried out by histone deacetylases (HDAC). HAT are often associated with transcription activators while HDAC are found with repressors (168). Non-methylated CpG clustered around promoters of actively transcribed genes are associated with hyperacetylated histones and the local chromatin structure does not stain strongly with DAPI (4,6-diamidino-2-methylindole) (22) whereas regions of the chromosome enriched with methyl-CpG are transcriptionally silent and produce a bright DAPI staining pattern due to the chromatin structure. The size of acetylated or deacetylated regions of the chromatin can range from a single nucleosome to the entire chromosome as seen in X chromosome inactivation (119). The majority of methyl-CpG DNA dependent transcription repressors typically recruit HDACs to condense the site of chromatin and

make it inhospitable for RNA polymerase transcription (Figure 1-3). For example, Kaiso is found to interact with HDAC3 in HeLa cells (236).

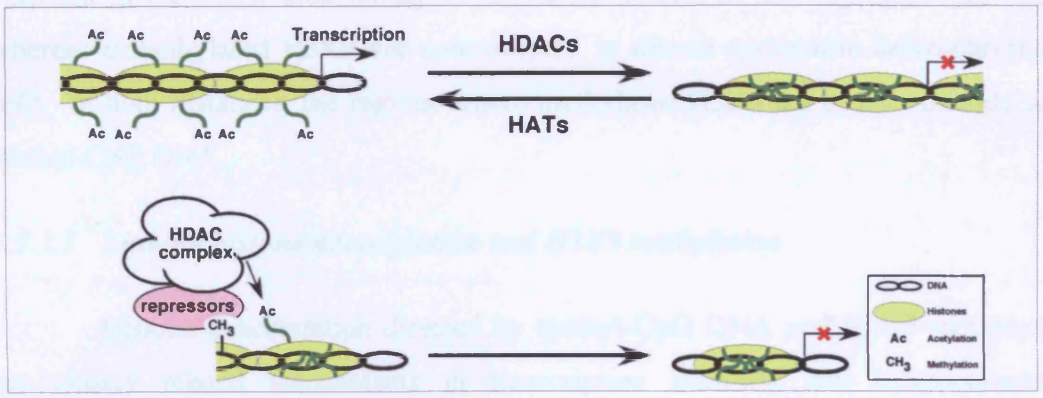

Figure 1-3 Actions of HDACs and HAT.

(Top) The interconversion between the acetylated (permissive) and deacetylated (repressive) chromatin structure by histone de/acetylases.

(Bottom) methyl-CpG DNA based repressors often recruit HDAC silencing complex to certain sites of chromatin and cause formation of transcriptionally silent chromatin.

1.3.3.2 Histone lysine methylation

Methylation of lysines on histone is another modification that is strongly linked with transcription regulation. Generally, methylation of lysine 4, lysine 36 and lysine 79 of histone H3 correlates with transcription activation while methylation of histone H3 lysine 9 (H3K9), lysine 27 and histone H4 lysine 20 is associated with heterochromatin and transcription repression (126). Notably, H3K9 is also the site for acetylation and effects of H3K9 methylation are in direct contrast with the transcriptional activation function of acetylation (156).

Specific lysines in histones can exist in three methylated states, mono, di and tri. The number of methyl groups on the lysine residue is believed to determine the transcriptional status (150). The diversity of histone lysine methylation can be further illustrated by the identification of different enzymes that catalyze the same modification. For example, methylation of H3K9 can be mediated by five known methyltransferases (Suv39h1 Suv39h2, G9A, Eset/SETDB1 and EuHMTase) (212). An enzyme that removes methyl groups from the methylated H3K9 was also recently characterized (118). The methylation signals on H3K9 are recognized by three isoforms of heterochromatin proteins (HP1α, β and γ) and this interaction is thought to

maintain the stable and heritable heterochromatin status (12, 122). Analysis of dimethylated H3K9 localizations in the genome shows that this modification is enriched in the inactivated female X chromosome and at the imprinted loci (181), whereas trimethylated H3K9 are concentrated in mouse pericentric heterochromatin (66). In both instances, the regions where methylated H3K9 are found coincide with methyl-CpG DNA.

1.3.3.3 Linking histone deacetylation and H3K9 methylation

Histone deacetylation directed by methyl-CpG DNA and H3K9 methylation are closely related mechanisms in transcription silencing and heterochromatin formation and both pathways show a certain degree of crosstalk. How one mechanism affects the other are still topics of intensive investigation, particularly in mammalian cells where various isoforms of DNMTs and histone methyltransferases exist to complicate the situation (212). H3K9 methylation is widely believed to be a prerequisite for CpG-DNA methylation as mouse cells derived from DNMT1 or DNMT3a/DNMT3b knockouts did not display mislocalization of trimethylated H3K9 whereas depleting SuV39h1 and SuV39h2, the enzymes responsible for H3K9 trimethylation at pericentric heterochromatin, results in loss of DNA methylation at the specific loci (131).

It is still unclear how particular regions of chromatin are recognized and tagged by these histone methyltransferases and DNA methyltransferases to result in the eventual transcription silencing. It is only certain that these processes are mediated by protein complexes acting together to perform their specific task. There are various examples that illustrate the interactions between DNMTs, HP1 and histone methyltransferases (75, 131). Furthermore, DNMTs are reported to interact with HDACs and this suggests that besides their catalytic role, DNMTs may also have non-enzymatic role in mediating stable transcription silencing and this adds on to the complexity (115).

Regardless of the factors involved, transcription repression mediated by methyl-CpG DNA is usually dependent on the collaboration between various cellular machineries that maintain the dynamic chromatin structure in the silent state (101). While the detail mechanistic insights of how transcription silencing by

heterochromatin formation is achieved, I look at the first clearest evidence of how methyl-CpG DNA leads to the changes in chromatin structure; using the MBD family (212).

1.4 Methyl-CpG DNA binding domain protein family

The first specific binding factor of symmetrically methylated double stranded CpG-DNA was identified in 1989 from gel filtration studies which indicated that a protein complex of 400 to 800 kDa was responsible, and it was assigned as MeCP1 (157). Repression activity of MeCP1 was subsequently demonstrated *in vitro* and *in vivo* (29).

Shortly after the discovery, a similar approach identified a *bona fide* methyl-CpG-DNA binding protein of 84kDa, and it was assigned as MeCP2. Antibodies raised against MeCP2 revealed that localizations of this protein coincide with the distribution of methyl-CpGs in mouse and rat genome (135). Using deletion assays, the minimal amino acid sequence within the MeCP2 that show preferential binding to methylated-CpG but not to non-methylated-CpG DNA was determined, and this sequence is defined as the methyl-CpG binding domain (MBD) (165). Further characterizations of MeCP2 also revealed a region of the protein that display independent transcription repression activity even when grafted onto irrelevant DNA binding protein and this region is named the transcription repression domain (TRD) (164).

By searching for sequences that are homologous to the MBD of MeCP2, additional members of the MBD family were identified. They are the MBD1 (53), MBD2, MBD3 and MBD4 (85) (Figure 1-4). The methyl-CpG DNA binding ability of these proteins were ascertained *in vitro* and MBD1, MBD2 and MBD4 are capable of binding DNA containing at least one methyl-CpG. The MBD3 protein is the only exception and it was illustrated to have different localization properties in the nucleus compared to MBD2, this is due to its inability to discriminate methyl-CpG from non-methyl-CpG DNA (85). Subsequent investigations revealed that inability of MBD3 in binding methyl-CpG varies from species to species; unlike the mammalian protein, MBD3 purified from *Xenopus* can bind methyl-CpG DNA. (224).

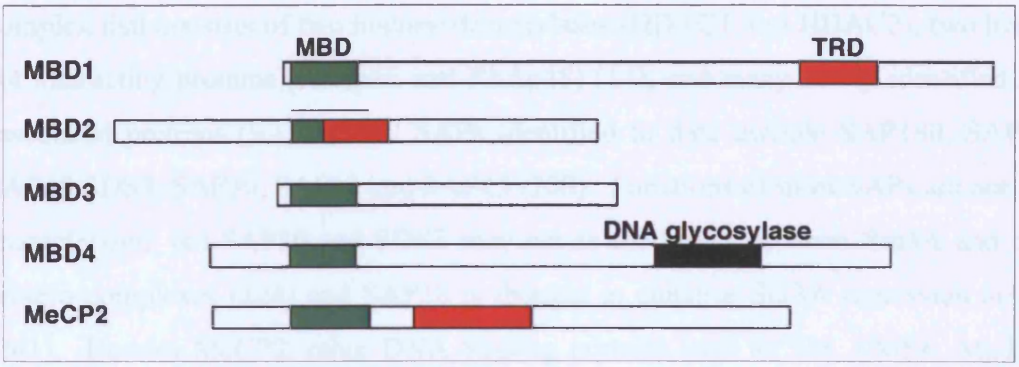

Figure 1-4 The mammalian methyl-DNA binding domain (MBD) protein family

The MBD (Green) of each protein shares both amino acid and functional homology. The transcription repression domain (TRD, Red) shares functional homology; it recruits histone deacetylase complexes to silence chromatin. The MBD of MBD2 overlaps the TRD and is indicated by a black line. The MBD of mammalian MBD3 has amino acid substitutions that do not allow it to recognize methylated DNA. MBD4 is a DNA glycosylase.

MeCP2 and MBD1-4 now form the founding members of the MBD family and more refined searches in the database had identified at least six novel members. Of which, three proteins had their MBD functionally verified (193). The MBD described here, together with their splice variants, illustrate the diversity of members within this family (10).

1.4.1 MeCP2

The first evidence of an MBD protein repressing transcription by chromatin remodeling comes from the study of MeCP2. In 1998, two laboratories reported the co-purification of MeCP2 with Sin3A protein in mammalian nuclear extracts (166) and *Xenopus* oocytes (105) (Figure 1-5). This interaction is mapped to the TRD described previously (164) and the purified MeCP2-Sin3A complex are found to posses histone deacetylase activity against synthetically acetylated histone peptide *in vitro*. In cells, the transcription repression activity of MeCP2 can be relieved by the presence of histone deacetylase inhibitor (105, 166).

Sin3A is a large multidomain protein that is postulated to be the scaffolding for assembly of other repressor complexes (119). Nucleosome remodeling factors involved with mouse Sin3A *in vivo* are: the core components of HDAC silencing

complex that consists of two histone deacetylases (HDAC1 and HDAC2), two histone H4 interacting proteins (RbAp46 and RbAp48) (14), and many newly identified Sin3 associated proteins (SAP) (58). SAPs identified to date include SAP180, SAP130, SAP45/SDS3, SAP30, SAP18 and SAP25 (208). Functions of most SAPs are not fully characterized, but SAP30 and SDS3 may act as the linker between Sin3A and other protein complexes (124) and SAP18 is thought to enhance Sin3A repression activity (241). Besides MeCP2, other DNA binding proteins such as Ski, UME6, Mad/Mnt and NCoR/SMRT also target Sin3A complex to chromatin (119).

1.4.1.1 Novel interacting partners of MeCP2

More recent biochemical studies of MeCP2 illustrate that the protein is not an exclusive and stable partner of Sin3A-HDAC silencing complexes but is involved in numerous chromatin remodeling machineries (116). The TRD of MeCP2 has been proposed to target DNMT1 in place of Sin3A complex to hemi-methylated CpG-DNA to perform maintenance CpG-DNA methylation (113). MeCP2 also co-purifies with DNMT1 and SUV39H1, a H3K9 methyltransferase. The interaction of this MeCP2/DNMT1/SUV39H1 complex with another corepressor complex, CoREST, is thought to reinforce and propagate the state of silent chromatin (76, 144). A recent model also implicates the MeCP2-Sin3A as part of a larger chromatin remodeling complex, the SWI/SNF. The mechanism by which this complex represses transcription is still unresolved (83).

1.4.2 MBD2 and MBD3

The MBD2 and MBD3 protein are the only two proteins from the MBD family that have homologues in organisms lower than vertebrates. Therefore, they are thought to be the ancestral proteins of the family. Genes coding for both proteins have identical genomic structure differing only in the size of their introns. Thus, both proteins might arise from a single gene duplication event (88).

The first characterization of the MBD2 coding sequence revealed an internal methionine codon that might be use as an alternative translation initiation site for a

shorter isoform of MBD2, this postulated isoform is termed MBD2b (85)[1]. In a subsequent report, an antibody was developed to reveal the presence of both MBD2 and MBD2b proteins in HeLa nuclear extract (170); another recently developed antibody suggested that both proteins might exist in a 1:1 ratio [2].

Biochemical analysis revealed that MBD2 is the specificity determinant for methyl-CpG DNA in the previously identified MeCP1 complex, which also contains histone deacetylase activity for its transcription repression (170). Purification of the endogenous MeCP1 complex in HeLa cells showed that it consists of MBD2 interacting with the nucleosome remodeling and deacetylation (NuRD) complex to form the 1 MDa MeCP1 (65)(see Chapter 1.4 and (157)).

Unlike the MeCP2/Sin3A, the molecular interaction between MBD2/NuRD is relatively more stable *in vivo* with majority of the MBD2 existing in the complex (65). However, similar to MeCP2/Sin3A, the MBD2/NuRD complex also contains the core components of HDAC silencing complex consisting of HDAC1, HDAC2, RbAp46 and RbAp48 (Figure 1-5). Proteins unique to the NuRD include the Mi2 (an ATP dependent helicase activity) (65), MTA2 (a modulator of HDAC activities) (242) and two isoforms of a zinc finger protein p66 and p68 that are speculated to be essential for proper targeting of the NuRD complex to the genome (64). Interestingly, the mammalian MBD3, which does not bind methyl-CpG DNA, is an integral component of the NuRD (242) and co-exists with MBD2 in the MeCP1 (219). However, a recent report that challenged the findings of the molecular interaction between MBD2 and MBD3 might redraw the position of MBD2 within the MeCP1 complex if positively verified (125). *Xenopus*, MBD3 protein has specificity for methyl-CpG DNA and plays a more active role in the organism to direct the NuRD complex to methyl-CpG DNA (224).

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[1] For comparison of MBD2 and MBD2b, see Figure 4-4.
[2] Please refer to Sigma , Anti-MBD2a,b (RA-18), Product Number M 7318.

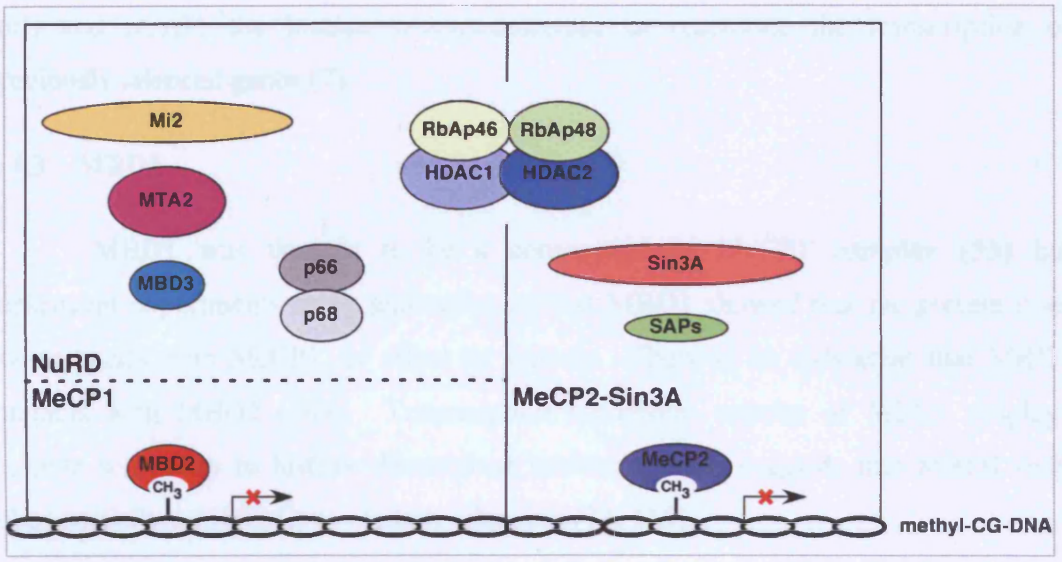

Figure 1-5 Components of chromatin remodeling complex on methyl-CpG DNA.

MeCP1 and MeCP2 HDAC silencing complexes share the core component of histone deacetylation (Middle). Well-characterized members of both complexes are shown, and MBD2 and MeCP2 target these protein complexes to methylated DNA to mediate transcription repression. MeCP1 complex consist of NuRD binding to MBD2.

While the majority of MBD2 are involved with NuRD, only a small fraction of cellular NuRD binds MBD2 (65). Other DNA binding factors that interact with the NuRD complex include the *Drosophila* hunchback protein and the mammalian Ikaros and Aiolos, which are all zinc finger protein (2).

1.4.2.1 Novel interacting partners of MBD2

Different from studies of endogenous MBD2 proteins, transiently overexpressed MBD2 in cells showed that the protein interacts with other chromatin remodeling factors. MBD2b, a predicted isoform of MBD2 that lacks the N-terminus[3] (85) is found to interact with the Sin3A complex via its TRD (23) (Figure 1-4). A novel MBD2 interacting partner MIZF (MBD2 interacting zinc finger) binds MBD2b and this interaction is postulated to direct sequence specificity in recognizing methyl-CpG DNA (206). Several groups illustrated that MBD2 can function as a transcription activator by its interaction with proteins such as the RNA helicase A (73) and the MBD*in*, a novel protein identified by its interaction with MBD2 (133). Notably, a recent finding indicates that MBD2 interacts with TACC3 (transforming-acid-coiled–

[3] For the map of MBD2b, refer to Figure 4-4.

coil) and pCAF, the histone acetyltransferase to reactivate the transcription of previously silenced genes (7).

1.4.3 MBD1

MBD1 was thought to be a component of MeCP1 complex (53) but subsequent experiments using antibodies against MBD1 showed that the protein does not co-purify with MeCP1, or affect its activity. There is no indication that MBD1 interacts with MBD2 (169). Transcription repression activity of MBD1 displays variable sensitivity to histone deacetylase inhibitor. This suggests that MBD1 only relies partially on HDACs to mediate silencing (73, 169).

MBD1 is the only member of the MBD family that contains the cysteine rich (CXXC) motif found in DNA methyltransferase (DNMT1) and histone methyltransferase (MLL) (88) (Figure 1-1). The numbers of CXXC motifs present depends on the isoforms of the protein, and the motif directs sequence specificity. In some mammalian MBD1 isoforms, three CXXC are present and these enable the *in vivo* repression of reporter plasmids containing non-methylated CpG (74, 107).

Similar to MeCP2, MBD1 is reported to form a stable complex with another H3K9 methyltransferase, SETDB1. This association is found to direct histone lysine methylation to methyl-CpG DNA at specific genes throughout the cell cycle. During DNA synthesis, the MBD1-SETDB1 complex transiently interacts with chromatin assembly factor, the CAF1, which is bound to the replication fork machinery. This interaction is thought to target the MBD1-SETDB1 to methyl-CpG DNA to form stable heterochromatin (201).

1.4.4 MBD4

MBD4 is the odd member of the MBD family as transcription repression activity remained undiscovered for many years. Instead, MBD4 is considered as a DNA repair enzyme. This is because, although the protein recognizes double stranded methyl-CpG DNA, it displayed a stronger preference for double stranded methyl-CpG DNA when the methyl-cytosine on the complementary strand is deaminated to thymine. Furthermore, a DNA glycosylase domain is identified at the C-terminus of

the MBD4, providing the ability to remove thymidine or uracil bases from damaged DNA strands (87) (see Figure 1-4).

As demaination of methyl-cytosine to thymine is the most common mutagenic base transition on the DNA (215), the repair function of MBD4 is much sought after because it represents a stabilizing agent of the genome. However, loss of MBD4 in mice that are deficient in mismatch repair activity does not increase tumor onset. This downplays the significance of this protein in defense of the genome against mutation (198).

The recently discovered transcription repression activity of MBD4 demonstrated that the central region of the protein interacts with Sin3A and HDAC1 (120).

1.5 Relevance of MBD to mice

In comparison to the embryonic lethality, transposon reactivation and loss of imprinting observed in DNMT knockout mice (79), mice with deletion of genes encoding for the MBD proteins generally produced viable and subtle phenotypes.

Loss of MBD in mice typically affects brain functions. This is best exemplified by MeCP2 knockout mice that display dysfunctional motor neurons and neurological symptoms after birth. These symptoms are similar to Rett's syndrome, a human neurodevelopmental disorder (38, 82). Rett's syndrome almost exclusively affects female patients and this correlates with the fact that MeCP2 is an X-linked gene. Male MeCP2 mutant mice can only be generated by conditional knockout as deletion of the only copy of the gene in the X chromosome resulted in embryonic lethality (218). The majority of Rett's patients are found to have mutations in the gene coding for MeCP2 and the mutations are commonly found within coding sequence for MBD and TRD (4) (Figure 1-4). As MeCP2 is most abundant in brain (164), failure to repress superfluous gene expression in the organ might be a plausible explanation (229).

Adult mice with deletion of the MBD1 have a smaller hippocampus, a region where the protein is highly expressed. Neuronal stem cells cultured from these mice

show a decreased potential in neuronal differentiation and increased genomic instability. Consistent with these findings, these MBD1 knockout mice exhibit spatial learning difficulties (243).

MBD2 homozygous deletion mice are viable and healthy except for the fact that the females have behavioral defects in nurturing their pups (86). A slightly more distinct phenotype involving MBD2 mutants was reported in a subsequent study in which these mice were crossed with intestinal tumor prone mice; it was found that the lifespan of their offspring increases when gene dosage of MBD2 decreases. This suggests that MBD2 might play a role in tumorigenesis (197).

The only lethal phenotype that is caused by the deletion of a MBD family member comes from mice with homozygous MBD3 deletion; embryos of these mice were retarded and were reabsorbed at E8.5. This demonstrates the importance of the MBD3 protein in embryonic development (86). Since the mammalian MBD3 does not bind methyl-CpG DNA (85), the severity of this mutation cannot be considered as the loss of factors that specifically recognize methyl-CpG DNA. This mutation most probably reflects on the loss of activity of the NuRD complex as targeted deletions of the other integral chromatin remodeling complex components produced similar embryonic lethal phenotypes (52, 123).

Studies of MBD4 gene deletion in mice by various groups produced inconclusive evidence of whether the loss of the DNA mismatch repair protein increases tumorigenesis or genomic instability in mice. However, all reports agreed on the observed rise in cytosine to thymine base transition (158, 198, 230). Investigations of these MBD4 knockout mice did not include the characterization of the behavioral or neurological aspects of this mutation.

1.5.1 Are MeCP2 and MBD2 functionally redundant?

The mild phenotypes of these MBD mutants compared to the DNMT mutants lead to the questions of whether the diverse members from MBD family play important roles in gene regulation. Double deletion mutant mice of MBD2/MeCP2 do not have altered survival or disease onset (82). This suggests that the proteins might not have a central role in controlling developmental gene expression. In the MeCP2

and MBD2 homozygous mutant mice, X chromosome inactivation and genomic imprinting appear normal (82, 86, 155). This could be due to the fact that DNMTs themselves posses alternative function such as targeting of chromatin remodeling complexes[4] (115), and the existence of MBD proteins are for fine-tuning of transcription repression, or that these MBD proteins are functionally redundant (88).

1.5.1.1 Genes controlled by MeCP2

Early biochemical characterization suggested that these proteins function as global transcriptional repressors (105, 166, 170) but microarray analysis of the general transcriptional profile from brain tissue of MeCP2 mutant mice affected by Rett's syndrome did not reveal the much anticipated genome wide gene misregulation. In fact, only slight changes were found when the transcription profiles of these mice were compared to wild-type mice (221). Therefore, approaches that are more precise were employed to identify the particular subset of genes controlled by the MBD proteins.

Using chromatin immunoprecipitation (ChIP), MeCP2 is found on the promoter of DLX5, an imprinted gene that is only expressed from the maternal allele. In MeCP2 null mice, it is found that the imprinting phenomenon is absent at the DLX5 locus and a two-fold increase in the transcripts was observed. Derepression of only one allele should not cause drastic changes in expression levels. Thus, a modest increase is expected (95). On the other hand, imprinted genes such as UBE3A and GABRB3 were found to have decrease expression in MeCP2 deficient cells but the changes are not linked to misregulation of imprinting (196).

1.5.1.2 Genes controlled by MBD2

MBD2 control of gene expression is probably crucial during cellular developmental stages. During T cell maturation, naive T-helper cells have the potential to differentiate into interferon-γ producing Th1 and interleukin-4 producing Th2 cells. T cells from MBD2 -/- mice express these cytokines at aberrant levels and this results in a difference in susceptibility to pathogens compared to wild-type mice (98). Although genes that control the lineage specificity of these T cells were found to

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[4] Refer to Chapter 1.3.3.3.

be appropriately repressed in the MBD2 mutant mice, ChIP analysis had identified that the MBD2 interact with the transcriptionally silent promoter of interleukin-4 and production of the cytokine was only possible when MBD2 is displaced by a transactivator protein, GATA3 (99). The data suggest that MBD2 might have a subtle role in controlling specific cellular development. Another study that supports this hypothesis uses MBD2 -/- mice to demonstrate the indirect overexpression of δ-globulin gene, which was otherwise specifically controlled in erythrocyte development (194).

Various other ChIP investigations had also located MBD2 on the promoter of genes such as GSTP1 (140), p16/INK4A and p14/ARF, which are all implicated with cancer (146). In all cases, treatment of cells with DNA methylation inhibitor and histone deacetylation inhibitor reverses the MBD2-mediated repression.

1.5.1.3 Genes controlled by various MBD proteins

In some instances, different members of the MBD family can be found on the same locus on methylated DNA. Both MeCP2 and MBD2 bind the promoter of MDR1, a gene that encodes a protein that confers drug resistance to cancer cells (57). The promoter of metallothionein stress response genes has been associated with MBD2, MBD4, MBD1 and MeCP2, while the former two proteins bind and mediate repression only when the promoter is CpG-methylated, the latter two interact and repress irrespective of the CpG- methylation status (148).

1.5.2 What are the specificity of MeCP2 and MBD2

Initial attempts to characterize the MBD using *in vitro* binding assay or immunofluorescence to locate the proteins on the genome were not sensitive enough to conclude whether these proteins have different sequence recognition specificity (85). However, more advanced experimental data accumulated over the years have suggested that these MBD are unevenly distributed along the genome and tend to cluster along precise chromosomal loci (10).

While most research concentrates on genomic screening and ChIPs to identify MBD target sequences, the direct characterization of MBD methyl-CpG DNA binding properties increases the understanding of these proteins tremendously. Measurement

of the MBD association with methyl-CpG DNA shows that each member of the family has a different affinity towards the same sequence of methylated DNA and that the spacing between methyl-CpGs along the DNA is critical for the interaction (67). A more recent investigation on the binding selectivity of these protein illustrated that the efficiency of binding of MeCP2 to methyl-CpG is dependent on the presence of an adenine or thymine adjacent to the CpG sequence. Genetic depletion of MeCP2 enables MBD2 to occupy its binding sites on methyl-CpG DNA but not vice versa (117). This supports the theory that MBD2 is the ancestral protein of the MBD family, possessing wider substrate specificity compared to the newly evolved MBD proteins that have more specialized functions (88).

Other than stably facilitating interactions between chromatin modifying complexes and methyl-CpG DNA, MBD proteins themselves can be governed by the dynamic equilibrium of cellular signaling to fine tune gene expression (88). The clearest example to date is shown by experiments in cultured mammalian neurons, where MeCP2 binds the promoter of bone-derived neurotrophic factor (BDNF) gene and represses transcription. When the cells are treated with a membrane depolarizing agent, MeCP2 becomes phosphorylated and lose its affinity for methyl-CpGs, resulting in BDNF expression (39, 151). Since then, the role of BDNF in the pathogenesis of Rett syndrome has been positively verified (36).

Therefore, it is only through the careful characterization of the molecular properties of MBD proteins and investigations of how they control their target genes that we can understand the phenotypic differences between DNMTs and MBDs deletion mice, and how these systems of chromatin control work together to govern the overall cellular gene expression program.

1.6 Aim of this study

Our laboratory's interest in studying the components of chromatin control comes from our recent discovery that a subset of DNMT and MBD proteins has intrinsic RNA binding properties (102). While the functional significance of MBD2-RNA interaction is not known, MeCP2 is shown to modulate alternative splicing of

reporter genes through its RNA dependent interaction with the Y-box binding protein, YB1 (238).

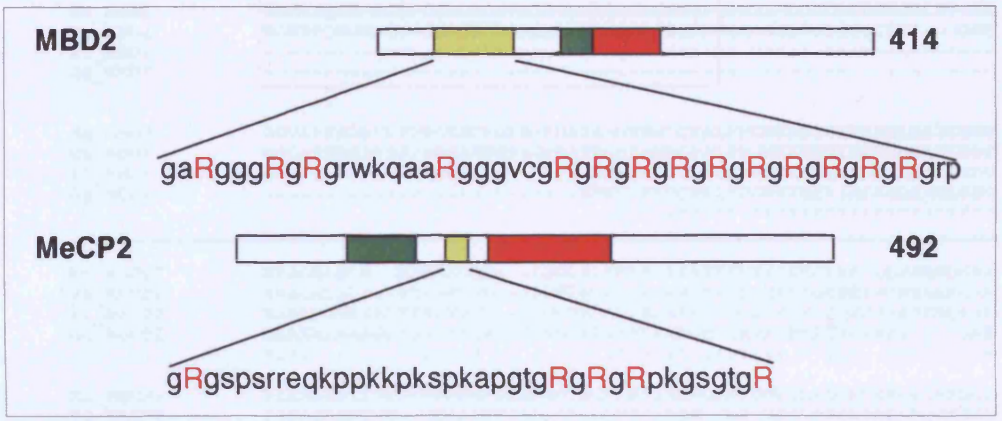

Figure 1-6 MBD2 and MeCP2 contain RG domains.

The RG domain of both proteins are illustrated (Yellow box) and the possible R are in red.

Within the MBD proteins, the MBD2 and MeCP2 bind RNA with the highest affinity and the physiological RNA target of MBD2 has been identified. It was found that MBD2 interacts with 7SK non-coding RNA in cells and that the interaction is dependent on the arginine and glycine (RG) rich domain within the MBD2 (114). While the RNA binding domain for MeCP2 was not fully characterized by Jeffery & Nakielny (102), it is highly postulated to be located in a similar RG domain found in the protein (Figure 1-6).

Although the RG domain of MeCP2 is less extensive than that of MBD2 (Figure 1-6), compared to the former, MeCP2 seemed to have a more conserved RG domain across species (Figure 1-7). Therefore, it would be interesting to characterize the functions of the RG domain of these two proteins and possibly address whether the RG domains of MBD proteins were acquired, or were lost during evolution. This may in turn unravel novel functions of MeCP2 in the pathology of Rett's syndrome.

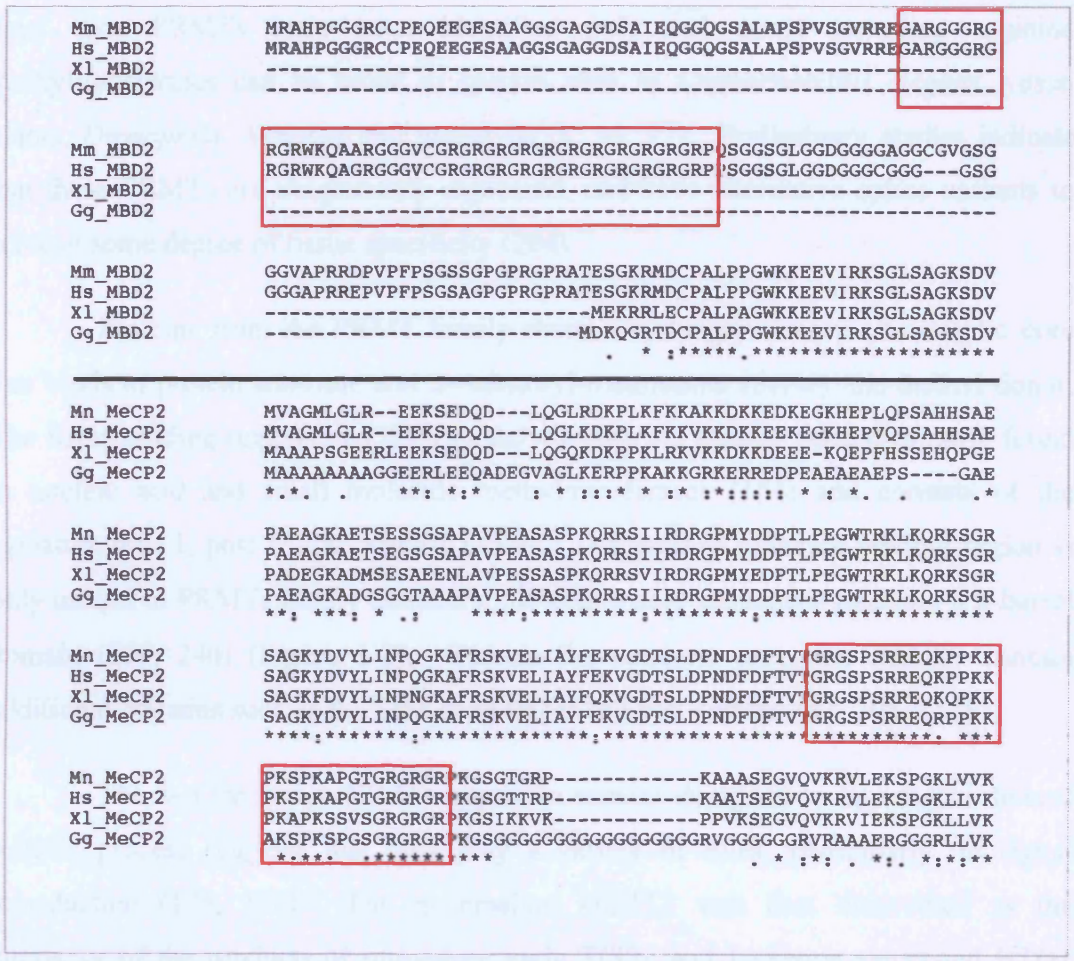

Figure 1-7 Comparison of N-terminus halves of MBD2 (residue 1-180) and MeCP2 (residue 1-240) sequences across different species. The RG domain indicated in Figure 1-6 is highlighted by the Red box.

RNA binding proteins with RG repeats are often targets of a novel post-translational modification catalyzed by a family of enzymes termed the protein arginine methyltransferases (PRMTs) (126, 141, 163), I test whether the arginines in the RG domain of MBD2 and MeCP2 can be post-translationally modified (see Figure 1-6). Moreover, I investigate which enzymes might catalyze such post-translational modifications and explore the biological consequences of these modifications at molecular and cellular level.

1.7 The PRMT family

Although it was established almost 40 years ago that arginine residues within proteins can be post-translationally modified with methyl groups (176), the enzymes that catalyze the modifications have only recently been identified (78, 89, 139). To

date, nine PRMTs have been identified (47) and genes encoding arginine methyltransferases can be found in species such as *Caenorhabditis elegans*, yeast, plants, *Drosophila*, *Xenopus* and mammals (9, 16, 27). Preliminary studies indicate that these PRMTs are ubiquitously expressed, and have alternative splice variants to achieve some degree of tissue specificity (204).

Proteins from the PRMT family share a strikingly conserved catalytic core that binds to protein substrate and S-Adenosyl-methionine (SAM), the methyl donor. The SAM binding regions of PRMTs catalytic core are closely related to those found in nucleic acid and small molecule methyltransferases (154) and consists of the signature post I, post II and post III motifs. The protein substrate-binding region is only unique to PRMTs family members, the amino acid sequences fold into a β-barrel domain (239, 240) (Figure 1-8). Outside the catalytic core, the PRMTs contain additional domains such as the SH3, zinc finger or Fbox domain (47, 205, 217).

The fact that most PRMTs appear in screens designed to investigate diverse cellular process suggests that they play a variety of roles, particularly, in signal transduction (126, 154). The mammalian PRMT1 was first discovered as the interactor of the products of immediate early TIS21 and leukemia-associated BTG1 gene, both of which modulate PRMT1 activity (139). PRMT4 is found in a complex with GRIP1, the p160 steroid receptor coactivator and therefore the enzyme is also termed the co-activator associated arginine methyltransferase (CARM1) (37). PRMT5, also known as JBP1 (Janus kinase binding protein 1) was initially identified as the interacting partner of Janus kinase (182) while PRMT7 was isolated in a genetic screen enriching for suppressor elements which confer resistance against cytotoxic agents (81). Subjecting the highly conserved PRMT catalytic core to homology searches has led to the identification of PRMT2, PRMT6, PRMT8 and PRMT9 (47, 69, 128, 205) and PRMT3 was uniquely identified by its interaction with PRMT1 (217).

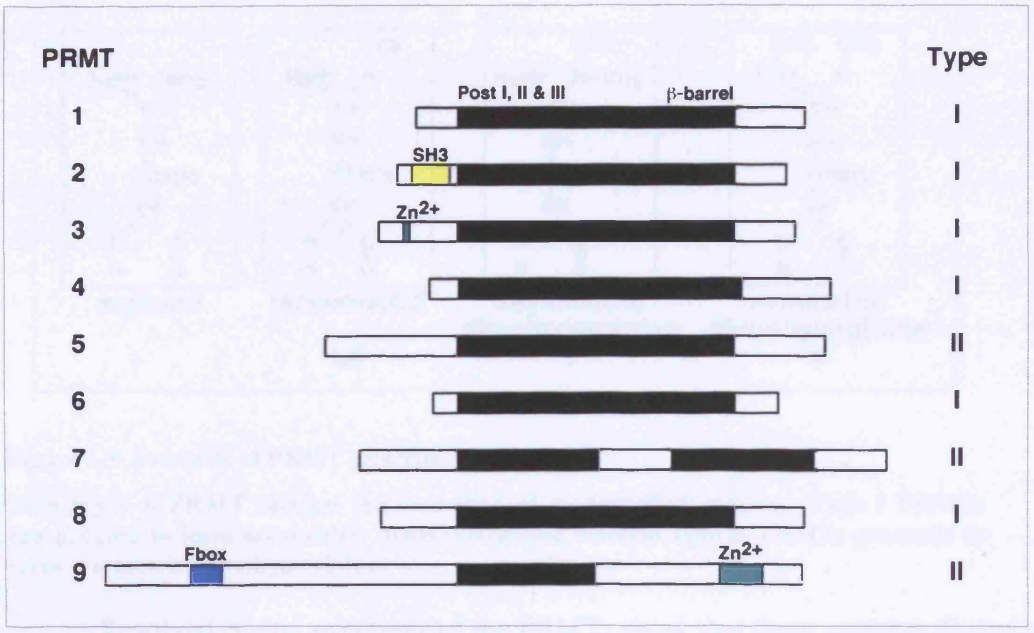

Figure 1-8 The protein arginine methyltransferase (PRMT) family.

A family of nine enzymes that catalyze methylation of arginines in proteins and contains a conserved catalytic domain (Black box) has been characterized. Their catalytic activity can be classified as type I or type II. Sequences outside the catalytic domain are unique to PRMT and may contain additional SH3 (Yellow), zinc finger (Green) and Fbox (Blue) domain.

1.7.1 Catalytic activity of PRMTs

Similar to lysine residues, there are three levels at which arginine residues can be methylated (see Chapter 1.3.3.2). The PRMTs are classified into two groups according to their catalytic end products on the target arginine residues. Both type I and type II PRMTs catalyze the modification of guanidino nitrogen atom on arginine residues to form a N^G-monomethylarginine, but type I PRMT proceeds with a further reaction to generate an N^G,N^G asymmetric dimethylarginine, and type II PRMT produces N^G,N'^G symmetric dimethylarginine (Figure 1-9). PRMT1, PRMT3, PRMT4, PRMT6 and PRMT8 are classified under type I PRMT and PRMT5, PRMT7 and PRMT9 belong to the type II grouping. No activity has yet been reported for PRMT2 (16, 126, 154) (Figure 1-8).

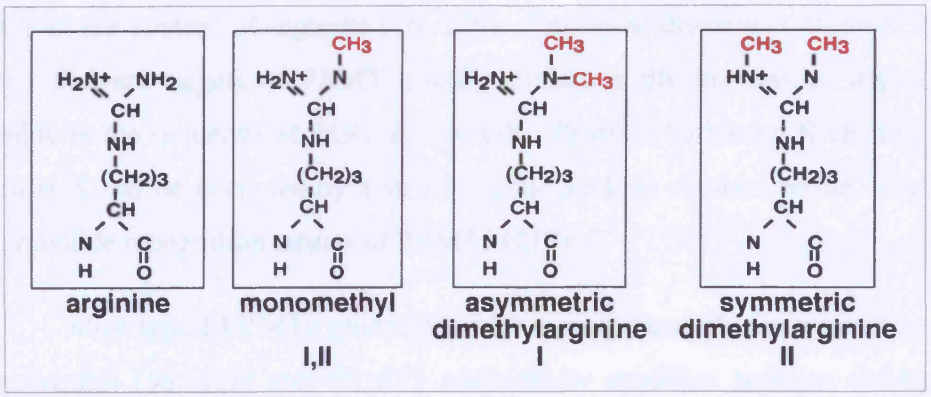

Figure 1-9 Products of PRMT catalysis.

Both types of PRMT catalyze the formation of monomethylarginine. Type I PRMTs can proceed to form asymmetric dimethylarginine whereas type II PRMTs proceeds to form symmetric dimethylarginine.

Resolved crystal structures of the PRMTs show that these proteins dimerize to become catalytically active (228). Deletion of the region in PRMT1 responsible for its dimerization resulted in its inability to bind SAM, which abolished the enzymatic activity (239, 240). The ability of PRMT1 to interact with PRMT3 and PRMT8 might also be attributed by the dimerization of the enzymes for active complex formation although further investigations are required to confirm this (128, 217).

Biochemical characterization of PRMT5 also indicates that the enzyme exists as homo-oligomers in cells and that the association is mediated by disulphite and non-covalent interactions. In sucrose density gradients, oligomers of PRMT5 co-migrate with its methyltransferase activity (188). This theory is further supported by the identification of two catalytic regions found in PRMT7, both of which are essential for its enzymatic activity (161) (Figure 1-8).

Dimerizations of the PRMTs are also believed to facilitate the formation of dimethylarginine end products. Monomethylated arginine substrates are postulated to transfer from the active site of one monomer directly into the active site of another without dissociation from the active catalytic complex (126).

1.7.2 Peptide motifs recognized by PRMTs

Despite the high degree of amino acid and structural conservation in the catalytic core, members of the family can recognize and modify different amino acid

motifs in the context of arginine (16, 126). The most distinctive arginine containing motifs that are targets of PRMT usually contain a glycine residue adjacent to the arginine in the sequence of RGG, RG or GR. Proteins harboring RXR motifs, where position X can be occupied by a small residue such as alanine, serine, or proline are also possible recognition targets of PRMTs (210).

Most type I PRMTs generally prefer arginine and glycine containing peptides as substrates (16, 128) and PRMT3 additionally modifies arginine residues within RXR motifs (24, 210). Currently, PRMT4 is the only exception in type I PRMT showing a higher degree of specificity. PRMT4 does not display affinity for RG containing sequences in proteomic screens. Alignment of peptide sequences methylated by PRMT4 has derived a loose consensus motif of RPAAPR (127). The crystal structure of the PRMT1 substrate binding domain revealed three peptide binding channels, possibly explaining the ability of a single PRMT protein to recognize different motifs on their substrates (126, 239).

For type II PRMTs, both PRMT5 and PRMT7 display efficient methylation for substrates containing a stretch of (RG) repeats but these sequences are not modified by PRMT9 (47, 129). Interestingly, while arginines in RG repeat sequences can be mono, asymmetrically and symmetrically methylated, arginines in RGG repeat are only reported to be mono and asymmetrically methylated (154). However, current data relies heavily on *in vitro* assays to determine the types of modifications and we await *in vivo* findings. With the increase in numbers of physiological PRMT modification reported, examples of RGG repeat being symmetrically modified may appear (153).

1.7.3 Reversibility of arginine methylation

One important feature of the regulation of cellular processes via post-translational modifications is that these changes are dynamic and reversible (e.g. de/acetylation and de/phosphorylation) (109). While knowledge of proteins that remove acetyl or phosphate groups from amino acids are abundant, enzymes that remove methyl groups from lysine or arginine remained elusive until two years ago (54, 207, 227).

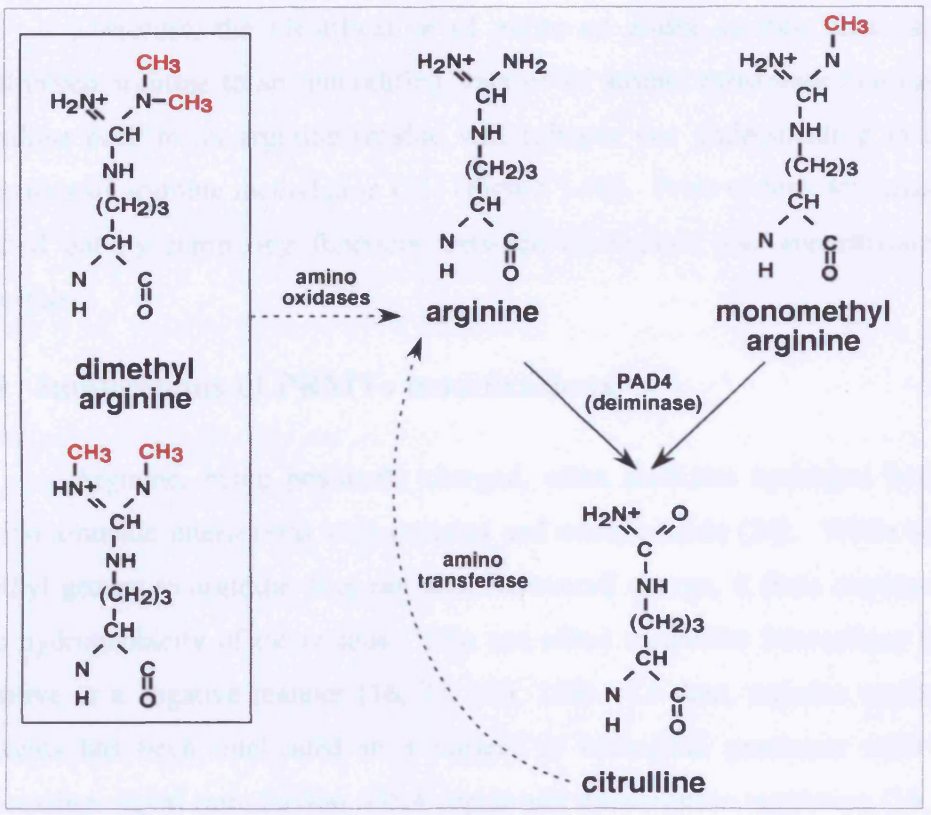

Figure 1-10 Demethylation of arginine.

Conversion of arginine and mono-methylated arginine to citrulline (Solid black arrow), and pathways that are speculated to exist to regenerate unmodified arginine residue from methylated species (Dash arrows).

For histone lysine methylation, the dimethyl group on histone H3 lysine 4 (H3K4) is demonstrated to be removable by an amine oxidase to give an unmodified lysine. Depletion of the enzyme increases H3K4 methylation in cells and led to the corresponding transcription activation (207).

However, for arginine methylation, several observations suggested that the reversal of this modification seems more complicated. Firstly, peptidyl arginine deiminase 4 (PAD4), the deiminase implicated with arginine is only reactive towards unmodified or monomethylated arginine residues but not dimethylarginine residue (54, 227). Secondly, the end product of PAD4 deimination is citrulline, and the reaction that converts the citrulline within a protein back to an arginine residue is not identified (16) (Figure 1-10). Due to the differences between the chemical properties of citrulline and arginine, the "demethylated" PRMT substrate is predicted to have impaired activity with respect to functional arginine residues (63).

Therefore, the identification of either an amine oxidase that can convert methylated arginine to an unmodified state or an amino transferase that can convert citrulline back to an arginine residue will enhance our understanding in biological functions of arginine methylation (54) (Figure 1-10). Prior to that, investigations are carried out by comparing functions between methylated and unmethylated PRMT substrates.

1.8 Implications of PRMTs modifications

Arginine, being positively charged, often mediates hydrogen bonding and amino aromatic interactions with proteins and nucleic acids (24). While addition of methyl groups to arginine does not alter its overall charge, it does increase the bulk and hydrophobicity of the residue. This can affect molecular interactions in either a positive or a negative manner (16, 77, 126, 154). To date, arginine methylation of proteins has been implicated in a variety of biological processes such as RNA processing, signal transduction, DNA repair and transcription regulation (16, 126). In most reports, involvement of PRMTs is largely inferred by the identification of proteins belonging to particular cellular processes as being substrates of PRMTs, and the molecular basis of the modifications remains unexplored (63). Therefore, the following examples are chosen based on the clarity of the molecular details of how arginine methylation affects the function of its target protein.

1.8.1 Arginine methylation modulates protein-protein interaction

RNA binding proteins such as heterogeneous nuclear ribonucleoproteins (hnRNPs) A1, A2, K, R and hnRNP U represent the major targets of PRMT. They contain RGG motifs and have been positively identified to be methylated (90, 141). In the nucleus of HeLa cells, hnRNP accounts for 65% of asymmetric dimethylarginines within the total pool of proteins (141). Although the significance of arginine methylation in RNA binding proteins is not well understood, it is proposed that the modification serves as the maturation signal. Many RNA binding proteins are mislocalized in their hypomethylated state (143, 211).

While the RGG motifs present on these proteins are known to be capable of mediating RNA interactions, mutations in the RGG motif of the hnRNP A1 decreases its ability to self associate (110).

Sam68, another RNA binding protein that is identified as a physiological target of PRMT1 contains asymmetrically modified dimethylarginine in its RGG motifs (48). Besides binding RNA, Sam68 is also the adaptor protein for Src kinase and its proline rich motifs adjacent to the RGG motif is known to facilitate interacting with a number of proteins containing SH3 and WW domains. *In vitro* studies using asymmetrically arginine methylated Sam68 peptides revealed that arginine methylation inhibits the interaction of proteins that are based on the proline rich-SH3 domains binding but does not affect protein interactions mediated by proline rich-WW domain binding (15).

The integral components of the pre-mRNA splicing machinery consist of seven RNA binding proteins. Four of the proteins are symmetrically methylated at the RG or GR motifs by PRMT5. They are, the SmB, SmB′, SmD1 and SmD3 (30, 31, 70). The efficient assembly of Sm proteins forms the core particle for RNA splicing and this is dependent on the interaction of these proteins with the SMN (survival of motor neuron) protein. The Sm-SMN association is mediated by the RG rich motif located at the C-terminal tail of most Sm proteins (71). It is found that SMN prefers binding to symmetric dimethylated Sm proteins and this interaction is mediated by a region in the SMN that contains Tudor domain. Convincingly, peptides containing symmetric dimethylarginine can compete with Sm proteins for the interaction with SMN but similar peptides containing mono or asymmetric dimethylarginines cannot (30, 70). Therefore, symmetric arginine dimethylation, in this case, serves to increase the interaction with proteins containing Tudor domain.

1.8.2 Arginine methylation modulates protein–nucleic acids interactions

Despite the prevalence of RNA binding proteins reported as PRMT substrates, there is a lack of strong evidence to address the roles of arginine methylation in protein-nucleic acid interactions (154). In salt elution columns, methylated species of hnRNP A1 proteins is found to have less affinity towards the RNA or single stranded DNA. They eluted from the immobilized nucleic acids at

slightly lower salt concentration (< 45 mM) compared to their unmethylated counterpart (185). The fragile X mental retardation protein (FMRP) binds mRNA and regulates translation. Within the FMRP, there are RGG motifs that contain asymmetric dimethylarginine. Artificially methylating the RGG motifs of recombinant FMRP proteins with PRMT1 resulted in a 60% decrease in association of FMRP with its interacting RNA (213).

Subtle effects of arginine methylation in modulating protein-nucleic acids interactions probably suggest that the modification serves to increase the specificity of the proteins towards their nucleic acid target (33, 153). The diverse secondary and tertiary structures of RNA that are not governed by Watson and Crick base pairing gives rise to a huge variety of surfaces for RNA-protein interactions (106). As arginine is one of the most common residues that functionally contacts RNA, methyl groups added to the residue might disrupt hydrogen bonding by steric hindrance. On the other hand, increasing the hydrophobicity of the arginine by methylation might also favor the ability of the residue to interact with the aromatic bases on RNA molecule (16). However, these hypotheses need to be supported by experimental data, as few reports exist to explore the issue.

Other than studying how arginine methylation can affect RNA binding properties, the reverse can also be investigated (153). *In vitro* methylation efficiency of substrate proteins within cell lysates can be positively or negatively altered when the cell lysate was treated with RNase prior to the labeling assay (68). Indeed, there are reports of PRMTs substrates losing potential to accept methyl groups when bounded to RNA, examples include the yeast Hrp1p (222) and NS3 protein encoded by Hepatitis C virus (189).

1.8.3 Arginine methylation and transcription

1.8.3.1 Arginine methylation of histone

Histones were the first proteins identified to contain methylated arginines (176), but investigations on how histone arginine methylation can regulate transcription begins only after the discovery of PRMTs. Currently, PRMT1, PRMT4 and PRMT5 are known to modify histones (16).

PRMT1 methylates histone H4 on arginine 3 (H4R3). This methylation facilitates the subsequent modification of the histone tail by HAT. Therefore, it has been correlated with transcription activation (226). The H4R3 residue, together with the histone H3 arginine 8 is also reported to be targets of PRMT5 (62, 177). However, there is no convincing evidence regarding the presence of symmetric dimethylarginines on these residues *in vivo*. This is because major conclusions were either based on *in vitro* methylation of histone peptide (178) or recognizing symmetrically methylated histone with antibodies generated against asymmetrically methylated arginine (62, 153). PRMT4 methylation of histone has also generated equally contradictory findings; histone H3 arginine 2, arginine 17 and arginine 26 are reported to be specific substrates of the PRMT *in vitro* (203) and H3R17 in particular, is strongly linked with transcription activation (145). However, antibodies generated to specifically recognize asymmetrically methylated H3R17 revealed that the residue remains asymmetrically methylated in PRMT4 knockout mouse cells (233).

1.8.3.2 Arginine methylation of transcription factors

While PRMT modification of histone remains confusing, findings that are more comprehensible are available to support the significance of PRMTs in transcription regulation. The cyclic AMP responsive element binding (CREB) protein is controlled by intracellular signaling to associate with its target DNA sequences, and this mediates gene expression. CREB recruits transcription co-activators such as CBP (CREB binding protein) or p300 to the site of chromatin, and these CBP/p300 proteins posses HAT activities. The CBP/p300 co-activators families are the first set of transcription factors reported as targets of PRMT (232). It is now a fact that PRMT4 modifies these proteins in at least three sites, leading to different downstream molecular and cellular effects (153).

Methylation of arginine residues in the CREB-interacting domain of CBP/p300 proteins result in the inability of the co-activators to bind CREB and mediate transcription activation (Figure 1-11). In cells, increasing expression of exogenous PRMT4 resulted in a corresponding decrease in transcription of reporter genes. This effect cannot be reproduced by PRMT4 catalytic mutants, implying that the arginine methyltransferase activity is required for this regulation (232).

The second site on the CBP/p300 that contains methylarginines is found near the C-terminus of the CREB-interacting domain. In this instance, arginine methylation is postulated to be a prerequisite for CBP to co-operate with GRIP1, a set of hormonally induced transcriptional activators (Figure 1-11). Substitutions of the arginines involved resulted in the loss in GRIP1 mediated transcriptional activation but the CBP-CREB activation pathway remains unaffected (37, 42).

Opposite effects on p300-GRIP1 interaction are observed when arginines within the C-terminus of CBP/p300 are methylated (Figure 1-11). The C-terminus of these proteins is the binding domain for GRIP1 and methylation of one particular arginine residue resulted in the loss of interaction between p300 and GRIP1, and mutating the arginine residue to lysine abolished p300 co-activation activity (130).

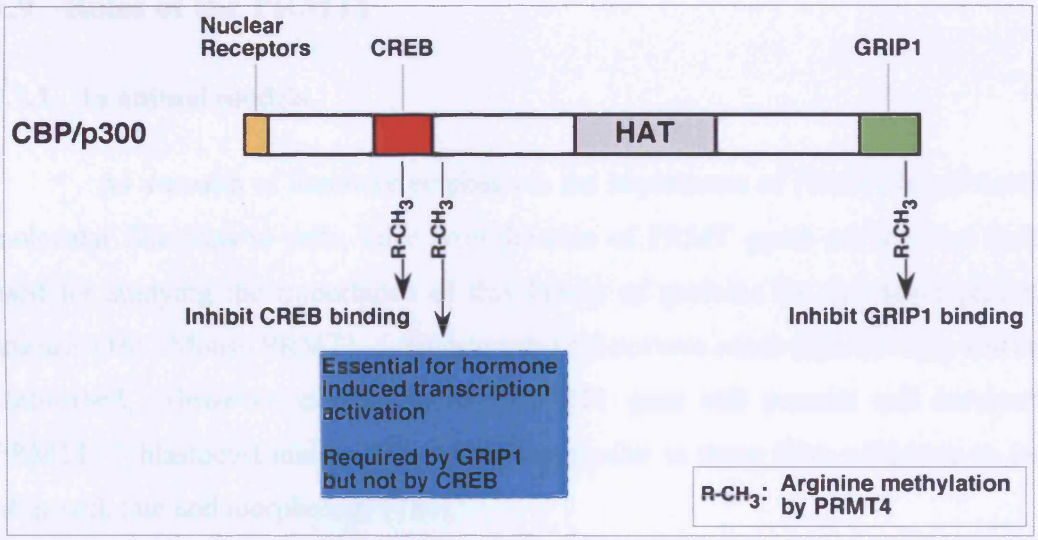

Figure 1-11 PRMT4 methylation of CBP/p300.

Basic map of the CBP/p300 proteins with the functional histone acetyltransferase domain (Gray box) and each co-activator interacting domain are labeled (Top). Arrows indicate sites of arginine methylation by PRMT4 and their effects (Bottom). Sites that are required for hormone dependent transcription activation are indicated in Blue box.

1.8.3.3 Arginine methylation of transcription elongation factors

Besides modulating gene expression by controlling transcription activation, PRMTs are also implicated in transcription elongation. SPT5 is a transcription elongation factor that determines the processivity of transcription by binding to RNA polymerase II (RNAP II). When RNAP II and SPT5 are not phosphorylated, SPT5 negatively regulates the basal level of transcription. Upon phosphorylation of SPT5

and RNAP II, SPT5 switches from inhibitory to stimulatory mode and positively regulates transcription elongation.

SPT5 is reported to be a substrate of PRMT1 and PRMT5 *in vivo* and methylation of arginines within SPT5 is thought to decrease its association with RNAP II. Substitutions of the arginines involved resulted in the increased association of SPT5 with its target promoters and enhancement in RNAP II/SPT5 transcription elongation activity (121).

A following study also identified FCP1 phosphatase, the enzyme that recycles RNAP II by dephosphorylation, as a substrate of PRMT5 but the significance of this modification remains unknown (3).

1.9 Roles of the PRMTs

1.9.1 In animal models

As a wealth of literature emphasizes the importance of PRMTs based on their molecular functions in cells, mice with deletion of PRMT genes are the best models used for studying the importance of this family of proteins for the development of animals (16). Mouse PRMT1 -/- embryos did not survive more than 6.5 days and were reabsorbed. However, deletion of the PRMT1 gene still permits cell survival as PRMT1 -/- blastocyst maintain in culture are similar to those from wild-type in terms of growth rate and morphology (180).

Consistent with reports of PRMT1 being the major type I arginine methyltransferase, PRMT activity in the mutant blastocyst is reduced by 84% and the asymmetric dimethylarginine content of cellular proteins is reduced by 50% (180, 216). Further analysis of these mutant blastocysts revealed that the loss of PRMT1 activity cannot be compensated by other type I PRMTs (179).

Homozygous mice with targeted disruption of PRMT4 died at birth and the embryos isolated were smaller and displayed breathing difficulties (233). Further analysis of the embryos also revealed an underdeveloped thymus containing fewer cells suggesting a role for PRMT4 in early T cell development (111). Mouse embryonic fibroblasts (MEF) of the PRMT4 -/- mutants were obtained and PRMT4

substrates were assayed for the loss of asymmetric dimethylarginines. While p300 becomes hypomethylated in the absence of PRMT4, histone H3 R17 is still recognized by the antibody specific for the methylated arginine (see Chapter 1.8.3.1). To ascertain whether the CBP/p300 hormonal induced transcription activation is dependent on its modification by PRMT4 (Figure 1-11), the MEF cells were transfected with estrogen responsive reporter and reporter expression was determined to be significantly lower than wild-type cells. The loss in expression can be rescued by adding exogenous PRMT4 (233).

Recently, the synergy between p300 and PRMT4 is implicated with another transcription regulator, nuclear factor kappaB (NF-κB). These three proteins form a complex *in vivo* and the enzymatic activities of PRMT4 and p300 are required to enhance the expression of a subset of genes controlled by NF-κB. In addition, these genes are misregulated in PRMT4 knockout MEF after NF-κB induction (50).

1.9.2 In human disease

As some PRMTs are known to be involved in nuclear receptors mediated transcription activation (42, 233), overexpression of these enzymes might result in hormone dependent cancer formation (16). It is observed that PRMT4 have aberrant expression in human prostate carcinoma (94) and inhibitors against PRMT1 and PRMT4 activity suppress hormone dependent transcription activation activity (41).

Increased arginine methylation of PRMT substrate proteins are also identified as a possible causes of human diseases. In multiple sclerosis, arginines in myelin basic protein (MBP) are observed to have higher levels of deimination and methylation (112). The pathology of this disease reveals that the autoimmune response triggered by methylated species of MBP causes damage to the components of the nervous systems. Similarly, hypermethylation of Sm proteins is also known to cause the autoimmune disease lupus erythematosus (34).

1.10 Studying the effects of arginine methylation on MBD

Being RNA binding proteins (102) as well as factors that bridge the association between methyl-CpG DNA and chromatin remodeling complexes, MBD2

and MeCP2 present themselves as good candidates for investigations into whether they can be controlled by PRMT. The emerging examples that illustrate how PRMT can participate in diverse cellular processes have led us to question whether the MBD can provide the first molecular evidence between CpG-DNA methylation and arginine methylation.

Here, I report that MBD2 and MeCP2 are methylated on arginines *in vivo* and I identify the PRMTs responsible for the modification of MBD2 in cells. I also show that MBD2 methylation regulates its affinity for co-repressor complex and methyl-CpG DNA. Consistent with the molecular effects of arginine methylation, MBD2 methylation blocks its transcription repression activity in cells. PRMTs function in this regulatory network as repressors of a repressor.

Chapter 2 Results I - Are MBD proteins methylated?

2.1 MBD proteins are methylated in vitro

2.1.1 Recombinant MBD2 and MeCP2 can be methylated.

To address whether the amino acid sequences of MBD2 and MeCP2 can be post-translationally modified by methyltransferases, recombinant forms of the proteins were produced in bacteria for *in vitro* methylation assay. These proteins served as good substrates because arginine methyltransferase activity is not reported in bacteria cells (141).

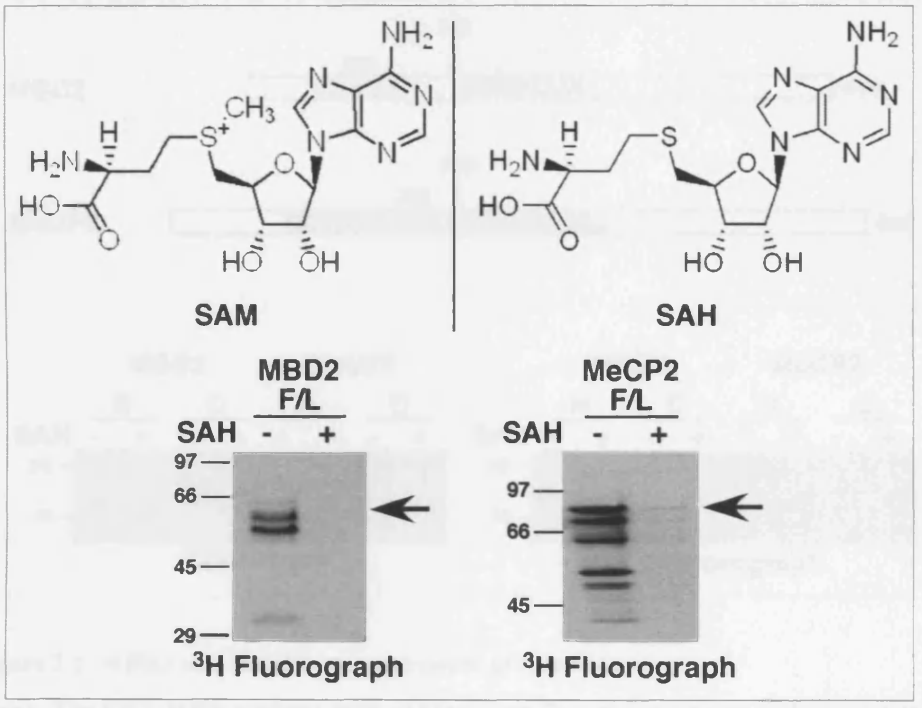

Figure 2-1 GST-MBD proteins are methylated *in vitro*.

(Top) Structure of the methyl donor (SAM) and the inhibitor (SAH).

(Bottom) Full-length GST-MBD2 and GST-MeCP2 proteins were subjected to protein methylation assay by incubating with rabbit reticulocyte lysate at 37°C for 90 min. The reactions were stopped by addition of SDS-PAGE sample buffer, resolved by SDS-PAGE and subjected to Fluorography. Arrows indicate the migration of the full-length proteins.

Full length GST-MBD2 and MeCP2 were tested for their ability to accept the ^{3}H-methyl group from S-adenosyl-methionine (SAM), the universal methyl donor for all methyltransferase reactions (Figure 2-1, Top). In this experiment, the source of methyltransferases was provided by rabbit reticulocyte lysate, which is known to contain PRMT activities (141). After the transfer of methyl group to substrate proteins, SAM is converted to S-adenosyl-homocysteine (SAH), the potent end product inhibitor of all methyltransferase reactions (162). Therefore, to demonstrate that the ^{3}H-label is incorporated into the proteins in Figure 2-1 (Bottom) were catalyzed by methyltransferases and not by other metabolic enzymes, SAH was included as a control. Only in the absence of SAH, MBD2 and MeCP2 were labeled by methyltransferases present in the lysate.

2.1.2 Recombinant MBD2 and MeCP2 are methylated at the N-terminus

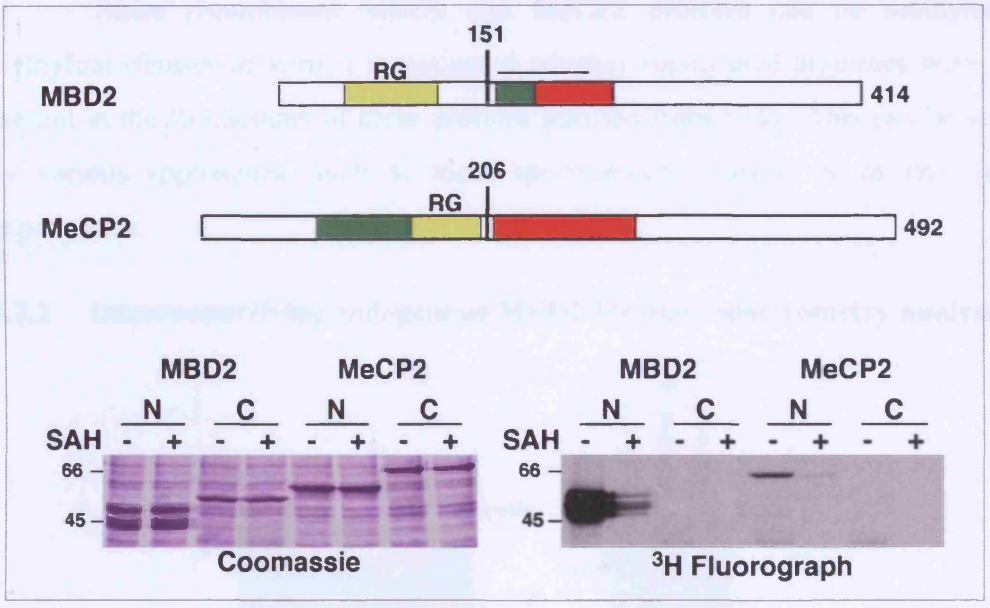

Figure 2-2 MBD2 and MeCP2 are methylated at N-terminus.

(Top) The GST-MBD proteins were expressed as N and C-terminus parts; position of black vertical lines separates the terminus.

(Bottom) The recombinant proteins are subjected to the *in vitro* methylation assay as for Figure 2-1, and the products were visualized by Coomassie and Fluography.

As the arginine and glycine repeats of both MBD2 and MeCP2 proteins are located nearer to the N-terminus, I tested whether the N-terminus halves of the proteins are the sites for methylation. The MBD proteins were expressed as GST-tagged N and C-terminus fusion proteins (Figure 2-2, Top), and were subjected to the

in vitro methylation assay using methyltransferase from Ramos cell lysate. The experiment was repeated with cell lysate from HeLa, 293T, Neuronal 2A and PC12 cells, and the outcome was consistent with Figure 2-2 (Data not shown). The N-terminus halves of both proteins containing the RG domain are the major ^{3}H-labeled products in the absence of SAH.

However, as the mammalian cell lysates used were robustly methylating the N-terminus halves of the two proteins, especially GST-MBD2, the SAH present was not able to fully suppress the methyltransferase activities. A titration assay was performed to determine the optimum SAH concentration required in future assays (see Figure 6-1 in Chapter 6.1), and all subsequent assays use 100 μM SAH.

2.2 MBD proteins are methylated in vivo

Since recombinant MBD2 and MeCP2 proteins can be methylated by methyltransferases *in vitro*, I investigated whether methylated arginines were indeed present in the RG domain of these proteins purified from cells. This can be achieved by various approaches, such as mass spectrometry analysis or *in vivo* labeling experiments.

2.2.1 Immunopurifying endogenous MBD2 for mass spectrometry analysis

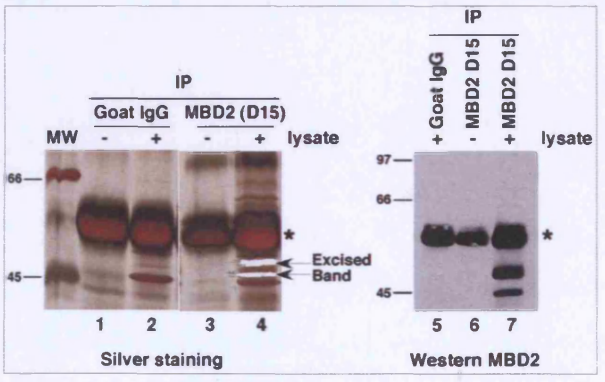

Figure 2-3 Large-scale immunopurification of MBD2 protein from Ramos cells[5].

MBD2 proteins were immunopurified using the optimized conditions stated in Figure 6-2 and the purified products were analyzed by Western blot (Right) or sliver staining

[5] For details of conditions used for immunoprecipitation of MBD2 protein in this experiment, refer to Chapter 6.2.

(Left). The silver stained band migrating at the same position as MBD2 in the Western blot was excised and analyzed by mass spectrometry. (*) Denotes antibody heavy chain.

The MBD2 protein was chosen for the mass spectrometry analysis as the primary amino acid sequence shows that the RG domain of MBD2 is more extensive than MeCP2 (Figure 1-6). Furthermore, compared to GST-MeCP2 N-terminus, GST-MBD2 N-terminus is also more robustly labeled in the *in vitro* methylation assay (Figure 2-2). This indicates that the MBD2 protein might have higher methylarginine content.

Using immunoprecipitation conditions optimized in Figure 6-2 and Figure 6-3 (see Chapter 6.2), endogenous MBD2 protein was isolated from 5 X 10^8 Ramos cells (Figure 2-3). From the silver staining, it was observed that the immunoprecipitations the presence of antibody without lysate (Lane 1 and 3) were cleaner compared to those in the presence of antibody and lysate (Lane 2 and 4). A distinct band of 45 kDa in lane 2 and 4 was confirmed to be a non-specific interaction of β-actin with goat antibodies. However, as lane 7 of the Western blot revealed a satisfactory yield of MBD2 protein, bands in lane 4 that correspond to the migrating positions of MBD2 proteins were excised and identified by mass spectrometry analysis. Although endogenous MBD2 was identified upon mass spectrometry analysis, peptide sequences of both β-actin and goat IgG heavy chain were also present. Therefore, it was unfeasible to analyze for the presence of methylarginines in the endogenous MBD2 proteins purified.

2.2.2 Post-translational labeling of transiently expressed MBD

2.2.2.1 Transient expression of MBD proteins

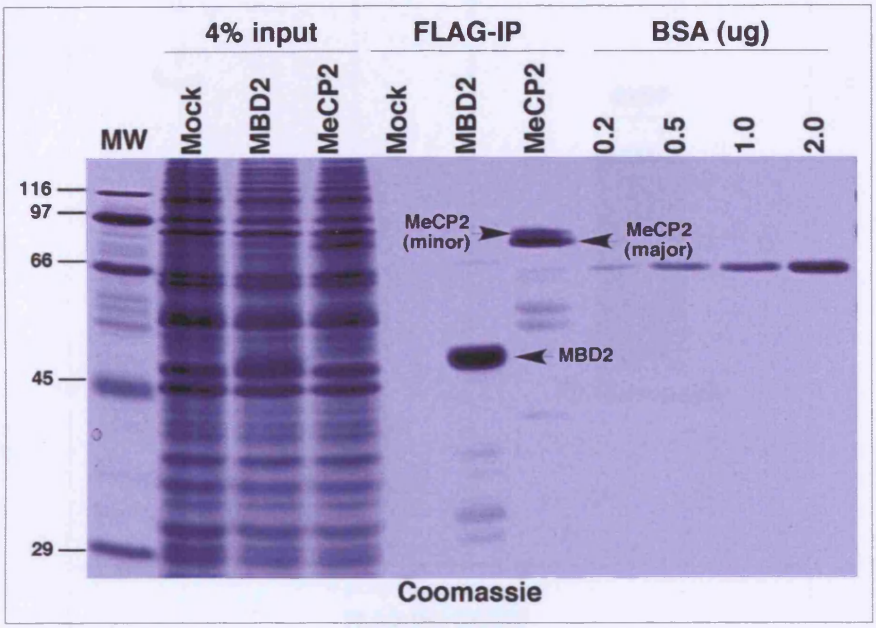

Figure 2-4 Purification of MBD proteins expressed in mammalian cells.

293T cells were transiently transfected with plasmids encoding the indicated FLAG-tagged proteins for 48 hr. The cells were then lysed and immunoprecipitated with FLAG antibodies and eluted with 3x FLAG peptide. Eluted FLAG-proteins were resolved by SDS-PAGE and visualized by Coomassie.

MBD proteins with purity of > 90% can be prepared by immunopurifying the FLAG-tagged proteins from transiently transfected 293T cells (Figure 2-4). As the FLAG-proteins were eluted from the agarose-conjugated antibodies by 3X FLAG peptide competition, problems of contaminating antibody heavy chain in the sample was avoided. The indicated MBD2 band was excised and analyzed by mass spectrometry for the presence of methylarginines. However, the result was inconclusive, as enzymatic digestion of the MBD2 band was not able to release MBD2 peptides at the RG domain. It is believed that the stretch of amino acid was either too large, or too hydrophobic to be resolved by the mass spectrometry.

2.2.2.2 In vivo *labeling of MBD proteins*

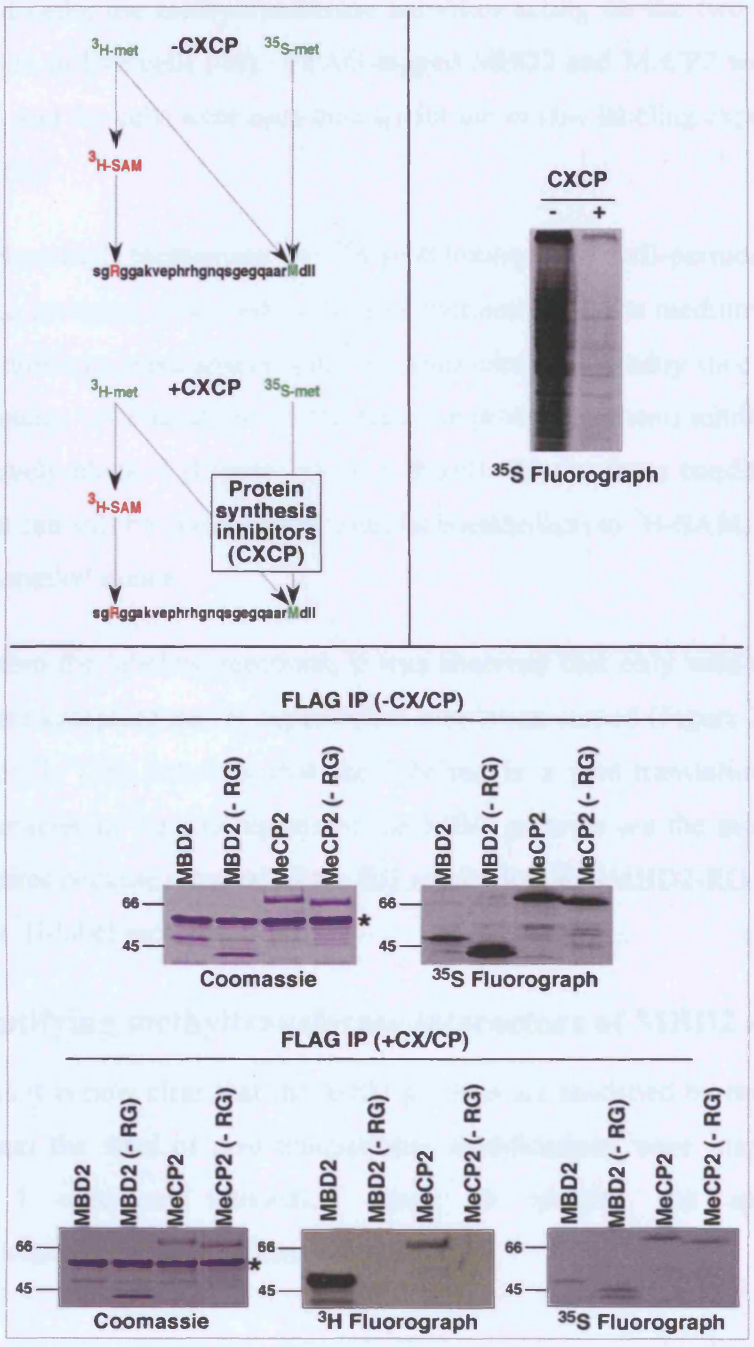

Figure 2-5 *In vivo* labeling of MBD proteins.

(Top Left) The scheme of the labeling experiment.

(Top Right) Lysate from cells treated with protein synthesis inhibitors cyclohexamide (CX) and chloramphenicol (CP) have translation effectively knocked down.

(Bottom) 293T cells were transiently transfected with plasmids expressing the indicated FLAG-proteins. The cells were treated with the indicated radioactive methionine labels and in the absence (Top) or presence (Bottom) of CX/CP. FLAG-proteins were then purified from total cell lysate by immunoprecipitation with anti-FLAG antibody, and methylated proteins were analyzed as for Figure 2-1. (*) Denotes antibody heavy chain.

Besides showing the presence of methylarginines in MBD purified from mammalian cells, the methyltransferase activities acting on the two proteins can be demonstrated in live cells (48). FLAG-tagged MBD2 and MeCP2 were expressed in 293T cells, and the cells were used directly for the *in vivo* labeling experiments (Figure 2-5, Top Left).

^{3}H-methyl methionine or ^{35}S-methionine, two cell-permeable radioactive labels, were included as the only source of methionine in the medium. When protein synthesis inhibitors were absent, the ^{35}S-methionine was readily incorporated into all cellular proteins by translation. In the presence protein synthesis inhibitors, translation was effectively blocked (Figure 2-5, Top Right). Under these conditions, ^{3}H-methyl methionine can still be converted by cellular metabolism to ^{3}H-SAM, which serves as the *in vivo* methyl donor.

From the labeling reactions, it was observed that only wild-type MBD2 and MeCP2 were accepting the ^{3}H-signal when translation ceased (Figure 2-5, Bottom, ^{3}H-Fluorograph). This indicates that the labeling is a post-translational event. The arginine residues in the RG repeats of the MBD proteins are the most likely methyl accepting sites because removal of the RG repeat domain (MBD2-RG and MeCP2-RG [6]) prevents ^{3}H-label incorporation.

2.3 Identifying methyltransferase interactors of MBD2 and MeCP2

As it is now clear that the MBD proteins are modified by methyltransferases in cells, and the sites of post-translational modifications were mapped to the RG domains, I used an interaction assay to identify the specific arginine methyltransferases catalyzing these modifications.

[6] The amino acid sequences of the RG domains removed from the two proteins are illustrated in Figure 2-6, top panel.

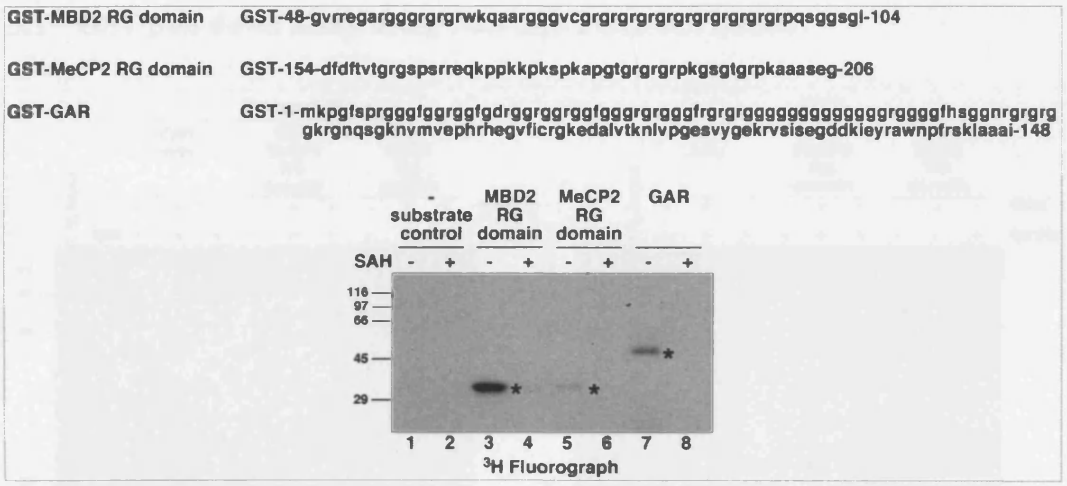

Figure 2-6 RG domain of MBD proteins are sufficient for methylation.

(Top) The RG domains of MBD proteins and fibrillarin (GST-GAR) were expressed as GST-proteins in bacteria. Amino acid sequences for each of the substrates are indicated, and the relative positions of the residues are shown as numbers.

(Bottom) The indicated proteins were subjected to methylation by HeLa cell lysate as for Figure 2-1. (*) Indicates the relative position of the proteins in the gel.

To create baits for identification of the interacting methyltransferase(s), the RG domains of MBD2 and MeCP2 proteins were expressed as GST-tagged proteins (Figure 2-6, Top). I needed to ascertain whether the RG domain alone was sufficient for interaction with, and could be modified by, the methyltransferase(s), or whether the interacting sites for the methyltransferase(s) is not dependent on the RG domain.

Therefore, proteins were tested for their capacity to be methylated by an *in vitro* methylation assay (Figure 2-6, Bottom). GAR (Lane 7 and 8), the RG containing domain of fibrillarin protein (217), was included as the positive control. To ensure that there was no auto-methylation of lysate proteins, controls without substrate were included (Lane 1 and 2). As expected, the RG domains of MBD2, MeCP2 and GAR were labeled by methyltransferases in HeLa lysate (Lane 3, 5 and 7). Therefore, the RG domains of the MBD proteins were considered suitable baits for the pull down assays.

2.3.1 GST pull down assay using N2A and PC12 cell lysates

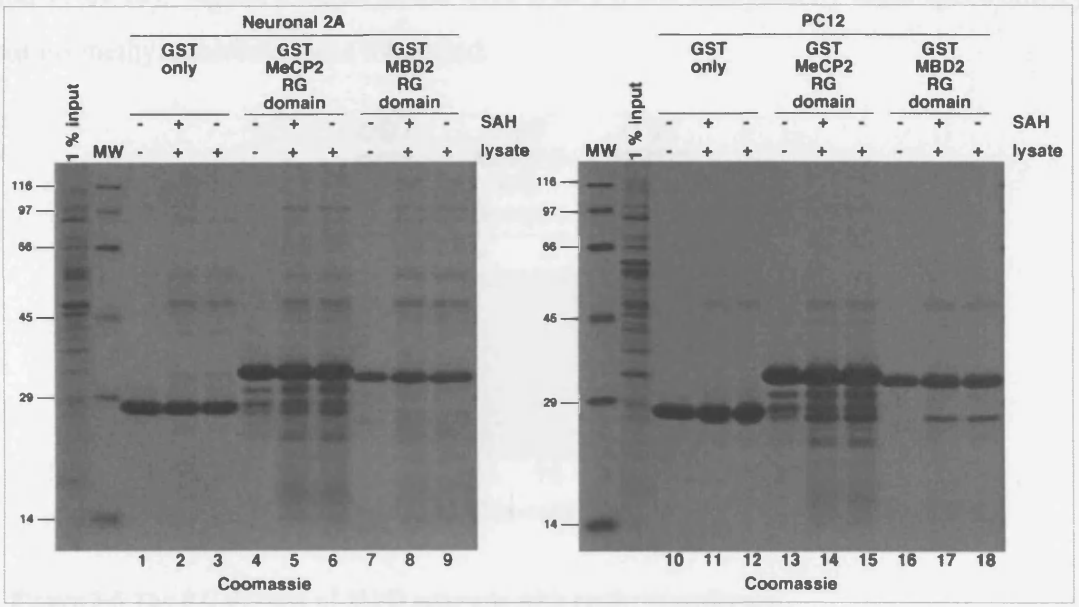

Figure 2-7 Identifying the interacting partners of the RG domain of MBD proteins [7].

GST protein or the GST-tagged RG domain of MeCP2 and MBD2 proteins were incubated in the absence or presence of methylation inhibitor and/or the indicated cell lysates, and the samples were prepared and analyzed as for Figure 6-4.

Mammalian cell lysates that posses *in vitro* methyltransferase activity towards MBD2 and MeCP2 proteins were used for GST pull down assays. In the initial experiments, HeLa and Ramos cell lysate were incubated with the RG domain of the two MBD proteins, and 35 proteins enriched in the interaction assay were sent for identification by mass spectrometry analysis. None of the interacting proteins was identified as a methyltransferase (see Figure 6-4 in Chapter 6.3).

As deficiency or mutations in the MBD proteins was linked to neuropathological disease/symptoms (4, 86), I repeated the pull down assay using transformed cell lines of neuronal origin (Figure 2-7). While the experimental setup and controls were similar to the previous experiment[8], I also wanted to test the hypothesis that SAH might affect the affinity of interaction between the target methyltransferases and the RG domain proteins, since it inhibits methyltransferase activity. However, addition of SAH did not produce any visible difference in the co-

[7] For details of the conditions used for GST pull down assay, refer to Figure 6-4. in Chapter 6.3.
[8] Refer to Figure 6-4.

purifying protein bands compared to samples without SAH (5 vs 6, 8 vs 9, 14 vs 15 and 17 vs 18). Again, distinct bands were selected and analyzed by mass spectrometry but no methyltransferase was identified.

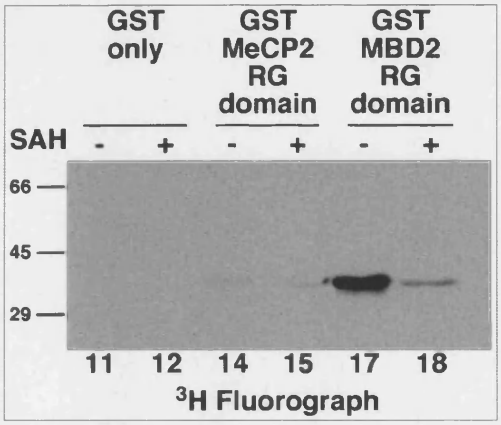

Figure 2-8 The RG domain of MBD interacts with methyltransferase.

The recombinant proteins captured after incubating with PC12 cell lysate as for Figure 2-7 were subjected to methylation assay by incubating at 37°C for 90 min in the presence of ^{3}H-SAM. The interacting methyltransferase present catalyzed the addition of ^{3}H-methyl groups to the recombinant protein, and the reactions were stopped and visualized as for Figure 2-1.

Due to the inability of the pull down assays to isolate specific methyltransferases, I asked whether the targeted methyltransferases were binding to the GST-bait. To ensure that this was the case, the experiment in Figure 2-7 was repeated (note similar lane numbers). Instead of visualizing the protein complexes by SDS-PAGE/Coomassie, the presence of methyltransferase activity was tested by adding ^{3}H-SAM to the purified complex (Figure 2-8).

From the Fluorography, it was confirmed that the GST-RG domain was attracting methyltransferase(s) that catalyze the addition of radioactive methyl groups to the recombinant substrate when a methyl donor was supplied (Lane 14 and 17).

I can also conclude that the presence of SAH in the lysate during the pull down assay had a detrimental effect on the interaction between the methyltransferase and the RG domain proteins (14 vs 15 and 17 vs 18). This is because the samples were washed extensively in SAH-free buffer before the methylation assay to prevent carry-over of the inhibitor.

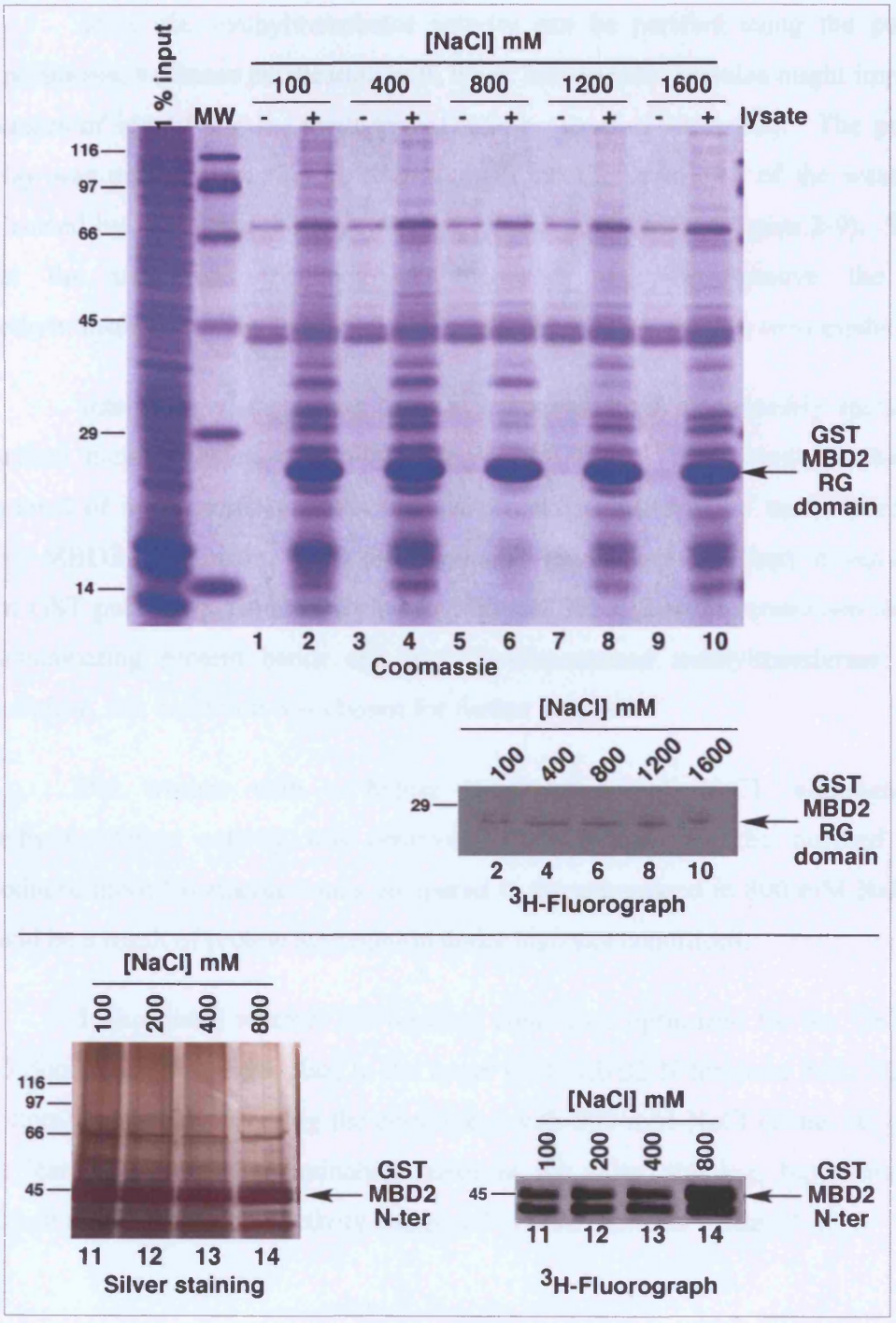

Figure 2-9 Optimization of washing conditions for the GST pull down assay.

(Top) GST-tagged RG domain of MBD2 was subjected to pull down assay using Ramos cells lysed in buffer containing 100 mM NaCl. The complexes were captured as for Figure 6-4, and were washed extensively (6 times) with the indicated concentrations of NaCl. 90% of the proteins were analyzed by Coomassie, while the remaining samples were assayed for co-purifying methyltransferase activity as for Figure 2-8.

(Bottom) The pull down experiment was repeated using GST-MBD2 N-terminus as the bait, and was analyzed as above.

Since the methyltransferase activity can be purified using the pull down experiments, a cleaner purification with fewer non-specific proteins might improve the chances of identifying the methyltransferase by mass spectrometry. The pull down assay was performed as before (Figure 6-4), but the stringency of the washes were enhanced by increasing the ionic strength of the wash buffer (Figure 2-9). To check that the increased stringency of the wash did not remove the desired methyltransferase(s), duplicates of the samples were assayed by *in vitro* methylation.

Interestingly, increasing the NaCl concentration reproducibly increases the purified methyltransferase activity (Figure 2-9, Top). This might be due to the removal of non-specific proteins that were masking the sites of methylation on the GST-MBD2 RG domain. From the Coomassie gel (Figure 2-9, Top), it was observed that GST pull down samples washed in 800 mM NaCl (Lane 6) contained the fewest contaminating protein bands and had uncompromised methyltransferase activity. Therefore, this condition was chosen for further assays.

For washes with a higher concentration of NaCl, although higher methyltransferase activity was observed (Lane 8 and 10), the purified samples produced more Coomassie bands compared to those prepared in 800 mM NaCl. This could be a result of protein aggregation under high salt conditions.

I also tested whether the washing conditions optimized for the GST-MBD2 RG domain can also be applied to the larger GST-MBD2 N-terminus bait (Figure 2-9, Bottom). Similarly, washing the complexes with 800 mM NaCl (Lane 14) produced the least number of contaminating bands in the silver staining, but contained the highest methyltransferase activity compared to other samples (Lane 11-13).

2.3.2 The RG domain of MBD2 interacts with PRMT1 and methylosome components

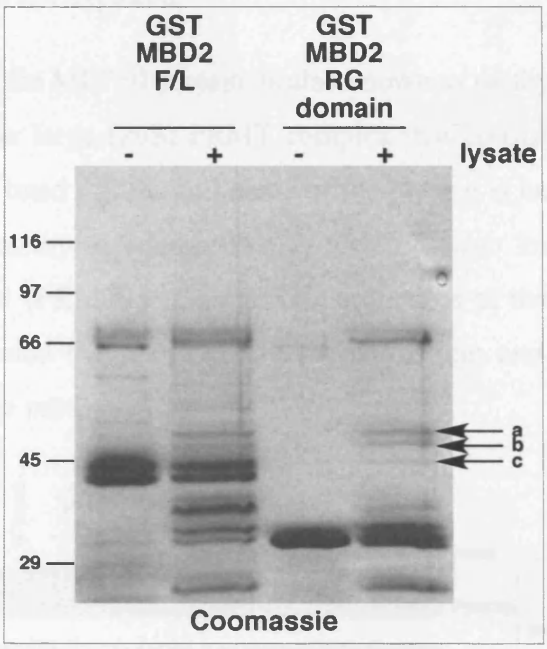

Figure 2-10 GST pull down using full-length and RG domain of MBD2.

GST-tagged MBD2 proteins were used in pull down assays with Ramos cell lysate containing 800 mM NaCl, the complexes were incubated and captured as for Figure 6-4, **and were washed extensively in buffer containing 800 mM NaCl. The purified complexes were eluted with SDS-PAGE sample buffer and resolved by SDS-PAGE. The indicated bands were analyzed by mass spectrometry.**

Using the optimized conditions previously worked out (Figure 2-9), the pull down assay was repeated for MBD2 protein (Figure 2-10). Three distinct bands were purified by the GST-MBD2 RG domain protein, and the identity of the bands is reported in Table 2-1.

Bands	Protein	Accession number
a	Eukaryotic transcription elongation factor δ	450348
b	MEP50	13129110
c	HMT hnRNP	4504497/1808644

Table 2-1 Results of mass spectrometry analysis.

Identity of protein bands from Figure 2-10 reported by mass spectrometry analysis.

From Table 2-1, band (a) was not a methyltransferase and was also reported in the previous experiments (Figure 6-4 and Figure 2-7). Hence, the significance of this interaction was not explored.

Band (b), the MEP50 protein, is also known as methylosome protein 50 and is a component of the large (20S) PRMT complex that consists of PRMT5, pICln and MEP50 (72). For band (c), the full name of the protein is human methyltransferase 1 (HMT1) hnRNP methyltransferase-like 2, which is also known as protein arginine methyltransferase 1 (PRMT1). The peptide sequences of the three proteins identified in Table 2-1 revealed that they were of human origin and this correlated with the source of cell lysate used.

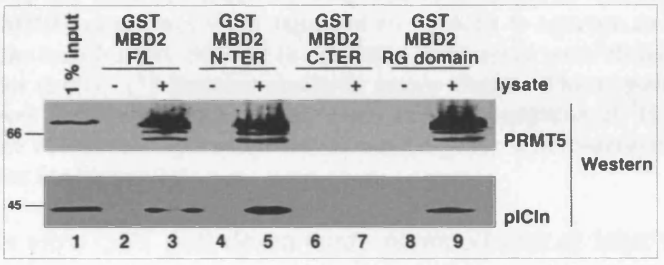

Figure 2-11 MBD2 interacts with components of the methylosome.

The indicated GST-tagged MBD2 proteins were used in pull down assays as in Figure 2-10, and interacting proteins were assayed for the presence of methylosome components by Western blot with the indicated antibodies.

Since MEP50 protein was identified in the pull down assay, I tested whether PRMT5 and pICln interact with GST-MBD2. Instead of using mass spectrometry analysis, protein complexes purified from pull down assays were subjected to Western blotting using antibodies raised against the two proteins (Figure 2-11).

MBD2 protein that contains the RG domain interacts with PRMT5 and pICln (Lane 3, 5 and 9), and GST-MBD2 C-terminus (Lane 7) was the only construct that did not interact with the methylosome proteins. This is consistent with the fact that *in vitro* arginine methylation was not reported in that section of the protein (see Figure 2-2).

2.4 MBD2 interacts with PRMT1 and PRMT5 in vivo

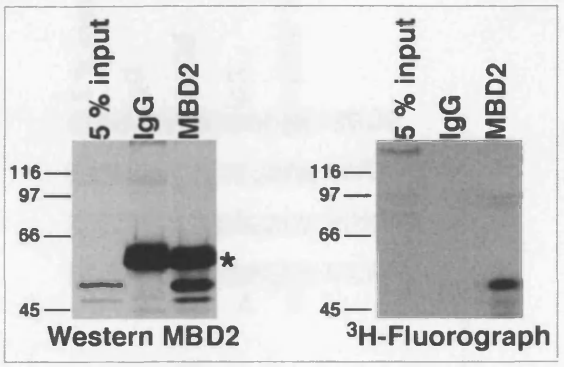

Figure 2-12 Endogenous MBD2 co-purifies with methyltransferase activity.

Control antibodies or antibodies against MBD2 were added to Ramos cell lysate in a buffer containing 800 mM NaCl, and binding was carried out at 4°C for at least 4 hr. The antibody-MBD2 complexes were captured by Protein G agarose and were washed extensively in the lysis buffer. 50% of the purified complexes were eluted and analyzed by Western blot (Left). (*) Denotes antibody heavy chain. The remaining antibody-MBD2 complexes were subjected to methylation assay by addition of ^{3}H-SAM to detect for the presence of interacting methyltransferase (Right). The reactions were stopped and visualized as for Figure 2-1.

The *in vitro* GST pull down assay revealed that at least two PRMTs interact with MBD2 via the RG domain (Figure 2-10). However, as amino acid repeats in the context of RG are the favored recognition motifs for most PRMTs (163), and MBD2 contains an extensive stretch of RG repeats, the authenticity of this molecular interaction required verification.

Using the optimized buffer system from Figure 2-9, I investigated whether endogenous MBD2 protein in cells can be immunopurified with methyltransferase activity. From Western blotting, I know that isolation of endogenous MBD2 protein is feasible using the high NaCl buffer (Figure 2-12). Thus, a duplicate of the immunopurified MBD2-protein complex was supplied with ^{3}H-SAM, as for Figure 2-8, to probe for any co-purifying methyltransferase activity.

From the Fluorography, a ^{3}H-band corresponding to the size of MBD2 protein was observed. As this band is the only enriched protein in the MBD2 immunopurification, it is believed to be the endogenous MBD2 methylated by the co-purifying PRMT(s).

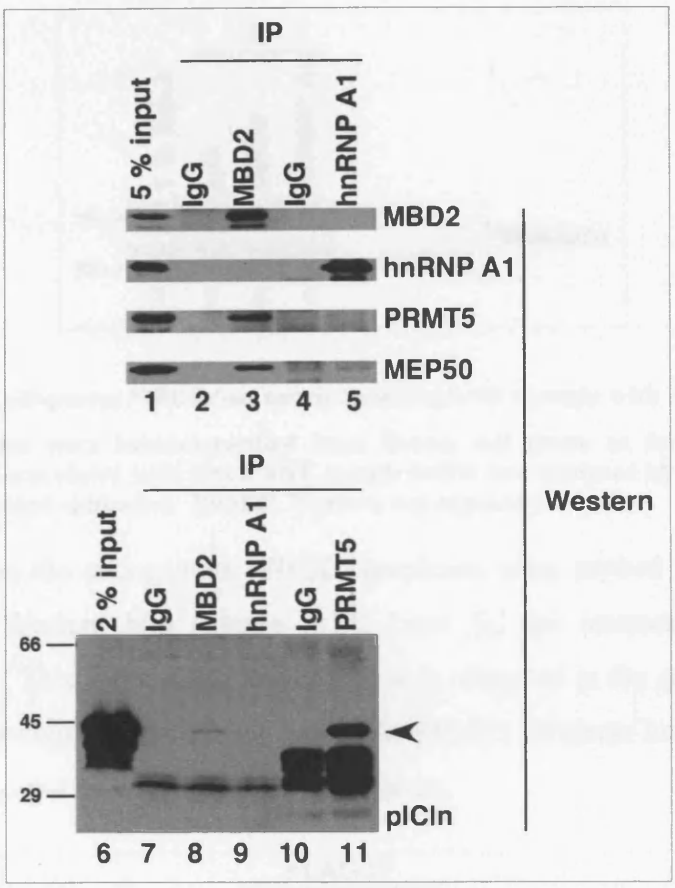

Figure 2-13 Co-immunoprecipitation of PRMT5 and MEP50 with endogenous MBD2.

(Top) MBD2 was immunopurified from Ramos cell lysate as for Figure 2-12. MBD2-complexes eluted with SDS-PAGE sample buffer were analyzed by Western blot with the indicated antibodies.

(Bottom) Western blot was exposed for 1 min. Arrow indicates position of pICln.

Since endogenous MBD2 co-purified methyltransferase activity, the isolated MBD2-protein complexes were tested for the presence of the PRMTs by Western blot. In Figure 2-13, MBD2 displayed a positive interaction with PRMT5 and MEP50 (Lane 3), whereas hnRNP A1, a well-characterized PRMT1 substrate found in the nucleus of cells (141), did not (Lane 5).

As for pICln, although it is present at high levels in the cell lysate (Lane 6), and displayed a strong positive interaction with PRMT5 (Lane 11), the existence of pICln in an endogenous MBD2-protein complex appeared minimal or insignificant (Lane 8).

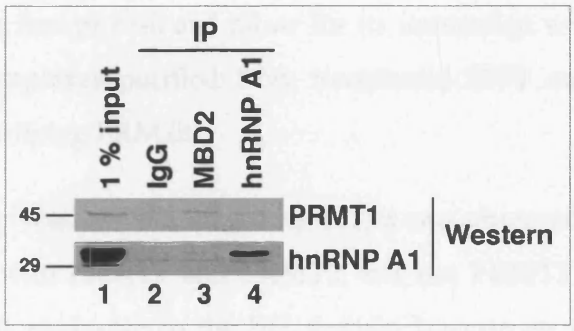

Figure 2-14 Endogenous MBD2 does not co-immunopurify strongly with PRMT1.

MBD2 proteins were immunopurified from Ramos cell lysate as for Figure 2-12. MBD2-complexes eluted with SDS-PAGE sample buffer and analyzed by Western blots with the indicated antibodies. PRMT1 Western was exposed for 1 min.

When the endogenous MBD2 complexes were probed for the presence of PRMT1 by Western blot (Figure 2-14, Lane 3), the interaction was weak and inconclusive. This is because PRMT1 was also observed in the control goat antibody immunoprecipitation (Lane 2). However, the PRMT1 substrate hnRNP A1 (141, 179), also did not co-purify with the methyltransferase.

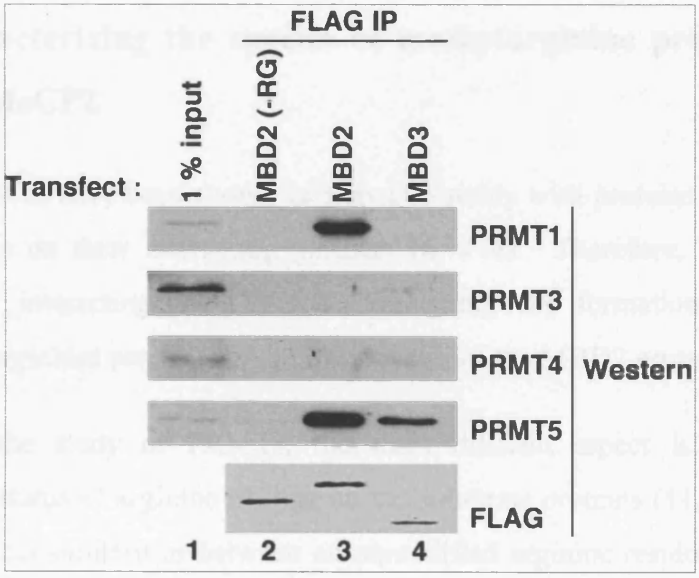

Figure 2-15 MBD2 proteins overexpressed *in vivo* co-purify with PRMT1 and PRMT5.

FLAG-proteins were immunoprecipitated with FLAG antibody, eluted with FLAG peptide, and normalized by titration against BSA in SDS-PAGE/Coomassie. Normalized protein complexes were analyzed for co-purifying PRMTs by Western blot with the indicated antibodies. 1% input represents pooled lysate used from all transfections.

As it was reported that the majority (> 90%) of MBD2 in cells exists in the 1 MDa MBD2/NuRD complex (65), expressing excess MBD2 in cells might increase

the amount of the free protein and allow for its interaction with the PRMTs. FLAG-MBD protein complexes purified from transfected 293T cells were tested for the presence of co-purifying PRMTs.

From the Western blot (Figure 2-15), it was observed that full-length MBD2 protein interacts with PRMT1 and PRMT5, but not PRMT3 and PRMT4 (Lane 3). This interaction is exclusive to the RG domain because an MBD2 mutant with the domain deleted (MBD2-RG, Lane 2) did not show any interaction with the two PRMTs.

MBD3, a protein sharing 70% sequence similarity with MBD2 in the MBD domain (85) interacts with PRMT5 but not PRMT1 (Lane 4). Although the presence of PRMT5 in the MBD3 immunoprecipitate could be due to their co-existence in the Brg1 complex in cells (56, 177), I can not rule out the possibility of an artifact, as PRMT5 was observed to non-specifically bind FLAG-agarose beads in some instances (Data not shown).

2.5 Characterizing the species of methylarginine present on MBD2 and MeCP2

PRMTs have been shown to associate stably with proteins without catalyzing modifications on their interacting partners (6, 178). Therefore, I needed to check whether the interacting PRMTs were catalyzing the formation of their specific methylated arginines products in the RG domain of the MBD2 protein.

In the study of PRMTs, the most difficult aspect is to determine the methylation status of arginine present on the substrate proteins (11, 220). This is due to the chemical similarities between an unmodified arginine residue and each species of methylarginine, which makes it difficult to generate antibodies that do not cross react. Therefore, any methylarginine specific antibodies used in this study needed to be carefully tested. For MBD2, since it is found to interact with both type I and type II PRMTs (PRMT1 and PRMT5 respectively), I would expect the two forms of dimethylarginines (asymmetric and symmetric respectively) to be present on the protein. For this reason, greater care was required to address the issue.

In the case of MeCP2, the interacting PRMT was still not identified. However, if I were able to determine the specific species of dimethylarginines present in the protein, I might be able to establish the particular type of PRMT that is responsible for catalyzing this modification. This would enable me to concentrate on a smaller subset of PRMT proteins.

2.5.1 Characterizing the antibodies that recognize methylarginine

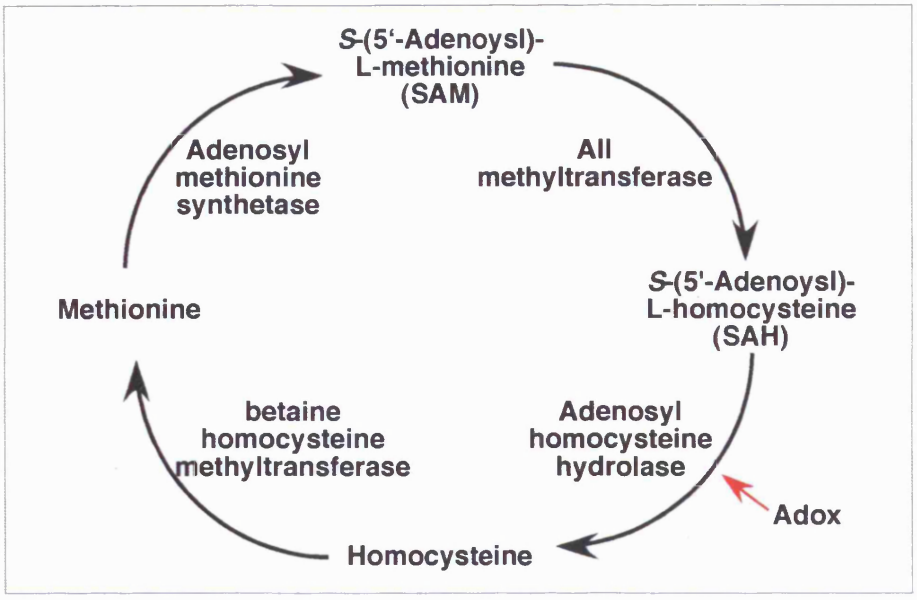

Figure 2-16 The methylarginine content of PRMT substrates can be depleted by using methyltransferase inhibitor.

Regeneration cycle of the universal methyl donor for all methyltransferases and the inhibitory effect of Adox in the cycle (43).

To generate MBD proteins with or without methylarginine contents, transfected 293T were treated with the cell permeable methyltransferase inhibitor, Adox. In cells, Adox prevents the regeneration of SAM by binding to Adenosyl homocysteine hydrolase. This causes the accumulation of intracellular SAH and inhibits all methyltransferase reactions (Figure 2-16). In this study, the *in vivo* post-translational labeling of MBD2 protein is consistently abolished by the addition of Adox[9].

[9] For an example illustrating the effects of Adox on the *in vivo* methylation of MBD2, refer to Figure 3-11, Lane 1 & 2).

With the ability to control the methylarginine content of proteins, commercially available antibodies can be tested against MBD2 and MeCP2 purified from cells. The outcome of the Western blot will be informative as to whether these antibodies are specific for methylated arginines or whether they are cross-reacting with unmodified arginine residues. The list of commercially available antibodies selected for screening the MBD proteins are listed in Table 2-2.

Antibody	Cat no.	Antigen	Species	Cross reaction	Company
7E6	ab412	MMA and Asym DMA	Mouse	unknown	Abcam
21C7	ab413	Asym DMA	Mouse	Sym DMA (unknown)	Abcam
16B11	ab414	MMA	Mouse	Sym DMA (unknown)	Abcam
5D1	ab415	MMA	Mouse	Sym DMA (unknown)	Abcam
Asym24	07-414	Asym DMA	Rabbit	No	Upstate
Sym10	07-412	Sym DMA	Rabbit	No	Upstate
Sym11	07-413	Sym DMA	Rabbit	Unknown	Upstate

Table 2-2 List of all commercially available antibodies that detect methylated arginine residues.

The characteristics and cross reactivity are based on information from the manufacturer. (MMA = monomethylarginine, DMA = dimethylarginine, Asym = asymmetric and Sym = Symmetric)

The cross reactivity and specificity of these antibodies were determined in the following experiments.

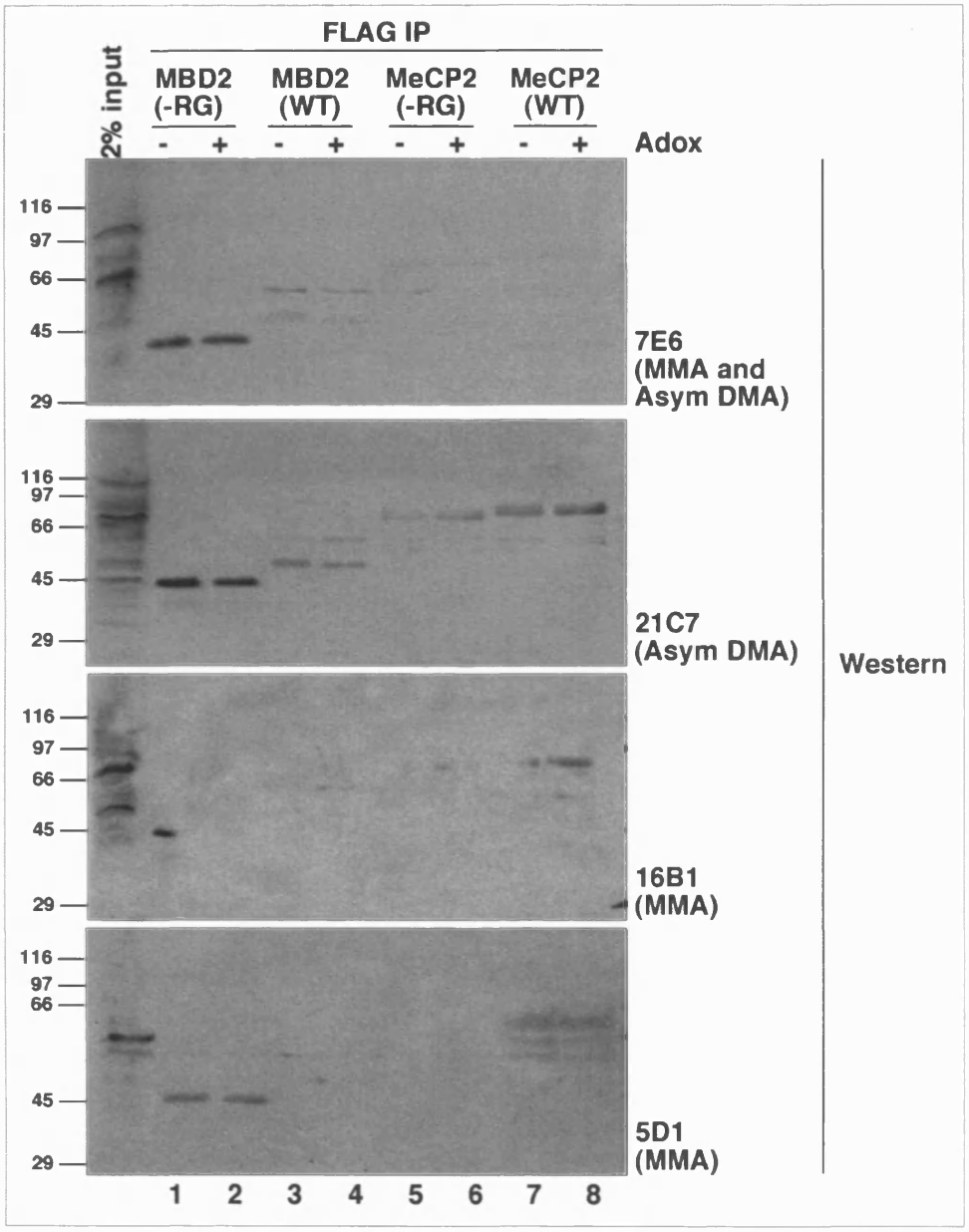

Figure 2-17 Screening mouse methylarginine antibodies against MBD proteins.

293T cells were transiently transfected with plasmids expressing the indicated FLAG-proteins for 30 hr and fresh medium with or without Adox were added to the cells for another 18 hr. The FLAG-proteins were purified and normalized as for Figure 2-15, and were analyzed by Western blots with the indicated antibodies stated in Table 2-2. Films for 7E6 and 21C7 were exposed for 20 sec, and for 16B1 and 5D1, exposure was > 2 min.

For Figure 2-17, the four proteins tested were prepared from cells treated with or without methylation inhibitor. Two of the proteins, (MBD2-RG; Lane 1,2 and MeCP-RG; Lane 5,6) were shown to be immune to protein methylation (see Figure 2-5), and therefore should not be recognized by the antibodies. Even if

methylarginines were indeed present in these –RG mutants, the Adox treated samples should not be detected in the Western blots (Lane 2 and 6).

In this experiment, the only samples that are expected to react with the antibodies were the wild-type MBD2 and MeCP2 proteins prepared from cells raised in Adox free medium (Lane 3 and 7). However, none of the antibodies used in Figure 2-17 seemed to be exclusive for these two samples (Lane 3 and 7). All proteins were recognized by 7E6 and 21C7 antibodies while 16B1 and 5D1 antibodies required lengthy exposure to reveal the non-specific bands and were thus considered unsuitable.

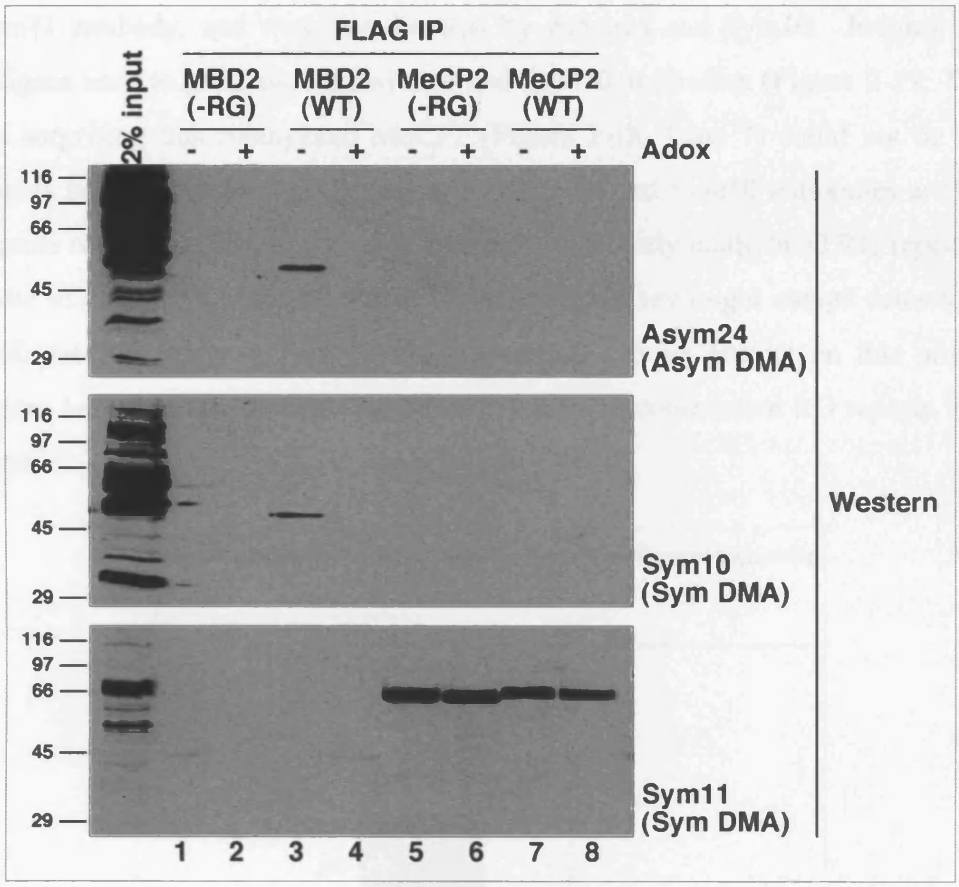

Figure 2-18 Screening rabbit methylarginine antibodies against MBD proteins.

FLAG proteins were prepared and purified from 293T cells as in Figure 2-17, and were screened for methylated arginines with the indicated antibodies stated in Table 2-2. All films were exposed for 20 sec.

Proteins prepared in Figure 2-17 were also tested with antibodies raised in rabbits immunized with peptides containing dimethylarginines. From the Western blots in Figure 2-18, it was observed that the Asym24 and Sym10 antibodies were specifically detecting wild-type MBD2 protein prepared from cells cultured in the

absence of Adox (Lane 3). The antibodies did not recognize the same protein prepared from Adox treated cells (Lane 4). This illustrates the ability of the two antibodies to discriminate between methylated and unmethylated arginine residues.

MBD2 protein with a deletion of the RG domain (MBD2-RG, Lane 1) was not recognized by the antibodies. This supported the findings in Figure 2-5, where I found that dimethylarginines were either non-existent or were present at an insignificant level in the C-terminus region of MBD2.

All variants of MeCP2 (Lane 4-8) were recognized non-specifically by the Sym11 antibody, and were not detected by Asym24 and Sym10. Judging from the antigens used to generate the Asym24 and Sym10 antibodies (Figure 2-19, Top), it is not surprising that methylated MeCP2 (Figure 2-18, Lane 7) could not be detected. This is because the immunogen used for Asym24 and Sym10 antibodies are based on peptide sequences with a row of at least 4 consecutively methylated RG repeats. Even if the RG domain of MeCP2 was fully methylated, they might escape detection by the antibodies, as there are only 3 consecutive RG repeats present on this protein (see Figure 1-6). MBD2, on the other hand, contains 10 consecutive RG repeats in the RG domain.

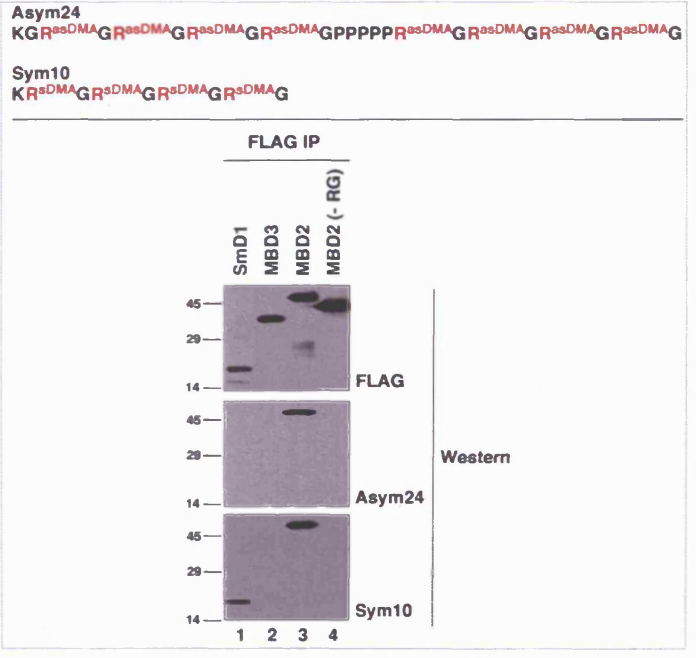

Figure 2-19 Asym24 and Sym10 antibodies are specific for arginine methylated MBD2.

(Top) The antigens used for generation of the antibodies, and the position and type of modification of the arginine residues are indicated. (as = asymmetric. s = symmetric. DMA = dimethylarginine.).

(Bottom) Plasmids encoding the indicated FLAG-proteins were transfected into 293T cells in the absence of Adox and the proteins were purified and normalized as for Figure 2-15. The proteins were tested for methylated arginines with the indicated antibodies.

Specificity of Asym24 and Sym10 antibodies for MBD2 was reconfirmed using MBD3 and SmD1, a protein reported to contain both species of dimethylarginines (Figure 2-19, Bottom) (31, 160). Both dimethylarginine antibodies did not recognize MBD3 (Lane 2) and MBD2-RG (Lane 4), but they detected the modified arginine residues on MBD2 protein (Lane 3), as observed previously (see Figure 2-18).

Although SmD1 proteins purified from the nuclear fraction were known to contain asymmetric dimethylarginines in mass spectrometry analysis (160), they were not recognized by the Asym24 antibody (Lane 1). This could be due to a bias of the purification scheme that might enrich for the cytosolic fraction of SmD1 that exclusively contains symmetric dimethylarginine. Alternatively, the proportion of asymmetrically methylated arginines in SmD1 might be too low for detection. The latter explanation is more appealing, because MBD2, the nuclear protein (85, 99) and was easily purified in the experiment using the high salt lysis buffer (800 mM NaCl). Furthermore, symmetric dimethylated species of SmD1 can also be found in the nuclear fraction, and the knowledge of its existence precedes the discovery of asymmetrically dimethylated SmD1 by years. These studies support the prevalence of the symmetric dimethylated species of SmD1.

While I cannot confirm whether Asym24 or Sym10 antibodies are only specific for their intended species of dimethylarginines, the uncertainty was partly resolved by the Asym24 Western blot on SmD1 proteins. Amino acid sequences of the RG domain in SmD1 and MBD2 are very similar in comparison (9 consecutive RG in SmD1 and 10 in MBD2). If Asym24 antibodies were to cross react with symmetric dimethylarginines, they should detect SmD1 proteins in the Western blot. However, since the specificity of the two dimethylarginine antibodies is of utmost importance, further experiments were designed to address this point.

2.5.2 MBD2 proteins expressed at endogenous levels contain methylated arginines

In previous experiments, the Asym24 and Sym10 antibodies were tested against overexpressed MBD2 purified from cells. As overexpression of proteins might sometimes disrupts cellular systems and lead to aberrant post-translational modifications, I generated stable cell lines that express controllable levels of FLAG-MBD2 and FLAG-MBD2(-RG) only upon tetracycline induction.

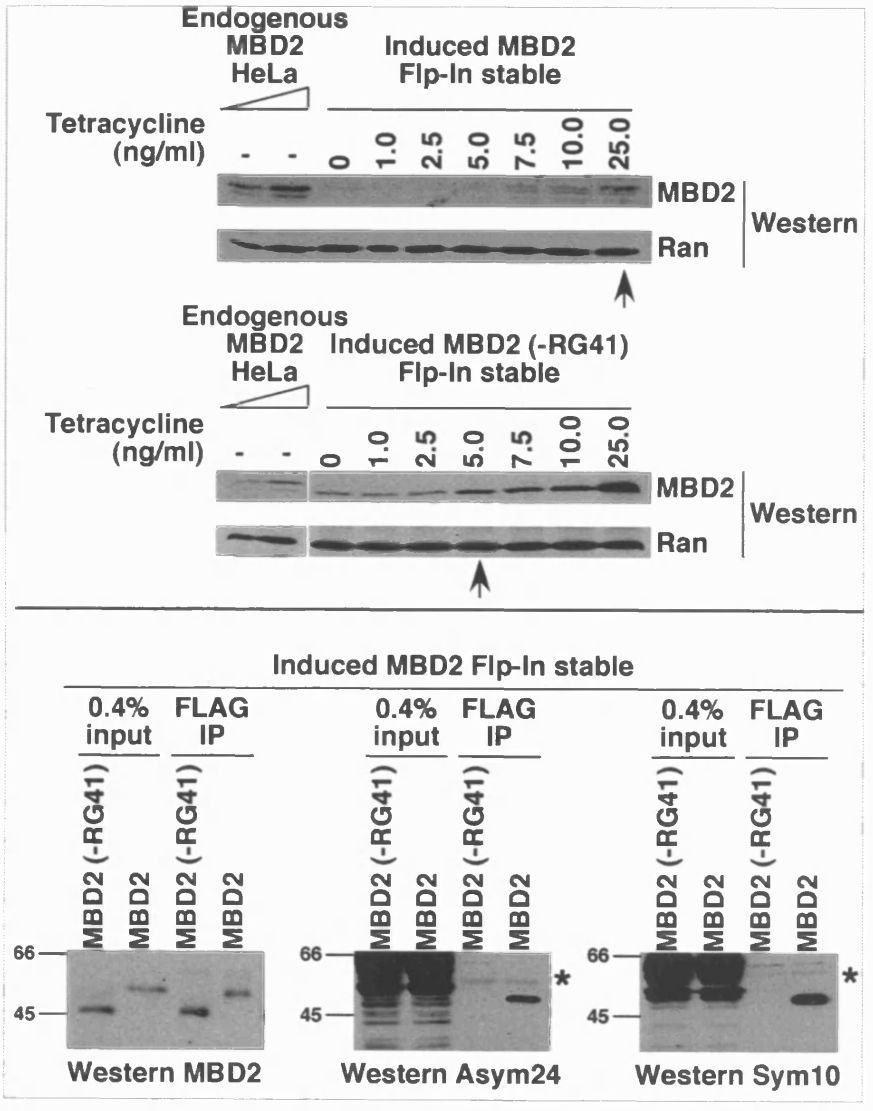

Figure 2-20 MBD2 expressed at endogenous levels is methylated.

Human (293T) cells from the Flp-In™ T-Rex™ system were transfected with plasmids containing the FLAG-MBD2 or FLAG-MBD2 (-RG41)[10] genes driven by a tetracycline inducible promoter.

(Top) Transfected cells were selected with antibiotics to obtain stable colonies (see Chapter 5.4.3.2) and these cells were titrated for tetracycline induction. Bradford normalized total cell lysates were prepared from the induced stable cells and HeLa cells to analyze for the expression level of MBD2. (↑) Indicates the tetracycline concentration selected for induction of each stable cell. Ran levels indicate protein loading.

(Bottom) FLAG-MBD2 proteins were purified from the selected tetracycline induction (↑) as for Figure 2-5 and analyzed by Western blot with the indicated antibodies (Bottom). (*) Denotes antibody heavy chain.

To show that MBD2 is unequivocally methylated *in vivo*, the FLAG-MBD2 proteins were expressed at levels comparable to endogenous MBD2 in HeLa cells (Figure 2-20, Top). These FLAG-MBD2 proteins were purified and were tested with the dimethylarginine antibodies. Again, full-length MBD2 but not MBD2-RG was determined to contain both asymmetric and symmetric dimethylarginines (Figure 2-20, Bottom).

2.6 Partial removal of the type I and type II PRMT specific for MBD2

It was demonstrated that removal of methylarginine content of MBD2 by Adox enabled us to test the ability of the antibodies to discriminate unmodified arginine residues from methylated arginine (Figure 2-16). However, to test if the Asym24 and Sym10 antibodies can differentiate between the two species of dimethylarginine residues, a more sophisticated system was needed.

To specifically remove the asymmetric dimethylarginine content of MBD2, the type I PRMT (PRMT1) responsible for this modification had to be removed from the cells. The same logic was applied to remove symmetric dimethylarginines by targeting PRMT5. PRMT knockout mice cells are most suitable for this purpose. However, only the attempt to delete PRMT1 in mice was documented, and the phenotype of PRMT1 -/- mice was embryonic lethal (180). Therefore, to obtain cellular systems that allow the study of the effects of PRMTs on MBD2, the siRNA

[10] In subsequent characterization, a better defined MBD2 mutant with deletion of the entire RG domain was created (MBD2-RG41, Figure 3-6). The amino acids that are removed from this MBD2 protein is illustrated in Figure 3-5.

approach was employed to reduce the normal levels of the two PRMTs in 293T cells. mRNA sequences of human PRMT1 and PRMT5 were analyzed by Extractor 5 software to select suitable regions of the sequence for design of siRNA .

Gene	Clone Number	siRNA sequence	Target sequence in mRNA (bp)
PRMT1	A	3'-TTTCGAATGATGAAACTGAGG	145-167
	B	3'-TTCTAGCAGTTTCGGTTGTTC	379-401
	C	3'-TTTCGGTTGTTCAATCTGGTA	389-411
	D	3'-TTTGTCGACCAGTGGTTGCGG	721-743
PRMT5	A	3'-TTCTTCCCTAAAGGACAAGAA	808-830
	B	3'-TTTCGAAAGAGGTACCTAAGC	342-364
	C	3'-TTTCCTGGGGTAGTTTATGAG	1074-1096
	D	3'-TTCCCTGACCTTATGCGATTA	315-337
p53	-	3'-TTGAGGTCACCATTAGATGA	774-796

Table 2-3 Design of siRNA sequences.

siRNA sequences designed based of mRNA sequences of human PRMT1 and PRMT5. Only the reverse strand of the targating sequences are shown. The vector encoding siRNA against p53 transcripts was included as a control (32).

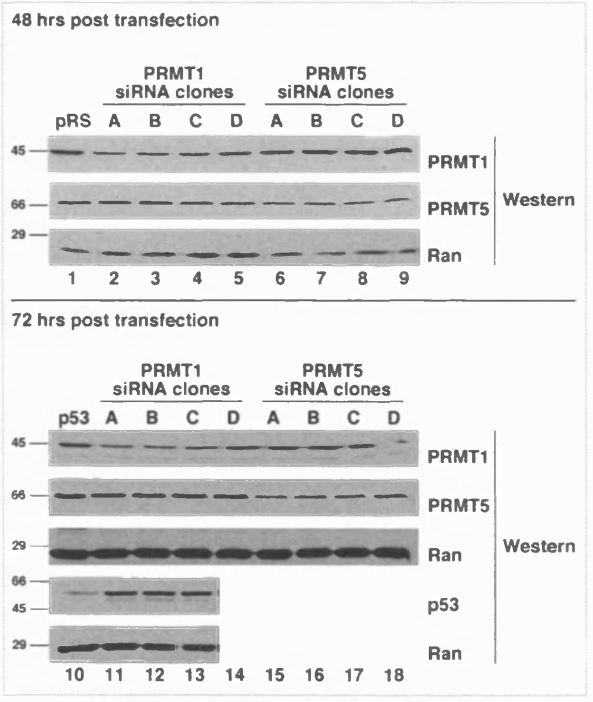

Figure 2-21 Transient expression of siRNA in 293T cells.

293T cells were transfected with plasmids encoding siRNA sequences specific for PRMT1 or PRMT5 (see Table 2-3) and were maintained in culture for 48 hr (Top) or 72 hr (Bottom). The cells were then lysed and the lysate were normalized by Bradford.

Normalized lysates were subjected to Western blot with the indicated antibodies. Western blot of Ran protein levels indicates loading control and a stable clone of 293T cell producing siRNA against p53 was included as control for siRNA knockdown. Lane 18 for the PRMT1 Western is a membrane transfer error.

The sequences were cloned into pRetro.Super plasmid for transient transfection in 293T cells. In cells, the constitutive H1 RNA promoter in the vector drives the transcription of short hairpin RNA (shRNA), which will be processed into the siRNA sequences listed in Table 2-3 (32).

At 48 hr post transfection, the endogenous levels of PRMT1 and PRMT5 were compared (Figure 2-21, Top). The pRetro.Super vector plasmid was included as the negative control because it does not generate any siRNA against protein transcripts. From the Western blots, there was a decrease of PRMT1 levels in cells transfected with the plasmids encoding PRMT1 siRNA (Lane 2-5), and similar PRMT5 decrease was observed for PRMT5 siRNA plasmids (Lane 6-9). However, the extent of knockdown was inadequate.

The minimal decrease in the PRMT protein levels might be attributed to the stability of the PRMT protein present prior to the knockdown. Therefore, the transfection was repeated and proteins were analyzed 72 hr post-transfection (Figure 2-21, Bottom). Instead of the pRetro.Super negative control, a plasmid encoding siRNA against p53 was included; p53 levels in cells transfected with this plasmid displayed distinct and significant knockdown (Lane 10). At 72 hr post transfection, there was a more effective knockdown of PRMT1 and PRMT5 proteins (Lane 11-14 and Lane 15-18 respectively) compared to the 48 hr time point.

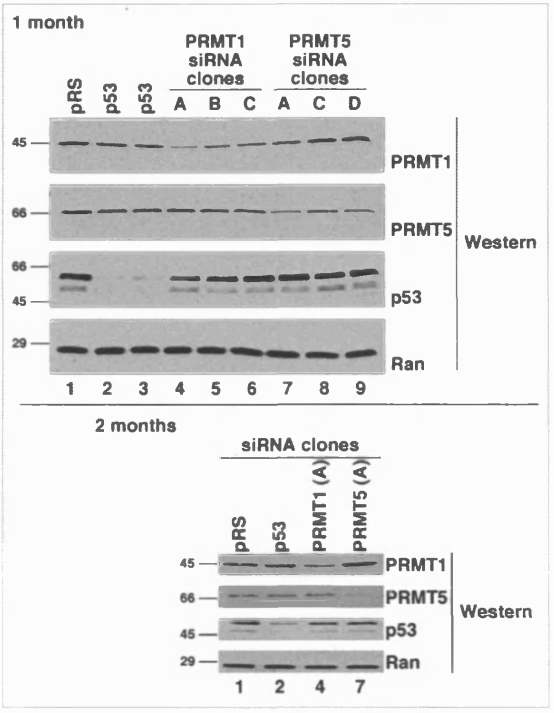

Figure 2-22 Generating the siRNA stable clones.

(Top) 293T cells were transiently transfected with plasmids encoding the indicated siRNA and were selected with puromycin. Positive clones were continuously selected for 1 month and were assayed for knockdown of targeted proteins as for Figure 2-21.

(Bottom) After two months of selection, the clones that displayed the highest level of protein knockdown were kept for further analysis.

Since PRMT1 and PRMT5 proteins appeared to be stable and required a longer time for the siRNA to be effective, stable cell lines producing the siRNA were generated to target the two PRMTs. siRNA sequences that displayed the highest level of knockdown of proteins in the 72 hr transient transfection were chosen to make the stable cells.

Transfected 293T cells were selected for 1 month with puromycin and were assayed for the endogenous level of target proteins (Figure 2-22, Top). The level of protein knockdown was very high in p53 siRNA cells (Lane 2 and 3); this is consistent with the findings of other laboratories (32). For the PRMTs, clone A of PRMT1 siRNA cell line (Lane 4) and clone A of PRMT5 siRNA cell line (Lane 7) were observed to produce the highest level of knockdown of their respective target protein. Thus, these cells lines were further selected for another month before the reanalysis of their protein levels (Figure 2-22, Bottom). After two months, knockdown of PRMT1 and PRMT5 (Lane 4 and 7) proteins were more than 50% and were therefore used for

further experiments. A stable clone containing the empty pRetro.Super vector plasmid (Lane 1) was also generated during the process to serve as the negative control in future experiments.

2.7 Analyzing the methylarginine content of MBD2 produced from PRMT1 and PRMT5 deficient cells

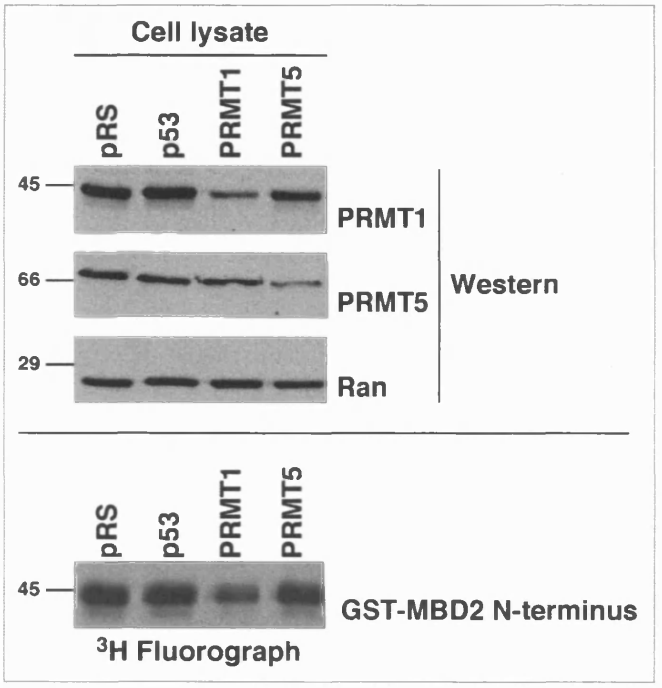

Figure 2-23 *In vitro* methylation assay using lysates from siRNA clones.

Cell lysates from siRNA stable clones in Figure 2-22 were normalized by Bradford assay and Western blot (Top), and were used as the source of methyltransferase for *in vitro* methylation of the GST-MBD2 N-terminus protein as for Figure 2-1 (Bottom).

To test whether the reduced PRMT levels in the lysate drastically affect the protein methyltransferase activity on recombinant MBD2 proteins, the *in vitro* methylation assay was performed (Figure 2-23, Bottom).

From the Fluorograph, it was observed that most lysates were able to methylate the GST-MBD2 N-terminus protein, and only the methyltransferase activity in the PRMT1 siRNA cell line was affected. This was expected, as PRMT1 is the predominant arginine methyltransferase in mammalian cells (180, 216). However, as *in vitro* methylation assay using bacterially produced MBD2 might not represent the

complex regulation of MBD2 arginine methylation in cells, the *in vivo* methylation reaction was performed in the siRNA clones.

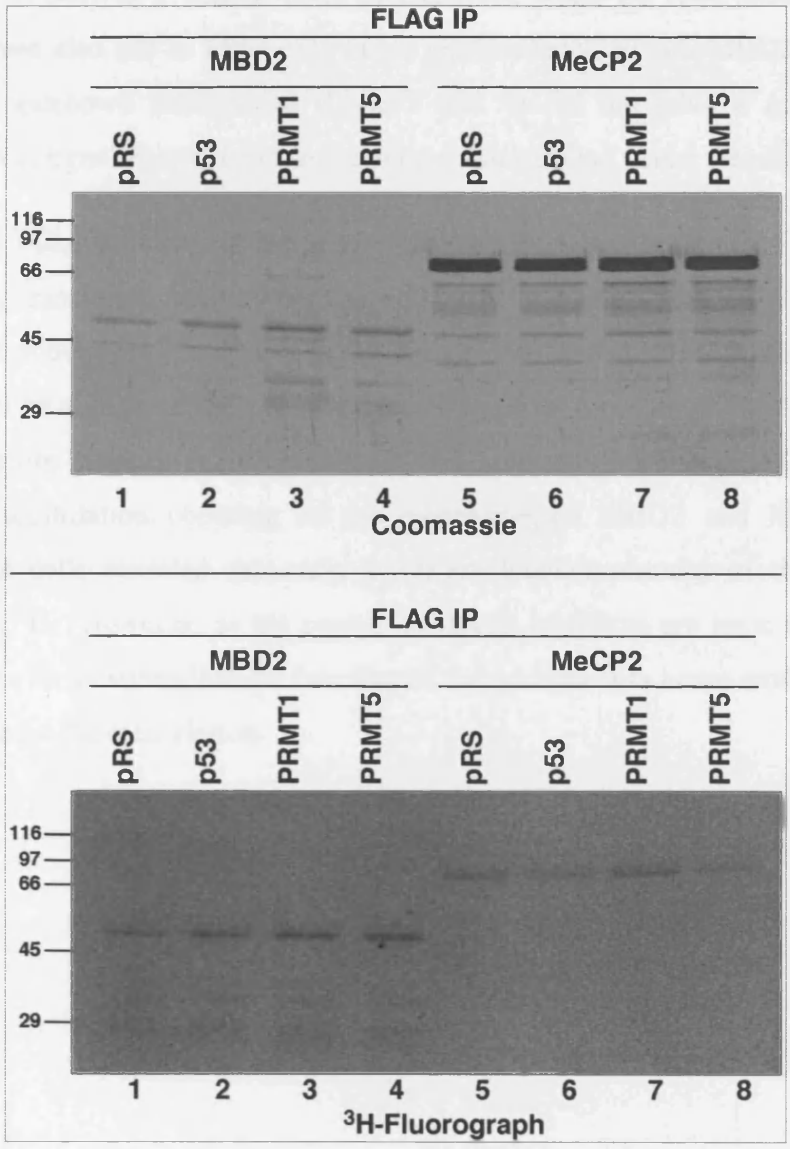

Figure 2-24 *In vivo* labeling assay using siRNA stable clones.

siRNA stable clones in Figure 2-22 were transfected with the indicated MBD constructs and cells were labeled with ^{3}H-methionine in the presence of protein synthesis inhibitors as for Figure 2-5. The proteins were FLAG immunoprecipitated, peptide eluted and normalized by Coomassie. Normalized protein (Top) was subject to Fluorography (Bottom) to detect the amount of ^{3}H-methylated arginine present.

Full-length MBD2 and MeCP2 were transfected into the siRNA clones and were post-translationally labeled in the cells (Figure 2-24). I included MeCP2 to check whether depletion of the PRMTs can have an effect on the methylation of the protein (Lane 7 and 8).

Although there was a reduction in ^{3}H-label on MeCP2 protein produced in PRMT5 knockdown cells (Lane 8), the protein produced in the p53 knockdown background showed a similar level of reduction (Lane 6). For MBD2 protein, the results were also not as clear cut, since, unexpectedly, FLAG-MBD2 labeled in the PRMT knockdown background (Lane 3 and 4) did not have a reduction in ^{3}H-methylation signal compared to in the control background (Lane 1 and 2).

Unlike inhibition of the *in vivo* labeling by Adox (Figure 2-16), where almost all methyltransferase activity in the cells was abolished, the siRNA clones only partially removed the PRMTs. The remaining PRMT1 and PRMT5 protein in the cells might still be able to saturate the available sites of methylation on the MBD2 proteins, and therefore produce no difference in the amount of labeling. This is possible because scintillation counting of the overexpressed MBD2 and MeCP2 proteins labeled in cells revealed that only 1:100 (molecule/molecule) of the total protein contained ^{3}H. However, as the protein synthesis inhibitors are toxic to the cells, the time given for posttranslational labeling of the proteins was never extended beyond 3 hr to increase the methylation.

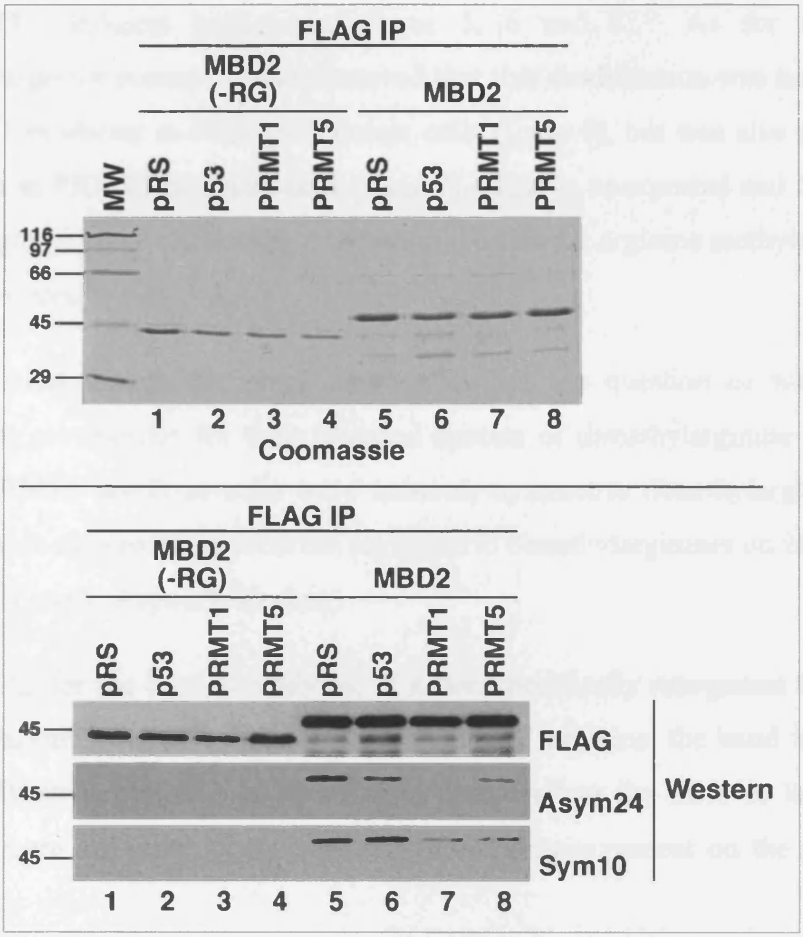

Figure 2-25 MBD2 expressed in PRMT deficient cells has reduced methylarginine content.

FLAG-MBD2 proteins were transiently transfected in to siRNA clones in Figure 2-22, and were purified and normalized as for Figure 2-15. Normalized proteins were analyzed with Coomassie (Top) and Western blot (Bottom) for the presence of methylarginine.

Using the Asym24 and Sym10 antibodies against purified MBD2 provides a more comprehensive assessment of the methylarginine content of the MBD2 proteins. In this instance, the siRNA clones were maintained under normal growth conditions throughout, and the FLAG-MBD2 and FLAG-MBD2-RG proteins were purified 48 hr post-transfection (Figure 2-25).

MBD2 with removal of RG domain was not recognized by the antibodies; this is similar to previous observations (Lane 1-4). Full-length MBD2 protein from the pRS or the p53 deficient cells was not affected in methylarginine content (Lane 5 and 6). The asymmetric dimethylarginine content of MBD2 from PRMT1 deficient siRNA cells (Lane 7) was completely abolished when compared to cells of control (pRS), p53

or PRMT5 deficient background (Lane 5, 6 and 8). As for the symmetric dimethylarginine content, it was observed that this modification was not only reduced in MBD2 produced in PRMT5 deficient cells (Lane 8), but was also reduced in that produced in PRMT1 deficient cells (Lane 7). This is unexpected and I speculate that there might be some regulation, or sequential order for arginine methylation of MBD2 protein to occur.

Even though the result was surprising, the question of whether the two antibodies are specific for their intended species of dimethylarginine was answered. When PRMT5 levels in cells were reduced, symmetric dimethylarginine levels on MBD2 were also reduced, whereas asymmetric dimethylarginines on MBD2 were not affected (Lane 8, Asym24 Western).

As for the Sym10 antibody, if it non-specifically recognizes the asymmetric dimethylarginine modifications present on MBD2 proteins, the band in lane 8 of the Sym10 Western blot should be stronger compared to the band in lane 7. This is because there are more asymmetric dimethylarginines present on the protein sample (Lane 8).

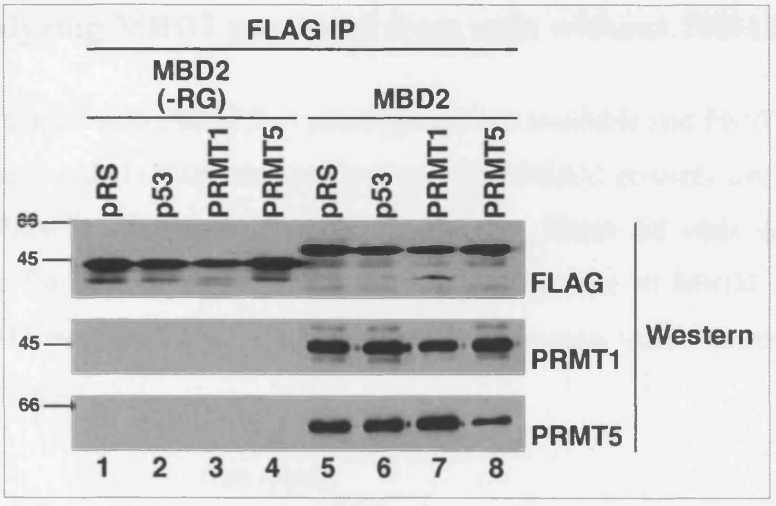

Figure 2-26 Reduction of PRMT1 in cells do not decrease MBD2-PRMT5 interaction.

FLAG-MBD2 proteins were transiently transfected to siRNA clones in Figure 2-22 and were purified and normalized as for Figure 2-15. Normalized proteins complexes were analyzed with Western blot for the presence of co-purifying PRMTs.

To understand how knocking down PRMT1 resulted in a decrease of symmetric methylation in MBD2 proteins, the MBD2 produced in PRMT deficient siRNA cells was tested for their ability to co-immunoprecipitate PRMTs (Figure 2-26).

As shown previously (Figure 2-15), MBD2 without the RG domain was incapable of binding PRMTs (Lane 1-4). Full-length MBD2 produced in PRMT1 deficient cells (Lane 7) displayed a decrease in the amount of co-purifying PRMT1. Likewise, co-purifying PRMT5 was distinctly decreased in cells deficient of the enzyme (Lane 8).

Since there was a decrease in symmetric dimethylarginines on MBD2 produced in PRMT1 deficient cells (Figure 2-25, Lane 7), I would expect to see a decrease in the levels of co-purifying PRMT5 (Figure 2-26, Lane 7). However, this was not observed. Therefore, I was only able to conclude that in PRMT1 deficient cells, PRMT5 was binding to MBD2 proteins but was unable to add symmetric dimethylarginine to the protein. To further understand the relationship between PRMT1 and PRMT5 in methylation of MBD2 protein, the PRMT1 knockout embryonic stem cells was employed.

2.8 Analyzing MBD2 produced from cells without PRMT1

As mice with PRMT5 -/- genotype are not available and PRMT1 mutant mice are embryonic lethal (180), characterization of the MBD2 proteins can only be carried out with PRMT1 -/- embryonic stem (ES) cells. These ES cells were difficult to manipulate for protein expression but are a good source of MBD2 protein (Figure 2-27, MBD2 western, Lane 1-4). Therefore, endogenous MBD2 protein was purified from these cells.

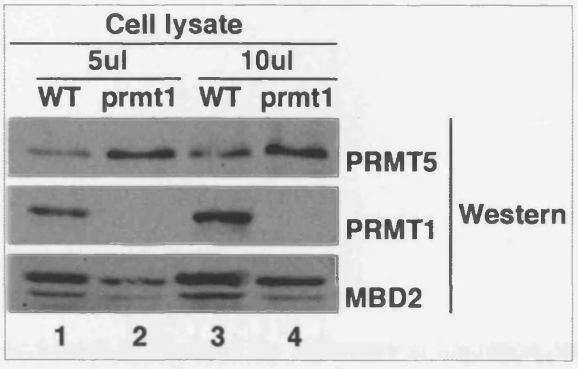

Figure 2-27 Analysis of PRMT levels in wild-type and PRMT1 -/- embryonic stem cells.

Bradford normalized ES cells were analyzed by Western blots with the indicated antibodies to detect for the presence of the proteins.

In the PRMT1 -/- ES (prmt1) cell lysates (Lane 2 and 4), the PRMT1 protein was not observed in the Western blot as expected. There was also an unexpected increase in PRMT5 protein level in the lysate compared to wild-type (Lane 1 and 3); this phenomenon was not reported previously, as researchers using the cells were seldom studying PRMT5.

With careful normalization of the samples for the Western blot, I determined that MBD2 protein levels in prmt1 cells (Lane 2 and 4) were slightly lower than wild-type cells. Furthermore, the MBD2 protein bands from prmt1 cells also seemed to migrate faster in SDS-PAGE gels. As arginine methylation does not drastically affect the overall charge or molecular weight of the target protein (77), arginine methylated proteins and their unmodified counterparts are generally believed to migrate similarly during one dimensional gel electrophoresis (97). However, the MBD2 protein contains an extensive RG domain that can be asymmetrically and symmetrically methylated, and since this is unique amongst most arginine methylated proteins, further tests were done to confirm the difference in migration of the two MBD2 proteins[11].

[11] For details of test performed to illustrate the difference in migration, refer to Figure 6-5 in Chapter 6.4.

2.8.1 MBD2 from prmt1 ES cells do not contain asymmetric dimethylarginine

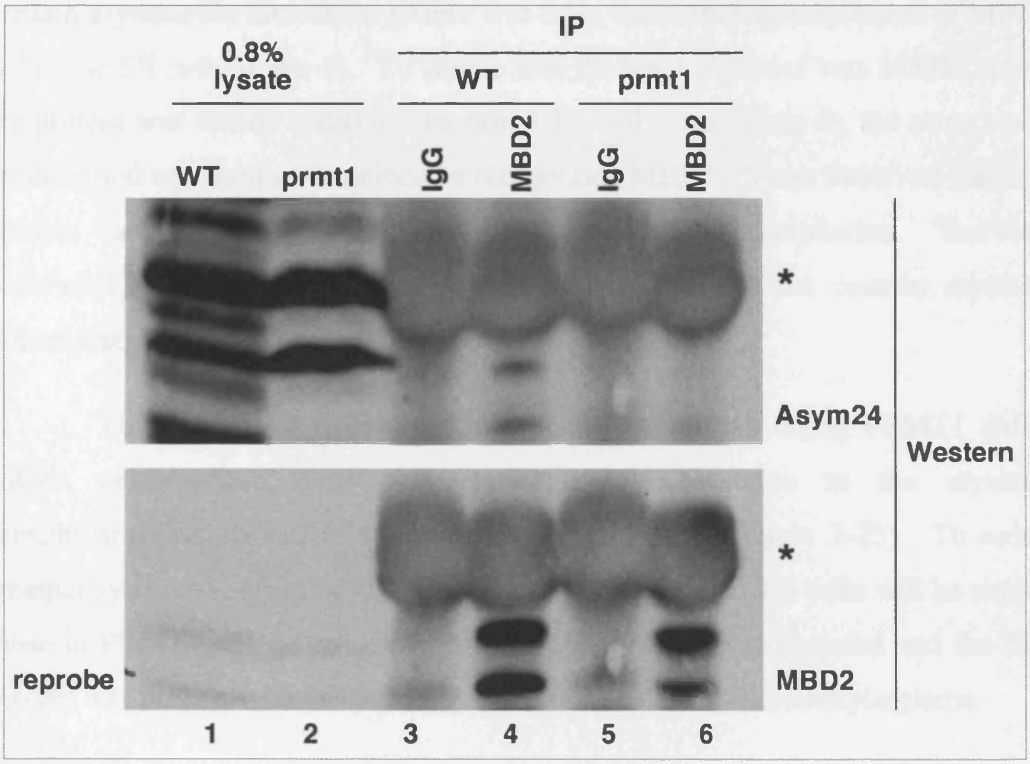

Figure 2-28 Analysis of the asymmetric dimethylarginine content of MBD2 from PRMT1 knockout cells.

(Top) Endogenous MBD2 protein was purified from wild-type and prmt1 ES cells (3 X 10^8) cultured under normal growth conditions. The purified MBD2 and control samples were analyzed by Western blot for the presence of asymmetric dimethylarginines using the Asym24 antibody.

(Bottom) After Asym24 Western blot, the membrane was stripped and reprobed with antibody against MBD2 protein.

(*) Denotes antibody heavy chain.

To thoroughly understand the relationship between PRMT1 and PRMT5 in methylation of MBD2 and the modifications present on MBD2 from the wild-type and prmt1 cells, endogenous MBD2 proteins were purified and analyzed for their asymmetric dimethylarginine content (Figure 2-28).

From the Asym24 Western blot, the lysate of prmt1 ES cells contained less protein carrying the asymmetric dimethylarginine modification (Lane 2) compared to lysate from wild-type ES cells (Lane 1). This probably reflects the loss of asymmetric dimethylarginine content in the cellular PRMT1 substrates proteins.

At the position where MBD2 migrates, the only band that was detected to contain asymmetric dimethylarginines was from the immunoprecipitation of MBD2 in wild-type ES cells (Lane 4). To ensure that the band observed was MBD2, and that the protein was indeed isolated from prmt1 ES cell lysate (Lane 6), the same blot was stripped and reprobed with antibodies recognizing MBD2. It was observed that MBD2 protein was purified from both cell lysates by immunoprecipitation. Therefore, I concluded that MBD2 purified from prmt1 ES cells did not contain asymmetric dimethylarginines.

This finding is similar to the previous experiment using PRMT1 deficient siRNA cells where there was an observable reduction in the asymmetric dimethylarginine content of the purified FLAG protein (Figure 2-25). To ascertain whether symmetric arginine methylation of MBD2 in prmt1 ES cells will be similar to those in PRMT1 siRNA cells, the immunoprecipitation was repeated and the MBD2 protein was probed with the antibody specific for symmetric dimethylarginine.

2.8.2 MBD2 from prmt1 ES cells have higher symmetric dimethylarginine content

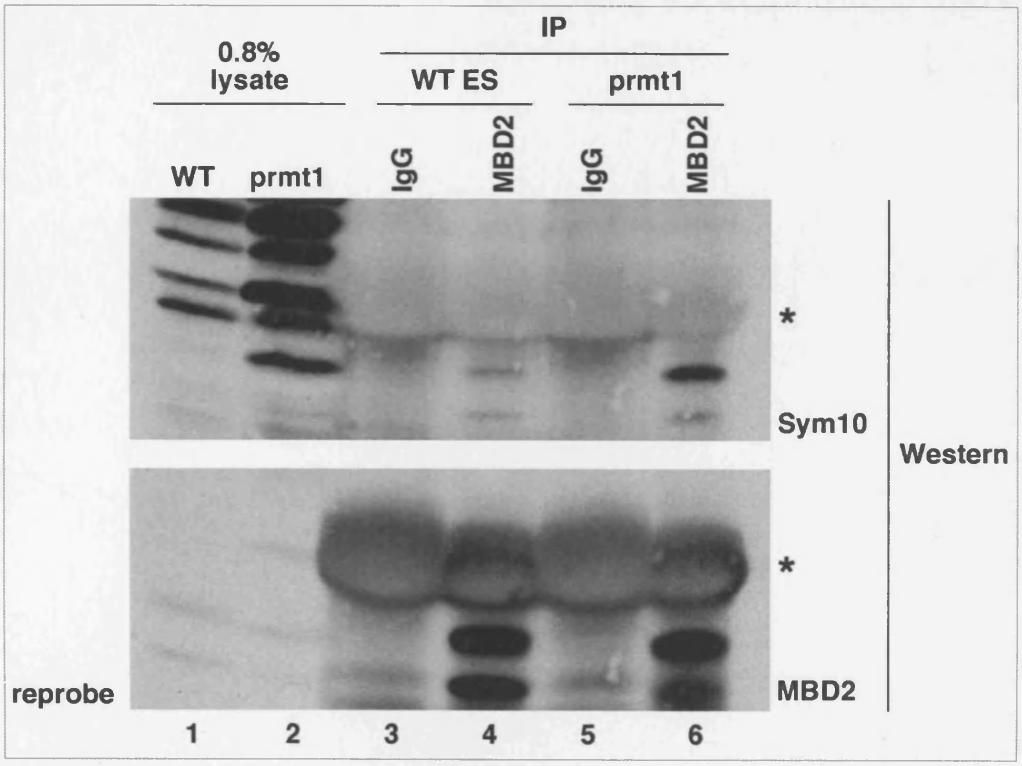

Figure 2-29 Analysis of the symmetric dimethylarginine content of MBD2 from PRMT1 knockout cells.

Endogenous MBD2 protein was purified as for Figure 2-28 and analyzed for the presence of symmetric dimethylarginine using Sym10 antibodies.

From the Sym10 Western blot (Figure 2-29), it was found that MBD2 immunopurified from prmt1 ES cells (Lane 6) contains higher content of symmetric dimethylarginine compared to protein from wild-type ES cells (Lane 4). The amount of immunoprecipitated MBD2 protein from both cell lines appeared similar when the Western blot was reprobed with MBD2 antibody. This result directly contradicts the findings from the siRNA cells (Figure 2-25), where a reduction in PRMT1 levels in cells caused an observed reduction in the PRMT5 ability to symmetrically methylate arginine residues in MBD2.

When comparing the normalized lysates of both ES cell lines in the Sym10 Western blot (Lane 1 and 2), it was observed that total proteins in prmt1 ES cells generally contains more symmetric dimethylarginines. As complete knockout of PRMT1 in ES cells resulted in an increase in PRMT5 protein levels (Figure 2-27), this might explain the increase in symmetric arginine methylation of all PRMT5 substrate proteins. Like the other cellular proteins observed in Lane 2, MBD2, a PRMT5 substrate protein, was most probably affected in the same manner.

Chapter 3 Results II - Functions of MBD2 methylation

3.1 Functional analysis of MBD2 arginine methylation

As the RG domains of the two MBD proteins are methylated *in vivo*, and the PRMT activity on MBD2 is characterized, further experiments were designed to investigate the effects of arginine methylation on the functions of the proteins.

Various roles of MBD2 in cells were reported in the literature, including the demethylation of methyl-cytosine in CpG-DNA (19), transcriptional activation (7, 133) and transcriptional repression (65, 170). Among these reports, evidence presented on the transcriptional repressor function is the most substantial. This is because in HeLa nuclear extracts the majority (> 90%) of endogenous MBD2 stably interacts with the HDAC silencing complex (NuRD) to form the 1 MDa MeCP1 (65, 157, 242) (Figure 1-5). Considering the ease in co-purifying components of the HDAC silencing complex with MBD2, I asked whether arginine methylation of MBD2 affects the stability of this interaction.

3.2 Arginine methylation of MBD2 and interaction with HDAC silencing complex

3.2.1 MBD2 with reduced methylated arginine content has higher affinity for HDAC silencing components

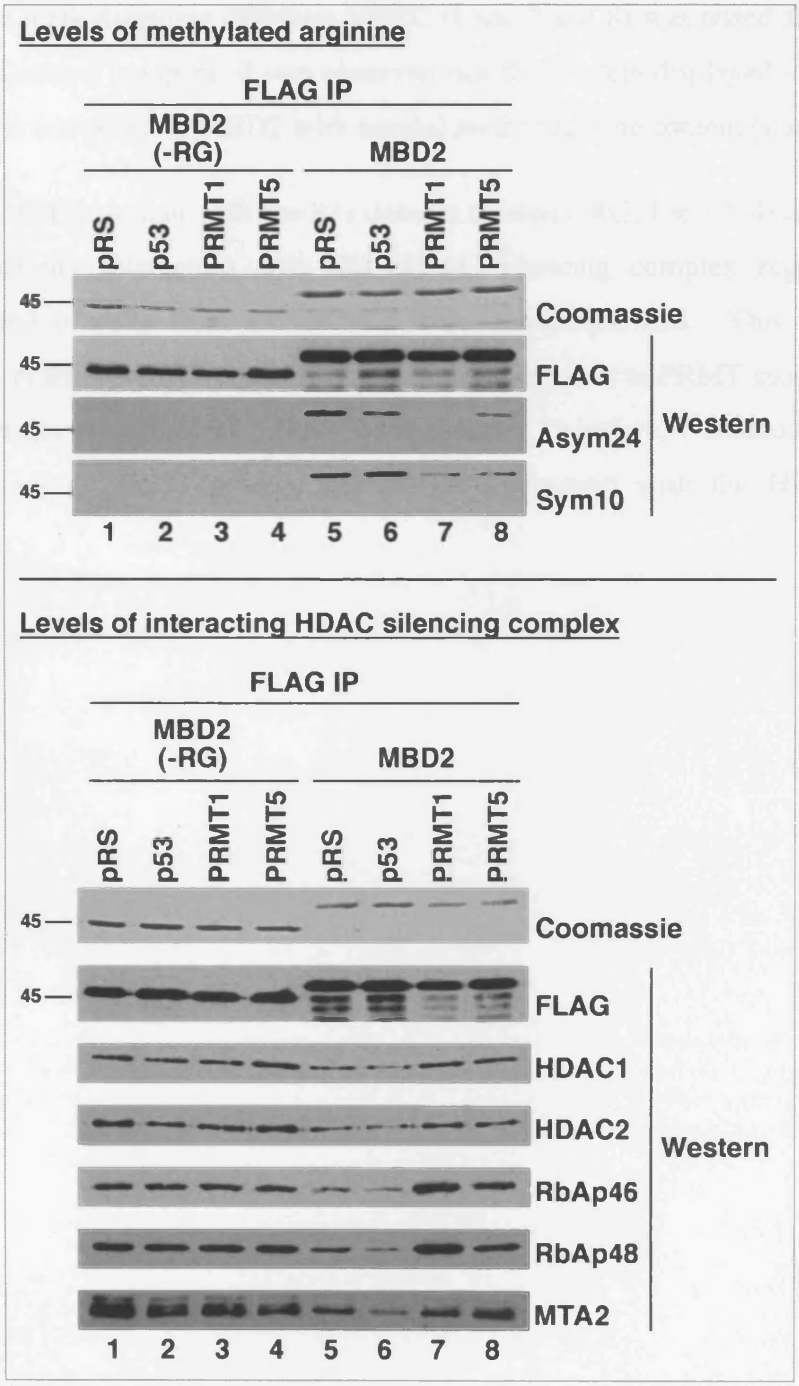

Figure 3-1 MBD2 with reduced methyl arginine content have has higher affinity for HDAC silencing complex.

FLAG-MBD2 proteins were transiently transfected in to siRNA stable cell lines (Figure 2-22) **and were prepared as for** Figure 2-15. **Normalized proteins complexes were analyzed by Western blot for the presence of co-purifying HDAC silencing complexes (Bottom).** Figure 2-25 **is reproduced here (Top) to show the level of methylated arginine on MBD2.**

As described previously (Figure 3-1, Top), expression of FLAG-MBD2 in PRMT deficient cells resulted in a decrease in methylarginine content of the protein. When the methylarginine deficient MBD2 (Lane 7 and 8) was tested for co-purifying HDAC silencing complex, it was observed that this protein displayed a higher affinity interaction compared to MBD2 with normal methylarginine content (Lane 5 and 6).

MBD2 protein with the RG domain deleted (-RG, Lane 1-4) also displayed a higher affinity interaction with the HDAC silencing complex regardless of the background of cells from which they were immunopurified. This is because the mutation in these MBD2 proteins made them impervious to PRMT modifications, and this overrides the effect of cellular background. Therefore, I conclude that arginine methylation of MBD2 protein disrupts its interaction with the HDAC silencing complex.

3.2.2 MBD2 with reduced methylated arginine content copurifies with higher HDAC activity

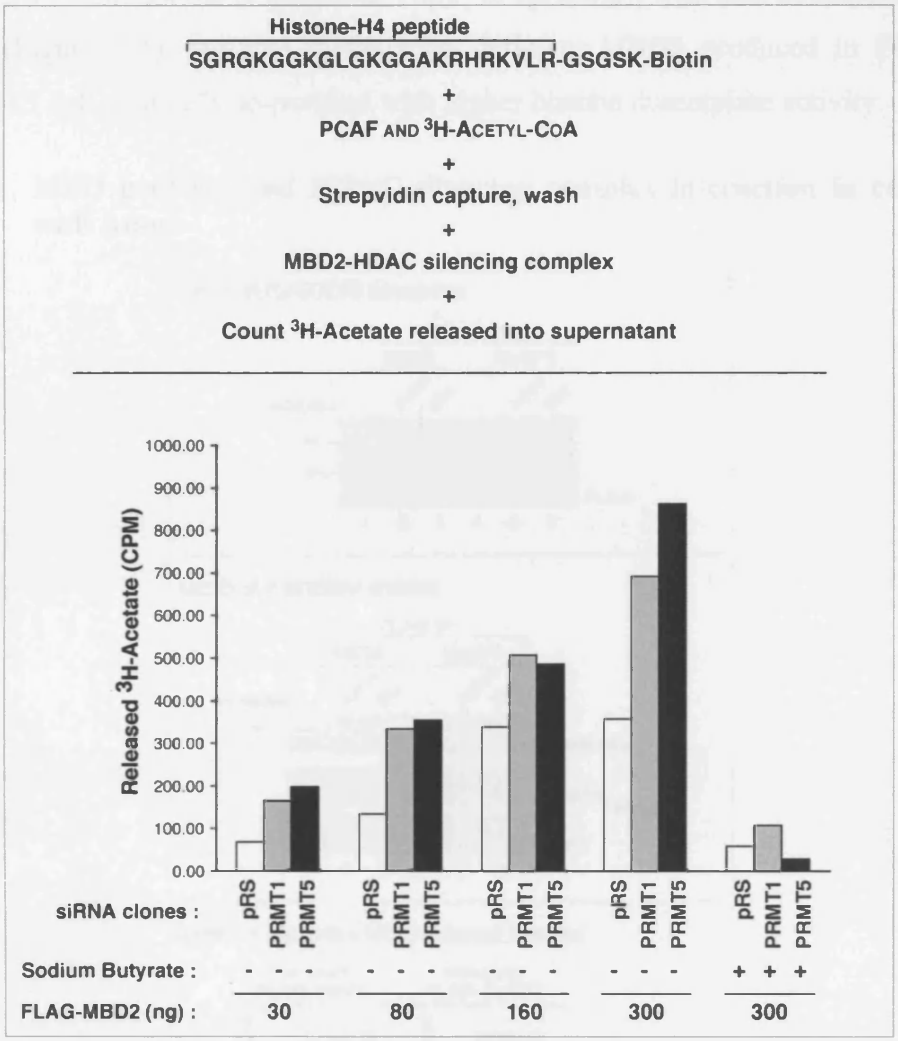

Figure 3-2 Measurement of histone deacetylation activity of MBD2-protein complexes.

(Top) The scheme of the histone deacetylation assay.

(Bottom) FLAG-MBD2 complexes prepared as for Figure 3-1 were incubated with ^{3}H-acetylated histone H4 peptide at 37°C for 3 hr with the indicated amount of protein (ng) and in the absence or presence of the HDAC inhibitor Sodium Butyrate (250mM). Release of ^{3}H-acetate (CPM) from the histone H4 peptide was determined by scintillation counting.

As the functional association of HDAC1, HDAC2, RbAp46, RbAp48 and MTA2 is essential for histone deacetylase activity (242), full-length MBD2 with reduced methylarginine content (Figure 3-1, Lane 7 and 8) was compared against normal MBD2 (Figure 3-1, Lane 5) for co-purifying histone deacetylase activity.

The immunoprecipitated native protein complexes were incubated with histone H4 peptide ^{3}H-acetylated *in vitro*, and the release of ^{3}H-acetate groups into solution was measured (Figure 3-2, Top). In agreement with data obtained by Western blot (Figure 3-1), arginine methylation deficient MBD2 produced in PRMT1 and PRMT5 deficient cells co-purified with higher histone deacetylase activity.

3.2.3 MBD proteins and HDAC silencing complex interaction in cells treated with Adox

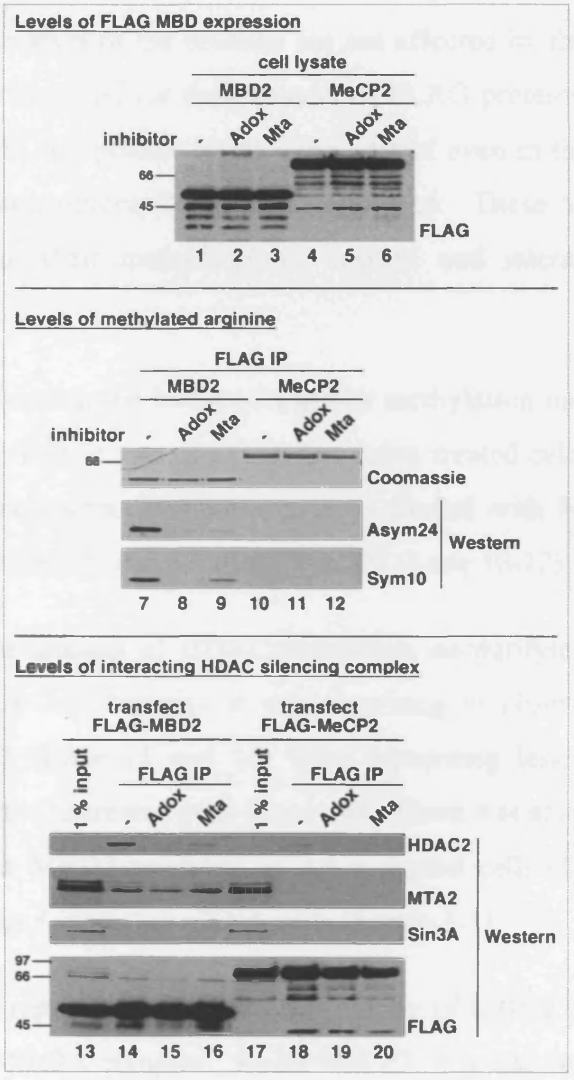

Figure 3-3 Creating arginine methylation deficient MBD proteins by treating cells with methyltransferase inhibitors.

(Top) 293T cells were transfected with the indicated MBD plasmids for 36 hr and were treated with the indicated methyltransferase inhibitors for another 12 hr before harvesting. Expression levels of the MBD proteins were checked in Bradford normalized lysate.

(Middle) FLAG-MBD proteins were prepared as for Figure 2-15 and were checked for methylation status using the Asym24 and Sym10 antibodies.

(Bottom) The co-purifying protein complexes were also checked for their interaction with HDAC silencing complex by Western blot with the indicated antibodies. 1% input represents the lysate from untreated cells used for immunoprecipitation of the protein.

As arginine methylation deficient MBD2 proteins have a higher affinity interaction with the HDAC silencing complex, I investigated whether unmethylated species of MeCP2 proteins would also produce the same effect.

Non-methylated species of both MBD proteins were produced by treating cells with methylation inhibitors, Adox or 5'-methyl-thioadenosine (Mta). To ensure that the expression level of the proteins are not affected by the inhibitors, normalized cell lysates were compared for their amount of FLAG proteins expressed (Figure 3-3, Top, Lane 1-6). FLAG protein levels were normal even in the presence of inhibitors and so FLAG immunoprecipitation was performed. These MBD protein complexes were analyzed for their methylarginine content and interacting HDAC silencing complexes.

When assessing the levels of arginine methylation on MBD2 protein (Figure 3-3, middle, Lane 7-9), it was observed that Adox treated cells produced MBD2 with less methylarginine content compared to cells treated with Mta (Lane 8 vs. 9). As expected, these antibodies did not detect MeCP2 (Lane 10-12).

When the amount of HDAC complexes co-purifying with the MBD2 was determined (Figure 3-3, bottom), it was surprising to observe that hypomethylated species of MBD2 (Lane 15 and 16) were interacting less strongly with HDAC2 proteins compared to untreated cells (Lane 14). There was also less MTA2 protein co-purifying with the MBD2 produced in Adox treated cells (Lane 15). This directly contradicts findings from using siRNA cells (Figure 3-1).

Sin3A is reported as an interacting partner of MBD2 (23), but I was unable to purify the MBD2-Sin3A complex. As for MeCP2, it is also reported to be in the same complex as Sin3a and HDAC2 (166), but in the experiment, I was only able to co-purify HDAC2 with the protein (Lane 18). This may be because the MeCP2-Sin3A complex does not exist stably (116). However, from the HDAC2 Western blot, it was observed that MeCP2 protein produced in cells treated with methylation inhibitors (Lane 19 and 20) also revealed a decrease in the level of co-purifying HDAC2 protein.

3.2.4 Adox disrupts the stability of the HDAC silencing complex

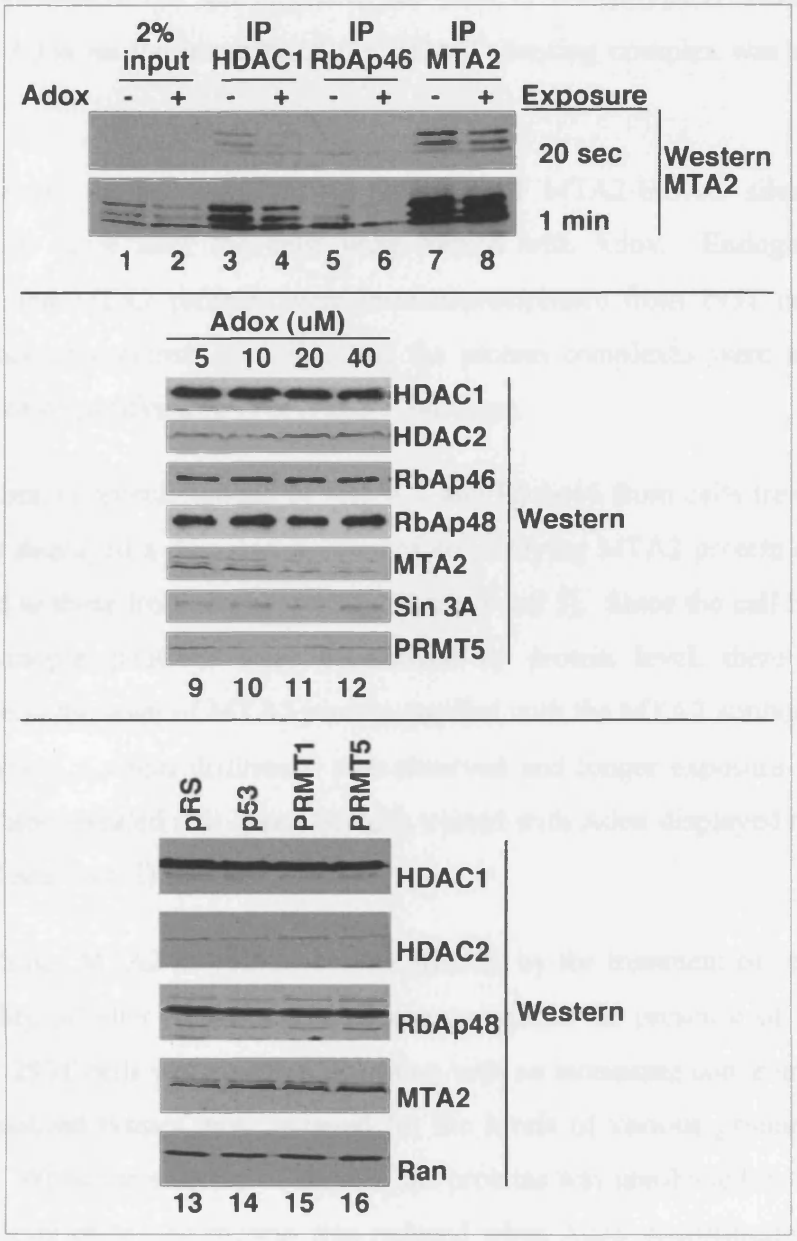

Figure 3-4 Adox affects NuRD integrity.

(Top) Control and 293T cells treated with Adox for 16 hr were lysed, normalized with Bradford, and subjected to immunoprecipitation for endogenous HDAC silencing complexes using the indicated antibodies. The immunoprecipitated complexes were analyzed by Western to detect for presence of co-purifying MTA2 protein. As the MTA2 signal is weak in Western blots, a longer exposure of the same blot was included.

(Middle) Adox affects MTA2 protein levels. 293T cells were subjected to an Adox titration assay for 16 hr and were lysed, normalized with Bradford and assayed with Western blots to check the level of each HDAC silencing complex component.

(Bottom) MTA2 levels in siRNA stable clones remain unchanged. siRNA stable clones as in Figure 2-22 were cultured under normal conditions and were subjected to Western blots with the indicated antibodies. Western blot of Ran indicates protein loading.

To determine whether using the methylation inhibitors or the siRNA cells is more representative of the physiological state of unmethylated MBD proteins, the effect of Adox on the integrity of the HDAC-silencing complex was studied (Figure 3-4).

First, I asked whether the integrity of MTA2-HDAC silencing complex remains the same after the cells were treated with Adox. Endogenous HDAC1, RbAp46, and MTA2 proteins were immunoprecipitated from 293T cells cultured in the absence or presence of Adox, and the protein complexes were assayed for the presence of co-purifying MTA2 (Figure 3-4, Top).

Immunoprecipitations of HDAC1 and RbAp46 from cells treated with Adox generally displayed a decrease in level of co-purifying MTA2 protein (Lane 4 and 6) compared to those from untreated cells (Lane 3 and 5). Since the cell lysates used for the immunoprecipitations were normalized by protein level, there should be no difference in the level of MTA2 protein purified with the MTA2 antibody (Lane 7 and 8). However, a slight difference was observed and longer exposure (1 min) of the Western blot revealed that lysate of cells treated with Adox displayed reduced MTA2 protein (Lane 1 vs. 2) as well.

Since MTA2 protein level was affected by the treatment of cells with Adox, the stability of other HDAC silencing components in the presence of Adox was also assayed. 293T cells were titrated in culture with an increasing concentration of Adox, and normalized lysates were assayed for the levels of various proteins (Figure 3-4, Middle). While the stability of most of the proteins was unaffected by Adox (Lane 9-12), the level of MTA2 protein was reduced when Adox concentration exceeded 20 μM (Lane 11), which is the usual concentration administered to cells. Therefore, I conclude that MBD2 produced in Adox treated cells are not suitable for assessing the levels of co-purifying HDAC-silencing complex. This is because the instability of one component protein most probably affects the integrity of the whole silencing complex.

The same comparison was also carried out from normalized lysate of the siRNA clones (Figure 3-4, Bottom). It was observed that the level of MTA2 and other cellular proteins was not affected by the genetic background of the cells. This

strengthens the conclusions in Figure 3-1 because the integrity of HDAC silencing complex components in the siRNA cells is not affected.

3.2.5 MBD2 hypermethylated on arginine copurifies less HDAC silencing components

3.2.5.1 Increasing the methylarginine content of MBD2 by increasing the amount of PRMT in cells

Since MBD2 protein with less methylarginine content tends to have higher affinity for HDAC silencing complex, the reverse was also tested by increasing the methylarginine content of MBD2. To achieve this, the level of PRMT in cells was increased by transfecting 293T cells that were expressing MBD2 proteins with the PRMT plasmids (see Figure 6-6 in Chapter 6.4). However, although there was an increase in PRMT protein levels, there was no observable increase in the methylarginine content of MBD2.

3.2.5.2 Increasing the methylarginine content of MBD2 by mutating the RG domain

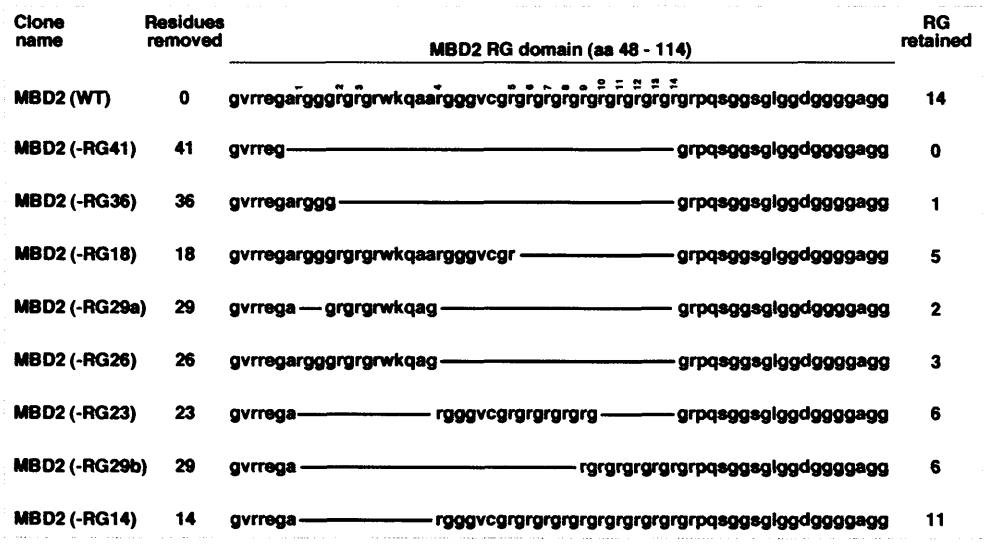

Figure 3-5 Mutants of the MBD2 RG domain generated in this study.

RG domain of the wild-type MBD2 protein (aa 48 – 110) and the 14 arginine residues within that fall in the context of RG. Sequences of the 8 MBD2 RG domain mutants are shown, black horizontal lines represent deleted residues and red letters represent substituted residues.

All experiments suggested that methylation of arginine residues within the extensive RG domain of MBD2 protein is a tightly regulated event. In an attempt to create intermediates between the wild-type MBD2 with 14 possible arginines for methylation, and the MBD2-RG mutant[12] containing no arginine for the modification, I modified the RG domain of the MBD2 protein to retain some arginine residues that are possible sites of PRMT modification (Figure 3-5). Unexpectedly, these proteins were found to have a higher stoichiometry of arginine methylation, and this is illustrated in the following sections. Biochemical properties of these hypermethylated mutants were also characterized.

[12] Refer to Figure 2-5 and Figure 2-20 for MBD2-RG mutants that do not contain the 14 arginines that are required for accepting methyl groups from PRMT.

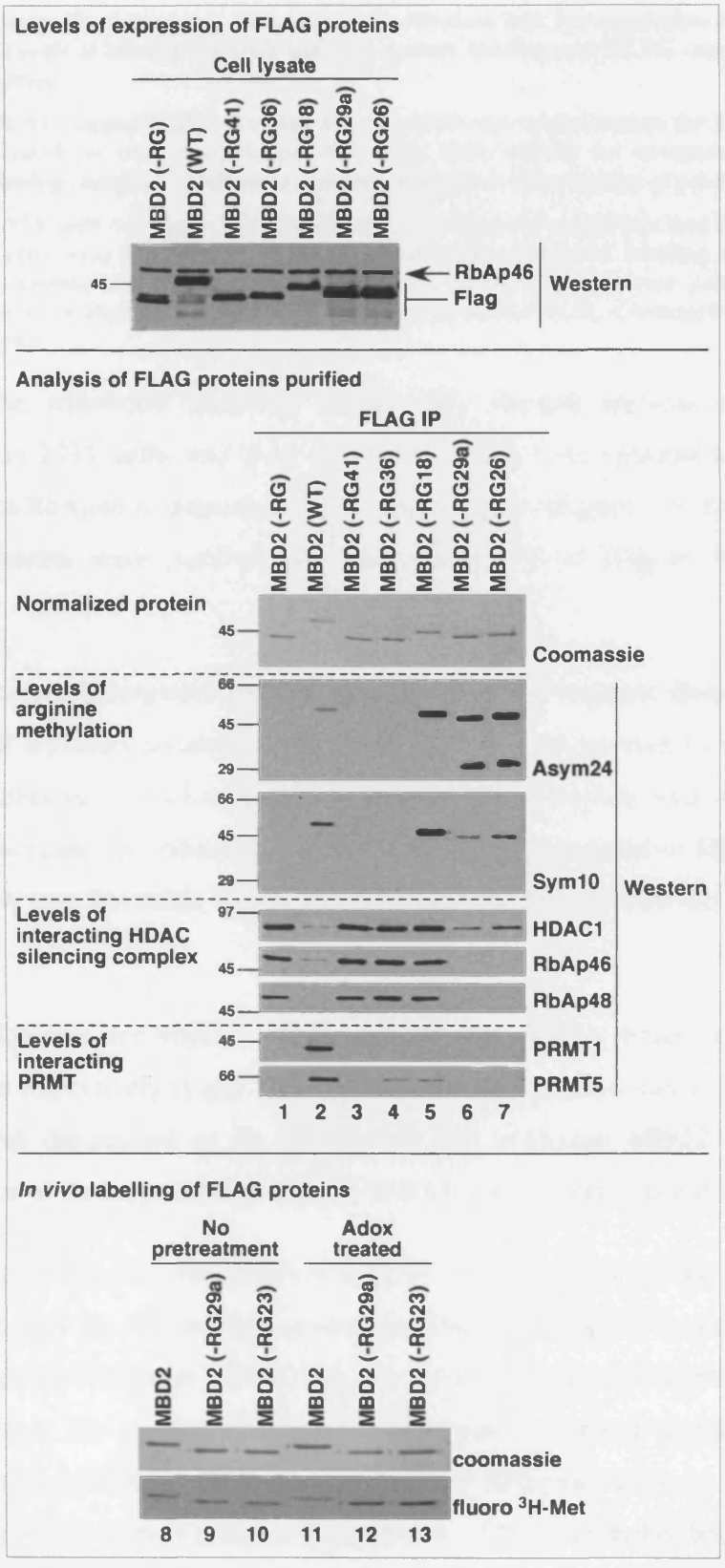

Figure 3-6 Highly methylated MBD2 have less affinity for the HDAC silencing complex.

(Top) 293T cells were transfected with plasmids encoding FLAG-tagged MBD2 and its RG deletion mutants described in Figure 3-5 for 48 hr. Cells were lysed and the

Bradford normalized lysates were analyzed by Western blot for expression level of the proteins. Levels of RbAp46 protein indicate protein loading and FLAG represents the MBD2 proteins.

(Middle) FLAG-tagged MBD2 proteins were purified and normalized as for Figure 2-15 and were tested for their methylation status and their affinity for components of the HDAC silencing complex. Coomassie staining indicates normalization of proteins.

(Bottom) 293T cells were also transfected with the indicated constructs and a duplicate set of the cells were treated with Adox prior to posttranslational labeling with ^{3}H-L-methyl-methionine and CX/CP as for Figure 2-5. FLAG-proteins were prepared and normalized as in Figure 2-15 **and were analyzed by SDS-PAGE, Coomassie stain and Fluorography.**

The constructs encoding various RG domain mutants of MBD2 were expressed in 293T cells, and their expression levels were checked together with the endogenous RbAp46 to provide sample normalization (Figure 3-6, Top). The FLAG-MBD2 proteins were purified and were characterized (Figure 3-6, Middle and Bottom).

For methylarginine content, the two deletion mutants described previously that had all arginines removed (Lane 1 and 3) were not detected by the Asym24 and Sym10 antibodies. Wild-type MBD2 protein was observed with normal levels of methylation (Lane 2). Methylarginine signal was not detected in MBD2 (-RG36) as there is only one RG motif, which is insufficient for detection by the antibodies (Lane 4).

However, for MBD2 –RG18, -RG29a and –RG26, which contain 5, 2 and 3 RG repeats respectively (Lane 5-7), their asymmetric and symmetric dimethylarginine content was determined to be higher than the wild-type MBD2 (Lane 2). The significance of the Asym24 degradation band (in Lane 6 and 7) is not known.

To ensure that the observed increase in recognition of the hypermethylated MBD2 mutants by the methylarginine specific antibodies is a result of increased methyl arginine content of MBD2, and not due to the nature of mutation that created a better epitope, the *in vivo* methylation assay was performed (Figure 3-6, Bottom). Under normal conditions, there was no dramatic difference between the ^{3}H-signal on the wild-type and mutant proteins (Lane 8-10). This is probably because the MBD2 proteins have reached their maximum potential to be methylated *in vivo*. A duplicate set of transfected cells was also inhibited for arginine methylation by addition of Adox to the medium post transfection, and the inhibitor was removed prior to labeling. The

Fluorograph shows that these hypermethylated mutants are indeed better acceptors of the ^{3}H-methyl groups compared to wild-type MBD2 (Lane 11-13).

The hypermethylated MBD2 mutants, together with data gathered from siRNA knockdown cells (Figure 2-25) and PRMT1 knock out ES cells (Figure 2-29), confirm the complexity of this modification in MBD2 and suggest that within the pool of transiently expressed MBD2, only a fraction contains methylated arginine.

Using the array of mutants with varying methylarginine content, the amount of co-purifying HDAC complex was investigated (Figure 3-6, Middle). As expected, mutant proteins (Lane 1, 3 and 4) that contain either low or no methylarginine displayed higher affinity for HDAC silencing complexes compared to wild-type MBD2. Hypermethylated mutants (Lane 5-7) generally have less affinity for the complex, with the exception of MBD2-RG18 (Lane 5), where the amount of co-purifying proteins seemed higher than for wild-type MBD2.

Since mutants in Lane 5, 6 and 7 were highly methylated, the presences of co-purifying PRMTs within the complex were also probed. Although these mutants were highly modified by the PRMTs, they did not interact strongly with PRMT1 or PRMT5. Wild-type MBD2 is the only protein that is capable of positive interaction with the two methyltransferases (Lane 3). The presence of PRMT5 in Lane 4 and 5 seems to be a result of non-specific binding as subsequent purification of the two mutant proteins did not observe any co-purifying PRMT5 (Data not shown).

3.2.5.3 Additional characterizations of the RG domain mutants

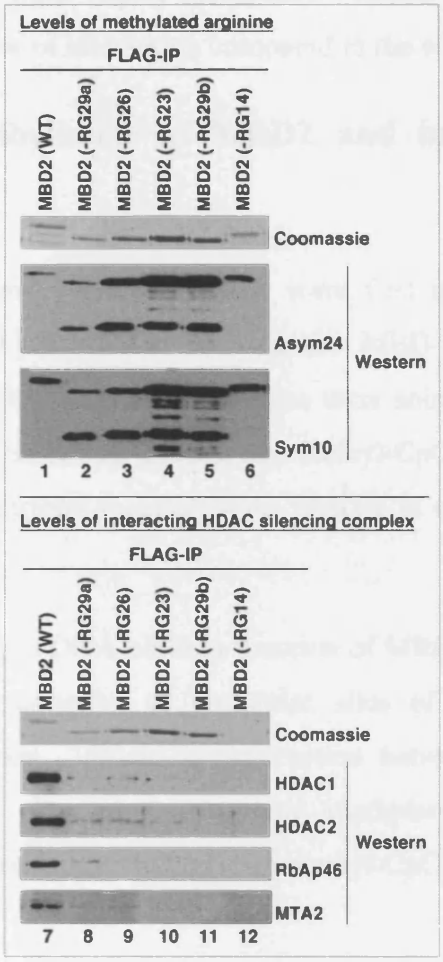

Figure 3-7 Highly methylated MBD2 have less affinity for HDAC silencing complex.

Experiment in Figure 3-6 was repeated to include remaining mutants stated in Figure 3-5. As normalization of FLAG proteins were not even (Top), they were repeated again and the Coomassie of the proteins were shown (Bottom).

As the MBD2-RG18 protein (Figure 3-6, Lane 5) is the only exception among the hypermethylated mutants with regard to the correlation between methylarginine content and interacting HDAC silencing complex, more mutants were tested.

Similar to the two mutants reported previously (Figure 3-7, Top, Lane 2 and 3), partial removal of amino acids from the RG domain of MBD2 protein resulted in an increase in both species of methylarginine on the protein (Lane 4-6). Moreover, some mutations also resulted in protein degradation (Lane 2-5).

When the levels of co-purifying HDAC silencing complex proteins were analyzed (Figure 3-7, Bottom), the Coomassie stain revealed that protein levels of

MBD2-RG14 were too low and thus no conclusions can be made regarding this mutant. However, all other highly methylated mutants of MBD2 (Lane 8-11) were displaying lower affinity of interaction compared to the wild-type MBD2.

3.3 Arginine methylation of MBD2 and interaction with nucleic acids

The MBD2 and MeCP2 proteins were first identified by their ability to specifically bind methyl-CpG DNA using the MBD-domain (29, 157). It was demonstrated *in vitro* that both MBD proteins were able to interact with a variety of double stranded DNA containing at least one methyl-CpG (85, 167). However, recent studies suggest that substrate specificity for MeCP2 in cells is different from MBD2 (117).

The methyl-CpG DNA binding function of MBD proteins allows targeting of the HDAC silencing complex to particular sites of the genome and mediates transcriptional repression. Since the interaction between MBD2 and the HDAC silencing complex is affected by arginine methylation, the effect of arginine methylation of MBD2 on its interaction with methyl-CpG DNA was investigated.

3.3.1 MBD proteins and methyl-CpG DNA interaction in gel shift assays

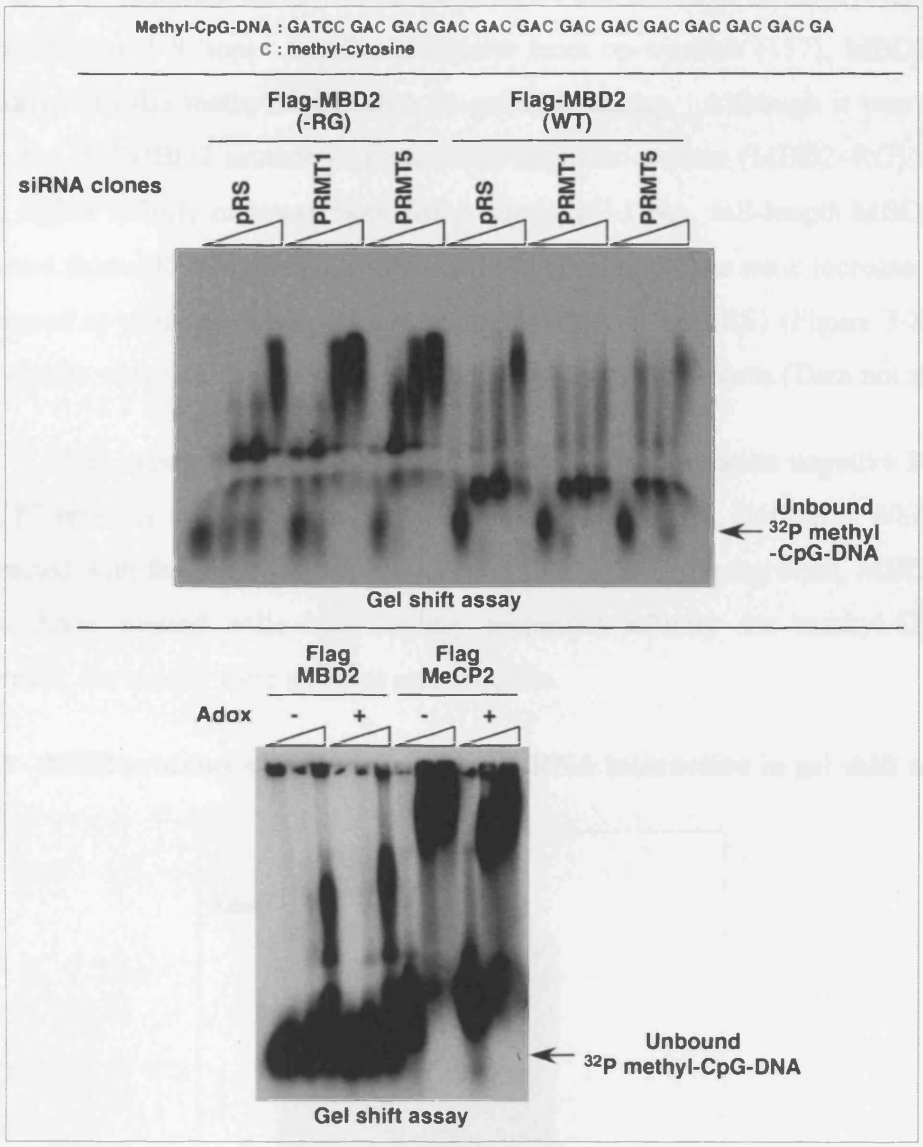

Figure 3-8 Interaction of arginine methylation deficient MBD2 with methyl-CpG DNA.

(Top) Sequence of one strand of the double stranded CpG-DNA used for the interaction assays is shown, and positions of methyl-cytosine are indicated in red. The complimentary DNA strand also contains the corresponding methyl-cytosine.

(Middle) MBD2 proteins from PRMT reduced background were prepared and normalized as for Figure 2-15. The normalized native proteins were incubated with the illustrated radiolabeled methyl-CpG DNA for 20 min and were resolved on 0.5% TBE agarose gels and autoradiograph to assay for shift in mobility of the nucleic acids.

(Bottom) MBD proteins purified from cells treated with control or methylation inhibitor were prepared and normalized as for Figure 2-15. Normalized FLAG proteins were used in gel shift assay as for above to detect for differential interaction with radiolabeled methyl-CpG DNA.

MBD2 proteins raised from siRNA stable cells were tested for their ability to interact with radiolabeled double stranded DNA that contains 12 methyl-CpG on each strand (Figure 3-8, top). Similar to reports from co-workers (157), MBD2 interacts robustly with the methyl-CpG DNA in gel shift assays. Although it was illustrated here that the MBD2 mutant without methylarginine content (MBD2–RG) seemed to have higher affinity of interaction with methyl-CpG DNA, full-length MBD2 proteins obtained from PRMT reduced background did not display the same increase in affinity compared to proteins obtained from control siRNA cells (pRS) (Figure 3-8, middle). The results were also not reproducible in subsequent experiments (Data not shown).

The experiment was repeated with arginine methylation negative MBD2 and MeCP2 proteins raised from Adox treated cells (Figure 3-8, Bottom). While MeCP2 interacted with the same affinity regardless of the cellular background, MBD2 isolated from Adox treated cells had slightly increased affinity for methyl-CpG DNA. However, the results were also not reproducible.

3.3.2 MBD proteins and double stranded RNA interaction in gel shift assays

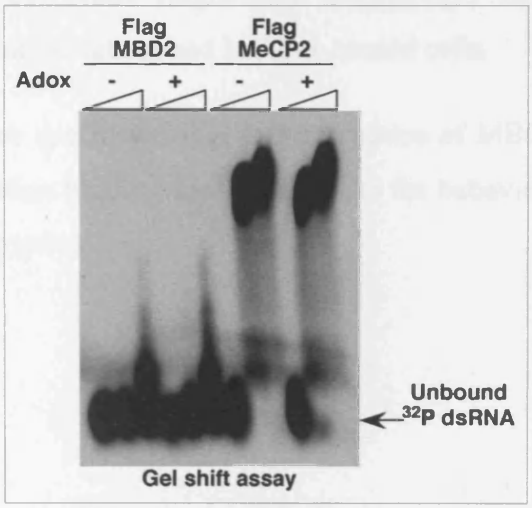

Figure 3-9 Interaction of arginine methylation deficient MBD proteins with RNA.

Proteins isolated from cells treated with methylation inhibitor were prepared and normalized as for Figure 2-15. **Normalized FLAG proteins were used in gel shift assays as for Figure 3-8 to detect for differential interaction with radiolabeled double stranded RNA.**

Since MBD2 and MeCP2 proteins also interact with double stranded RNA in gel shift assays (102), I tested whether the methylated species of the MBD proteins display a different interaction affinity with RNA compared to unmethylated proteins

(Figure 3-9). It was found that Adox treatment had no effect on the interaction of MBD proteins with double stranded RNA. A plausible explanation for this observation could be because throughout the purification of the proteins, no RNase treatment was included, the unspecific binding of cellular RNA to the MBD proteins made it impossible to measure the binding of radiolabeled RNA to the Adox treated proteins. If this is the case, it might also explain the observed higher degree of association between the radiolabeled double stranded RNA and MeCP2, which have a less extensive RNA binding domain (RG) compared to MBD2.

3.3.3 MBD2 and methyl-CpG DNA interaction in solution

The gel shift assays did not give a clear conclusion of whether arginine methylation of the MBD proteins resulted in a change of affinity of the proteins towards nucleic acids. Therefore, the experimental approach for addressing this issue was modified. Data from Chapter 3.2.5.2 suggested that the methylarginine content of wild-type MBD2 protein is much lower compared to the various MBD2 mutants that had a portion of the RG domain removed. Therefore, wild-type MBD2 raised from cells cultured without Adox might bind methyl-CpG DNA at a similar affinity compared to the same protein raised in Adox treated cells.

Since it was speculated that only a fraction of MBD2 purified is methylated on arginines, a solution binding assay that tracks the behavior of these MBD2 species might be more appropriate.

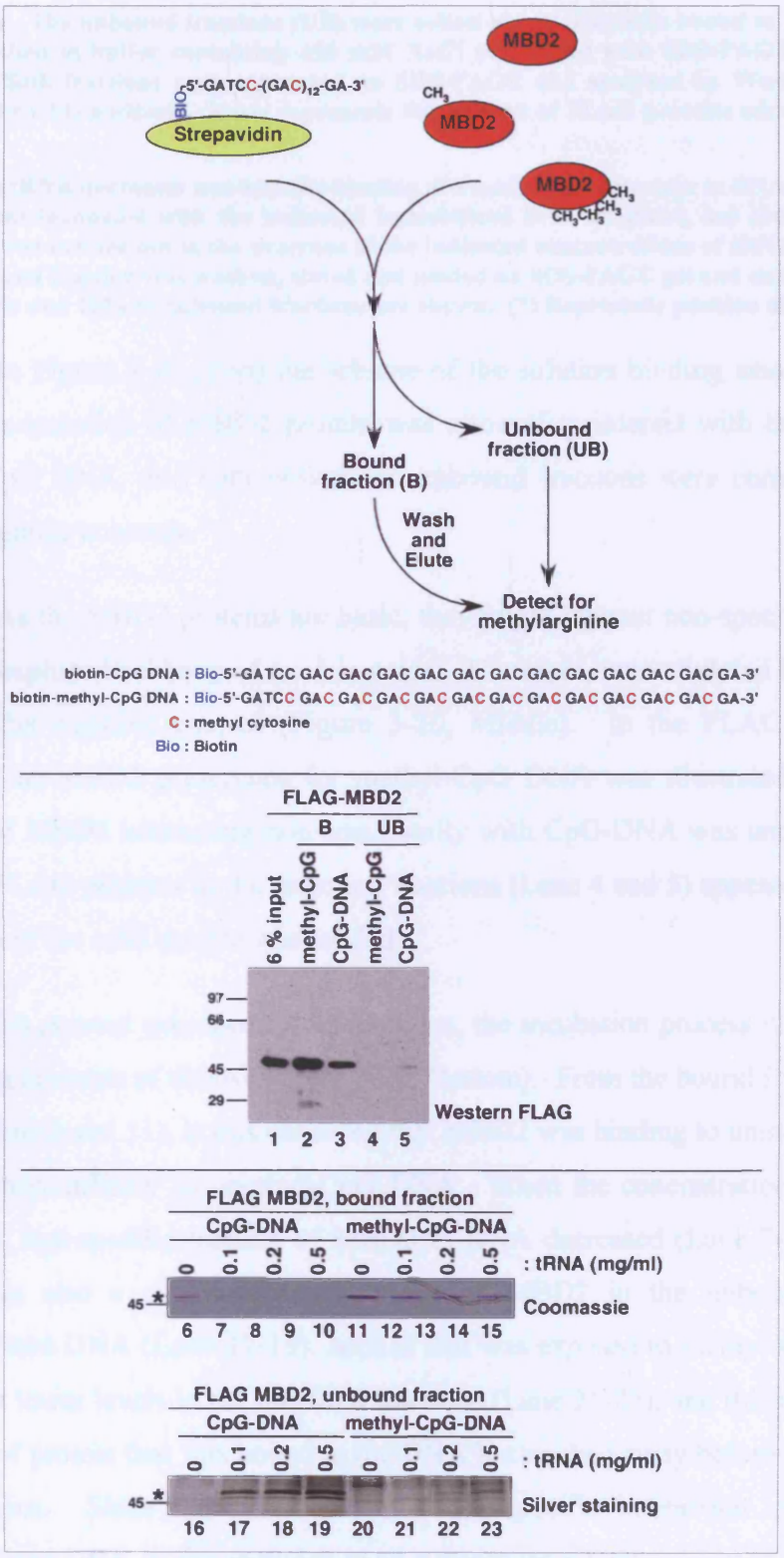

Figure 3-10 MBD2-DNA solution binding assay.

(Top) The scheme of the binding assay.

(Middle) Basic proteins interact non-specifically with DNA. Biotinylated DNA were synthesized, annealed *in vitro*, and immobilized on strepavidin agarose. The FLAG proteins were prepared as for Figure 2-4 and were incubated with the immobilized DNA

for 2.5 hr. The unbound fractions (UB) were collected and fractions bound to resin (B) were washed in buffer containing 350 mM NaCl and eluted with SDS-PAGE sample buffer. Both fractions were separated on SDS-PAGE and analyzed by Western blot with anti-FLAG antibody. Input represents the amount of FLAG proteins added to the resins.

(Bottom) tRNA decreases non-specific binding of FLAG-MBD2 protein to DNA. FLAG MBD2 was incubated with the indicated immobilized DNA as above, but the binding reaction was carried out in the presence of the indicated concentrations of tRNA. 100% of the bound fraction was washed, eluted and loaded on SDS-PAGE gel and analyzed by Coomassie and 10% of unbound fractions are shown. (*) Represents position of MBD2.

In Figure 3-10 (Top) the scheme of the solution binding assay is described; the total population of MBD2 protein was allowed to interact with the immobilized methyl-CpG DNA, and both bound and unbound fractions were compared for their methylarginine contents.

As the MBD2 proteins are basic, they might interact non-specifically with the acidic phosphate backbone of nucleic acids. Therefore, unmethylated CpG-DNA was used as the negative control (Figure 3-10, Middle). In the FLAG Western blot, although the MBD2 preference for methyl-CpG DNA was illustrated (Lane 2), the amount of MBD2 interacting non-specifically with CpG-DNA was undesirable (Lane 3). The FLAG proteins in the unbound fractions (Lane 4 and 5) appeared low because only 10% of the total sample was loaded.

To prevent non-specific interactions, the incubation process was titrated with increasing amounts of tRNA (Figure 3-10, Bottom). From the bound fractions without tRNA (Lane 6 and 11), it was observed that MBD2 was binding to unmethylated DNA but had more affinity for methyl-CpG DNA. When the concentration of tRNA was increased, non-specific binding of MBD2 to DNA decreased (Lane 7-10 and 12-15). There was also a corresponding increase of MBD2 in the unbound fraction of unmethylated DNA (Lane 17-19). MBD2 that was exposed to methyl-CpG DNA was present at lower levels in the unbound fractions (Lane 21-23), and this might represent the pool of protein that was bound to the DNA but washed away before the recovery of the proteins. Since the tRNA prevents non-specific interaction of MBD2 with unmethylated DNA, it was included in all subsequent assays.

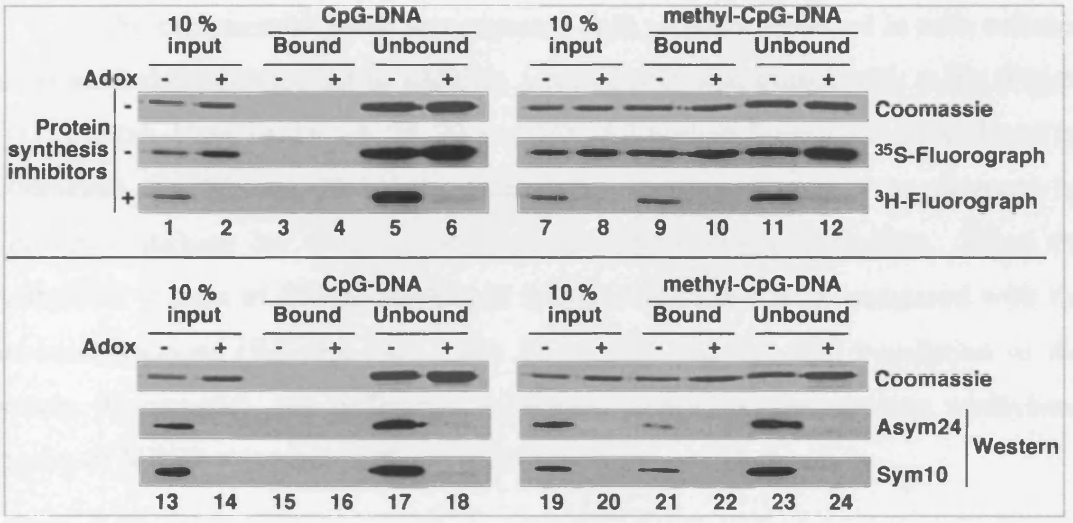

Figure 3-11 Specific MBD2-methyl-CpG DNA interaction.

(Top) Co- and post-translationally labeled MBD2 as for Figure 2-5 **were FLAG immunoprecipitated and eluted with 3x FLAG peptide. The labeled proteins were loaded onto the indicated immobilized DNA as for Figure 3-10 in the presence of 0.8 mg/ml tRNA. 100% of the bound and unbound fractions were precipitated, and analyzed by Coomassie, ^{35}S-Fluorograph or ^{3}H-Fluorograph accordingly.**

(Bottom) To reveal the species of dimethylarginine bound to DNA, proteins cultured under normal conditions were analyzed with Western blot with the indicated antibodies.

In both assays, Adox was included to produce non-methylated species of MBD2.

Using the optimized conditions, the ability of MBD2 to discriminate methylated from unmethylated-CpG DNA was illustrated (Figure 3-11, all Coomassie). In the ^{35}S-Fluorograph, all MBD2 proteins were labeled regardless of Adox. It was observed that there was also no ^{35}S-MBD2 signal binding to CpG-DNA (Lane 3 and 4), but at least 40% of total MBD2 protein interacted with methyl-CpG DNA (Lane 9 and 10). Being more sensitive than Coomassie, the ^{35}S-Fluorographs further support the interaction specificity of MBD2 in this experiment.

When post-translationally modified proteins in the experiment were visualized (Figure 3-11, ^{3}H-Fluorograph), the input fractions show that only proteins that were produced in cells cultured without Adox were labeled (Lane 1 and 7). As expected, the labeled MBD2 species only interacted with methyl-CpG DNA (Lane 9), but not CpG-DNA (Lane 3). However, it was difficult to interpret whether the ^{3}H-arginine methylated MBD2 (Lane 9) or the unmethylated MBD2 (Lane 10) have better affinity for methyl-CpG DNA.

The experimental setup was repeated with proteins produced in cells cultured under normal conditions, but in addition, a set of cells was treated with Adox (Figure 3-11, Bottom, Lane 14, 16, 18, 20, 22 and 24). All protein fractions were analyzed by Coomassie and Western blot. As usual, Adox treated proteins were observed by Coomassie staining but were not detected by methylarginine antibodies. When the methylated species of MBD2 binding to methyl-CpG DNA were compared with the unbound fractions (Western blot, Lane 21 vs. 23) and the total population of the protein (Coomassie), the difference in elution pattern of the arginine methylated species of MBD2 was again difficult to deduce.

3.3.4 Arginine methylation controls the affinity of the MBD2 and methyl-CpG DNA interaction

Even with conditions that clearly defined the specificity of MBD2 affinity towards methyl-CpG DNA and not CpG-DNA, I was unable to determine whether arginine methylation resulted in a change in affinity between MBD2 and methyl-GC DNA. Therefore, the solution binding assay for MBD2 and methyl-CpG DNA was modified to account for the behavior of all MBD2 proteins added to the methyl-CpG DNA.

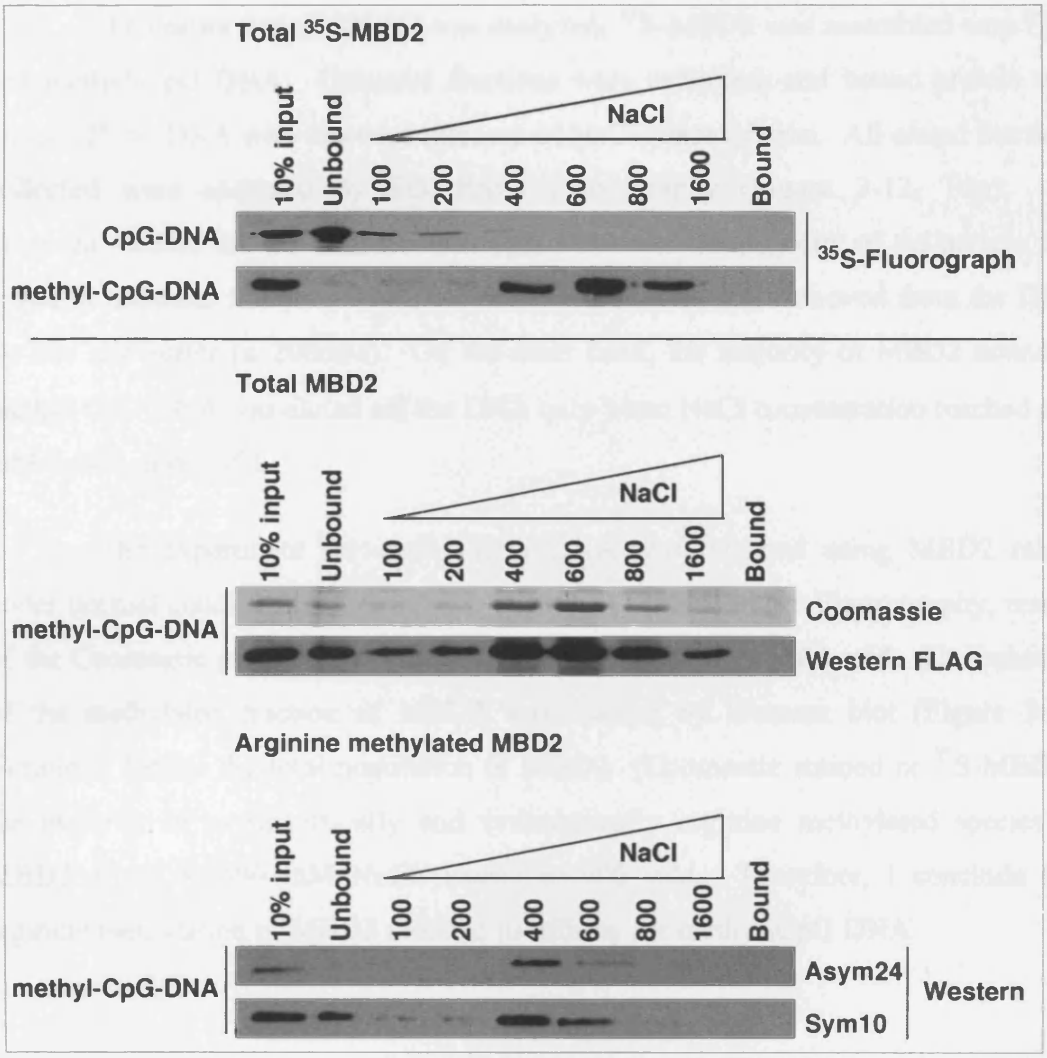

Figure 3-12 Analysis of MBD2-methyl CpG DNA affinity using step salt elution.

(Top) Co-translationally labeled MBD2 prepared as for Figure 2-5 was FLAG immunoprecipitated and eluted with 3x FLAG peptide. The labeled proteins were loaded onto the indicated immobilized DNA as for Figure 3-10 in the presence of 0.25 mg/ml tRNA. MBD2 proteins bounded to the DNA were salt eluted in steps using the indicated concentrations of NaCl (in mM). All elutions including the unbound fractions were precipitated and analyzed by Fluorography.

(Bottom) Arginine methylated MBD2 species have less affinity for methyl-CpG DNA. To differentiate the affinity of methylated and unmethylated forms of MBD2 towards CpG-methylated DNA, FLAG MBD2 produced under normal conditions was incubated with the immobilized methyl-CpG DNA as for above. Coomassie and Western blots with FLAG antibody indicate the total population of MBD2 proteins and Western blots with methylarginine antibodies represent the modified species of MBD2.

In the previous experiment, (Figure 3-10, Bottom), it was observed that in presence of tRNA, washing the methyl-CpG DNA-MBD2 complex with 350 mM NaCl probably resulted in unwanted loss of proteins in the procedure, as the unbound fraction did not contain a significant amount of the remaining protein.

To ensure that all MBD2 was analyzed, ^{35}S-MBD2 was assembled onto CpG and methyl-CpG DNA. Unbound fractions were collected, and bound protein was eluted off the DNA with stepwise increase of NaCl concentration. All eluted fractions collected were analyzed by SDS-PAGE/Fluorography (Figure 3-12, Top). As observed, MBD2 did not interact with CpG-DNA and the majority of the protein was found in unbound fractions while the remaining protein was removed from the DNA by low salt buffer (≤ 200mM). On the other hand, the majority of MBD2 bound to methyl-CpG DNA was eluted off the DNA only when NaCl concentration reached 400 mM to 600 mM NaCl.

The experiment for methyl-CpG DNA was repeated using MBD2 raised under normal conditions (Figure 3-12, Bottom). Similar to the Fluorography, results of the Coomassie gel revealed that elution of MBD2 peaks at 600 mM. The behavior of the methylated fraction of MBD2 was tracked by Western blot (Figure 3-12, Bottom). Unlike the total population of MBD2 (Coomassie stained or ^{35}S-MBD2), the majority of asymmetrically and symmetrically arginine methylated species of MBD2 eluted at 400 mM NaCl instead of 600 mM. Therefore, I conclude that arginine methylation of MBD2 reduced its affinity for methyl-CpG DNA

3.3.5 Control of MBD2 and methyl-CpG DNA association by arginine methylation is a mechanism exclusive to wild-type MBD2

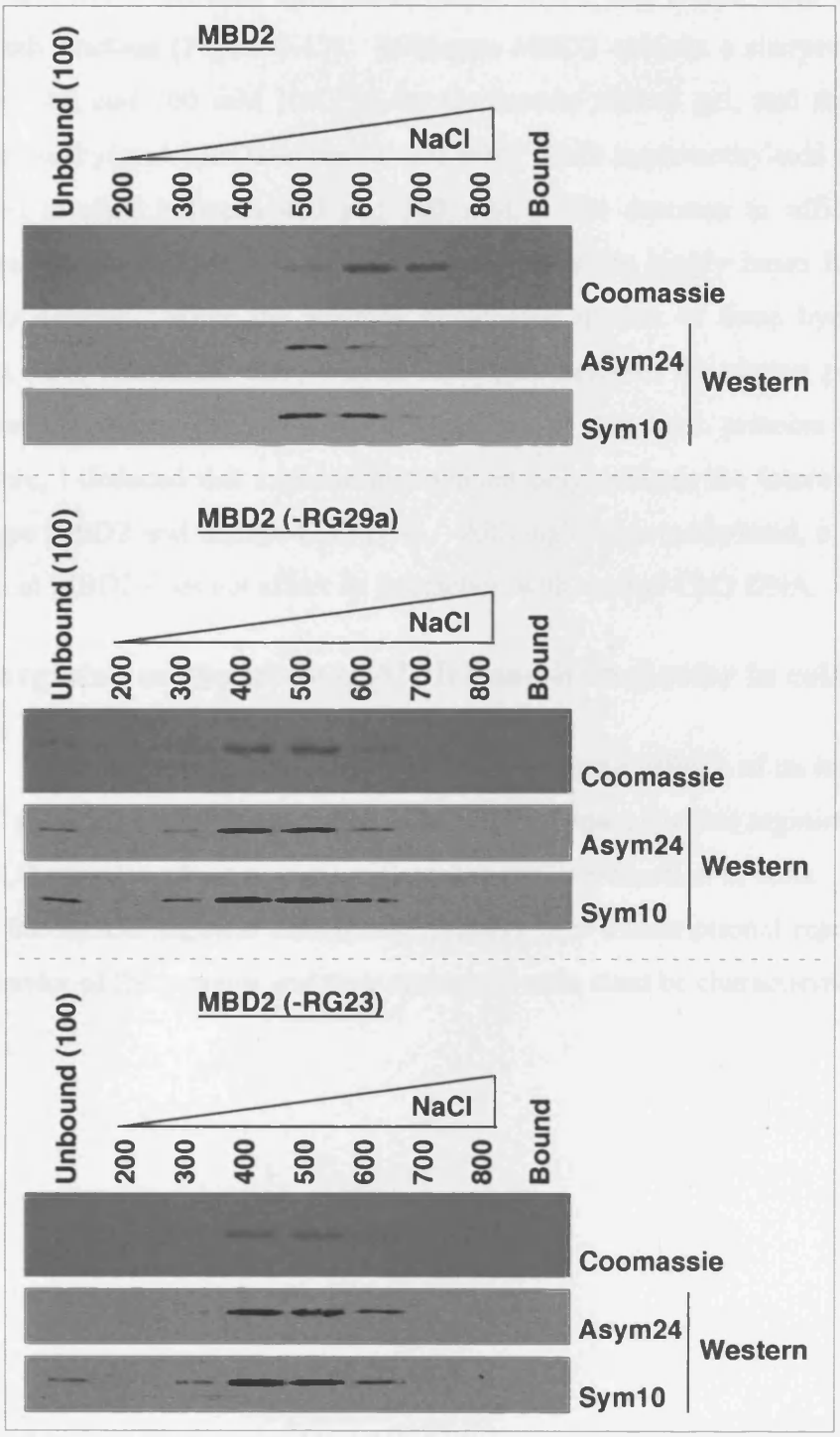

Figure 3-13 Step elution of hypermethylated MBD2 mutants from methyl-CpG DNA.

The experiment in Figure 3-12 was repeated to compare the elution of wild-type MBD2 and hypermethylated MBD2 mutants from Figure 3-5. Coomassie staining represents the total population of MBD2 and Western blots represent the modified species of MBD2.

To check whether this effect of arginine methylation applies to the hypermethylated MBD2 mutants, I assembled the wild-type MBD2, MBD2-RG29a and MBD2-RG23 proteins onto methyl-CpG DNA, and eluted them using a more gentle salt gradient (Figure 3-13). Wild-type MBD2 exhibits a sharper elution peak between 600 and 700 mM NaCl in the Coomassie stained gel, and the majority of arginine methylated MBD2 elutes at 500 mM. Both hypermethylated mutants were observed to elute between 400 and 500 mM. This decrease in affinity could be attributed to the reduction in pI of the proteins as the highly basic RG domain is partially deleted. When the arginine methylated species of these hypermethylated mutants were visualized, there was no difference between the elution patterns of the methylated proteins (Western blot) compared to the total proteins (Coomassie). Therefore, I deduced that arginine methylation only controls the interaction between wild-type MBD2 and methyl-CpG DNA. Although hypermethylated, a disrupted RG domain in MBD2 does not affect its interaction with methyl-CpG DNA.

3.4 Arginine methylation of MBD2 and its behavior in cells

Since arginine methylation of MBD2 affects the affinity of its interaction with HDAC silencing complex and methyl-CpG DNA, I speculate that arginine methylation of MBD2 can also affect its transcriptional repression function in cells. To be able to utilize the MBD2 arginine methylation mutants in a transcriptional repression assay, the behavior of the proteins and their mutants in cells must be characterized.

3.4.1 Subcellular localization of MBD2 and arginine methylation mutant proteins

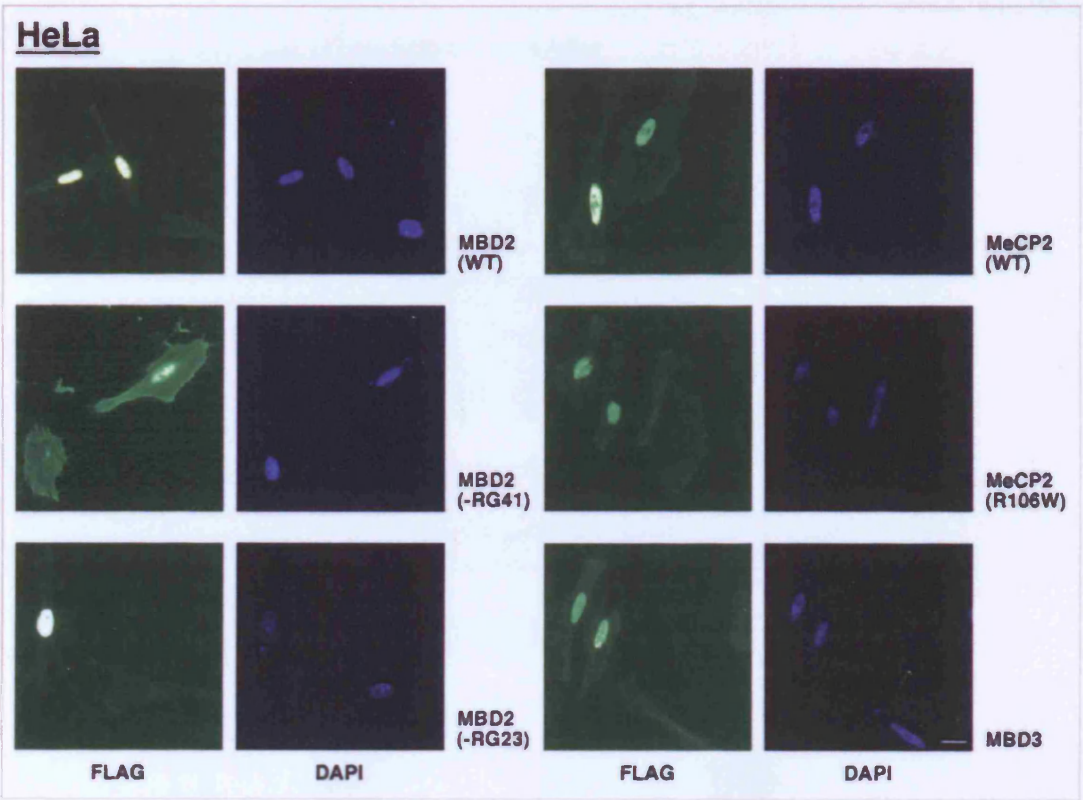

Figure 3-14 Immunofluorescence of MBD proteins.

HeLa cells were transfected with the indicated FLAG-MBD plasmids for 18 hr and were stained with anti-FLAG/FITC or DAPI. Scale bar: 10 μm (Bottom right)

Although the biochemical properties of MBD2 arginine methylation mutants are conclusive, their subcellular localization has yet to be addressed. Wild-type MBD2 is reported to localize in the nucleus of mammalian cells (85, 99). If any of the MBD2 mutant proteins fail to be imported into the nucleus, they will be unable to repress transcription.

Therefore, non-methylated (MBD2-RG41) and hypermethylated (MBD2-RG23) mutant protein was compared against wild-type MBD2 for their nuclear localization properties (Figure 3-14). Similar to wild-type protein, all MBD2 mutants localized in the nucleus. This indicates that they are at least structurally competent in terms of recognition and transport by nuclear import factors. As the MeCP2 and MBD3 were used as controls in subsequent assays, they were also included in this experiment. Similar to studies from co-workers, these proteins localize in the nucleus

(135, 167). MeCP2 R106W, a mutation that cripples the binding affinity of the protein to methyl-CpG DNA *in vitro* (10), is also found in the nucleus.

3.4.2 Transcriptional repression activity based on methyl-CpG DNA binding and recruitment of the HDAC complex

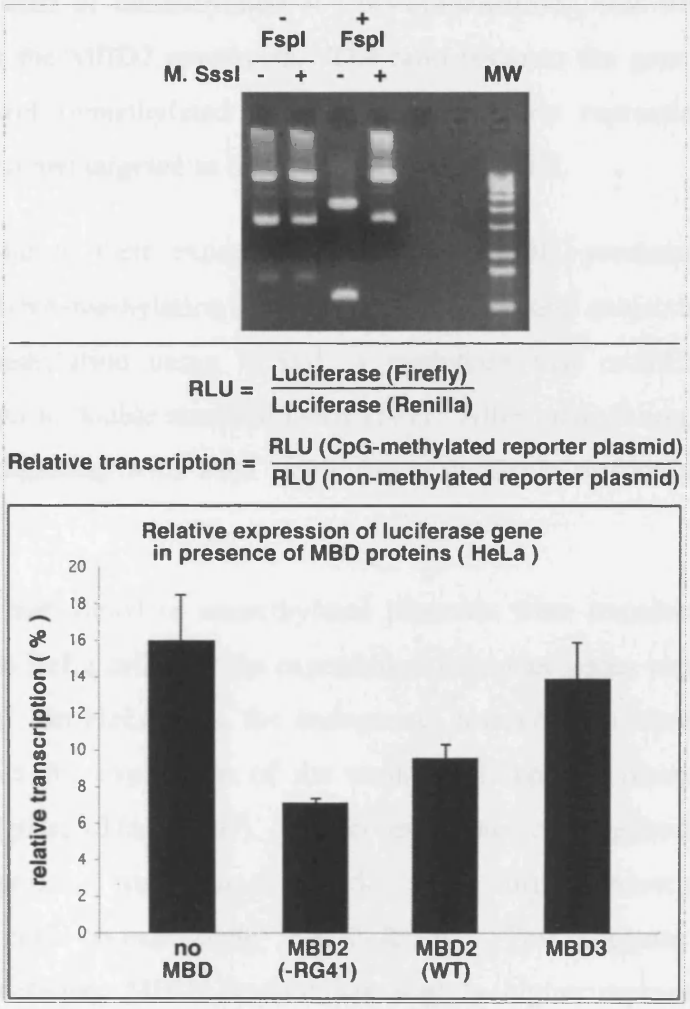

Figure 3-15 Repression activity of MBD2 on methyl-CpG DNA.

(Top) DNA methylation of a reporter plasmid. The pGL2-luciferase reporter plasmid was incubated in the absence or presence of DNA methylase M.SssI and the plasmid was precipitated and purified. FspI was used to check for completion of the methylation reaction.

(Bottom) Arginine methylation deficient MBD2 has higher repression activity than methylated MBD2. HeLa cells were transfected with the unmethylated or the methylated luciferase reporter plasmid, the renilla control plasmid, and the indicated FLAG-MBD plasmids. 24 hr after transfection, cells were assayed for their firefly luciferase and renilla luciferase activity and relative luciferase unit (RLU) is expressed as a function of firefly/renilla luciferase. To obtain the relative transcriptional activity in presence of the indicated MBD, the ratio of RLU of cells transfected with methylated plasmid and unmethylated plasmid was taken. Each value represents an average from 3 independent experiments.

The repression activity of MBD towards methylated CpG-reporter-DNA can be demonstrated in cells (29, 86). In a repression assay described by Hendrich *et al.*, expressing exogenous wild-type MBD2 protein in MBD2 knockout mice cells rescued the repression of a methylated reporter plasmid. In their experiment, reporter DNA, either methylated or unmethylated at CpG-dinucleotides, was transfected into cells together with the MBD2 constructs. The ratio between the gene products from the methylated and unmethylated reporter represents the repression activity of the exogenous proteins targeted to methyl-CpG DNA in cells.

Similar to their experiment, I used the pGL2-promoter reporter plasmid produced in DNA-methylation deficient *E. coli* cells and subjected them to mock or CpG-DNA-methylation using M.SssI, a methylase that modifies cytosine in the context of CpG in double stranded DNA (171). After methylation, the plasmids were checked by digesting with FspI (methylation sensitive restriction enzyme) (Figure 3-15, Top).

The methylated or unmethylated plasmids were transfected with the MBD constructs into HeLa cells and the expression of reporter genes was quantified (Figure 3-15, Bottom). In HeLa cells, the endogenous transcription repressors present were able to restrict the expression of the methylated reporter plasmid to 16% of the unmethylated plasmid (no MBD). With co-expression of exogenous repressor (MBD2 WT), the expression was reduced to 9.5% and a further reduction to 7.1% can be achieved in cells co-expressing MBD2–RG41. This suggests that the arginine methylation deficient MBD2 mutant has slightly higher repression activity. The mammalian MBD3, which does not bind directly to methyl-CpG DNA (85) (see Chapter 1.4.2), did not significantly reduce expression of the reporter when co-expressed.

Although the experiment was consistently reproduced, the increase in repression activity of the MBD2-RG41 protein compared to the WT never exceeds 1.4 fold. To demonstrate a greater difference in repression activity, the expression level of the MBD proteins in HeLa cells was taken into consideration.

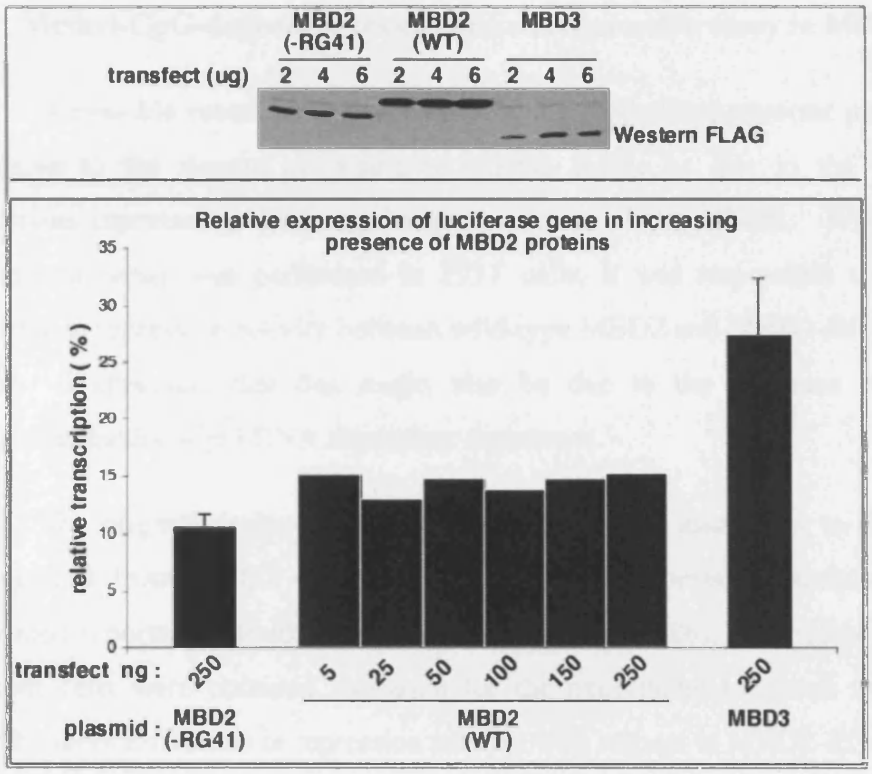

Figure 3-16 Expression levels of MBD proteins are different in HeLa.

(Top) HeLa cells were transfected with different amount of the indicated FLAG-MBD plasmids for 48 hr and Bradford normalized cell lysates were used to determine the expression level of MBD proteins by Western blot with FLAG antibody.

(Bottom) Relative transcription activity of the MBD proteins was determined as for Figure 3-15. Transfection of MBD2-RG41 and MBD3 were fixed at 250 ng of plasmid and values represent 3 independent experiment. For wild-type MBD2, expression of the protein was titrated in cells with increasing ng of plasmid, each value represents one transfection.

When expression of the MBD proteins were checked in HeLa cells by titrating the amount of plasmid in transfections (Figure 3-16, Top), it was observed that wild-type MBD2 protein were generally expressed at higher levels compared to MBD2-RG41. MBD3, the negative control for the experiment had the lowest level of expression among the 3 proteins.

To ensure that the relative transcription assay is correlative to the levels of MBD repressors proteins present in cells, the experiment was titrated with increasing amount of wild-type MBD2 plasmids (Figure 3-16, Bottom). Although the assay had showed a similar trend of MBD3 being the least capable of repression, and MBD2-RG41 had the highest repression activity, expression of wild-type MBD2 plasmid in the system did not show a dose dependent increased in repression.

3.4.3 Methyl-CpG-dependent transcriptional repression assay in MBD2 -/- cells

A possible reason why expression of the methylated reporter plasmid is not responsive to the dosage of wild-type MBD2 might be due to the presence of endogenous repressor proteins masking the effects of the MBD2. When the same experimental setup was performed in 293T cells, it was impossible to illustrate a difference in repression activity between wild-type MBD2 and MBD2-RG41 (Data not shown). I speculate that this might also be due to the presence of saturating endogenous methyl-CpG DNA dependent repressors.

The original design of the relative transcription assay was to illustrate that cells derived from MBD2 -/- mice were unable to repress transcription of CpG-methylated reporter plasmid as well as wild-type cells (86). Therefore, the MBD2 knockout cells were obtained and used for the experiment to check whether there would be more difference in repression activity with respect to MBD2-RG41 and wild-type MBD2 protein.

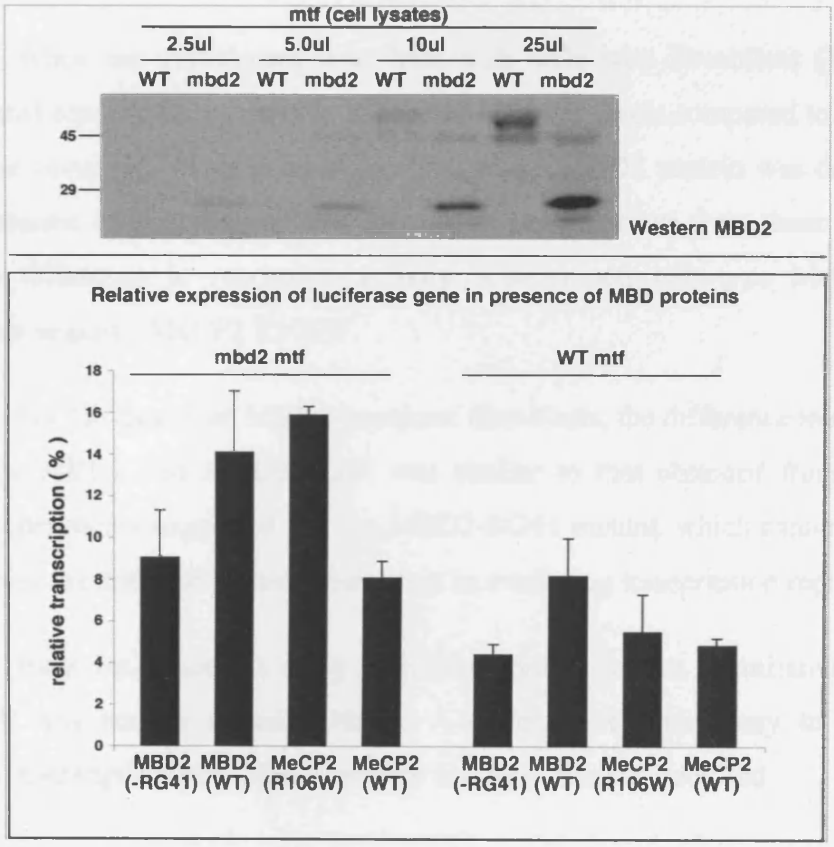

Figure 3-17 Using MBD2 knockout cells for repression assay.

(Top) Mouse tail fibroblast (mtf) cells from MBD2 knockout mice were compared to wild-type mice cells for the presence of MBD2 protein. The indicated amounts of Bradford normalized cell lysate were analyzed by Western blot with antibodies against MBD2.

(Bottom) Both cell types were transfected with reporter plasmids to assay for relative transcription in the presence of MBD as for Figure 3-15.

Cell lysate from the MBD2 knockout mice cells was checked for the presence of endogenous MBD2. Similar to the report by Hendrich *et al.* (2001), targeted disruption of the MBD2 allele produced a truncated protein of 25 kDa (86), whereas the wild-type cell line was producing the normal MBD2 protein doublet observed in Western blots (Figure 3-17, Top).

Applying the relative transcription assay to MBD2 knockout fibroblast (mbd2 mtf, Figure 3-17, Bottom), I again observed that MBD2-RG41 reduced expression of the methylated reporter to a lower level (9.0%) compared to wild-type MBD2 (14%). Wild-type MeCP2 also demonstrated higher repression of the methylated reporter (8.0%) when compared to MeCP2 R106W, a mutant incapable of binding methyl-CpG DNA (10), which allowed the methylated plasmid to express at 15.8%.

When the experiment was done with wild-type fibroblasts (WT mtf), the methylated reporter was generally expressed at lower levels compared to in the MBD2 knockout fibroblast. This is because endogenous MBD2 protein was contributing to the repression of the reporter. No conclusion can be drawn from these cells as there was no difference in repression activity between the wild-type MeCP2 and the dominant-negative MeCP2 R106W.

For the data from MBD2 knockout fibroblasts, the difference in repression for wild-type MBD2 and MBD2-RG41 was similar to that obtained from HeLa cells. These experiments suggested that the MBD2-RG41 mutant, which cannot be modified by arginine methylation is more competent in mediating transcription repression.

However, since the assay was illustrated to be not quantitative (see Figure 3-16), it was not optimized further. A different reporter assay to measure the repressive strengths of the MBD2 protein and its mutants is required.

3.4.4 Transcription repression activity based on interacting HDAC complex

The relative transcription assay used previously measures the functional activity of both the MBD and TRD domains of the exogenous MBD2, and this is highly relevant to this study. However, the data was obtained by indirect calculation of the ratio between the expression of CpG-methylated and non-methylated reporter plasmids in different transfections, and values were not correlative to the dosage of MBD2. Therefore, a more direct approach that measures the repression activity of MBD2 with respect to its TRD domain in cells was employed.

3.4.4.1 Hypermethylated MBD2 mutant has impaired repression activity

Using the cell based tethering assay, MBD2 fused to Gal4 DNA-binding-domain protein was illustrated to be capable of repressing reporter plasmid with a 5X Gal4 binding site upstream of the promoter in a HDAC dependent manner (22, 170).

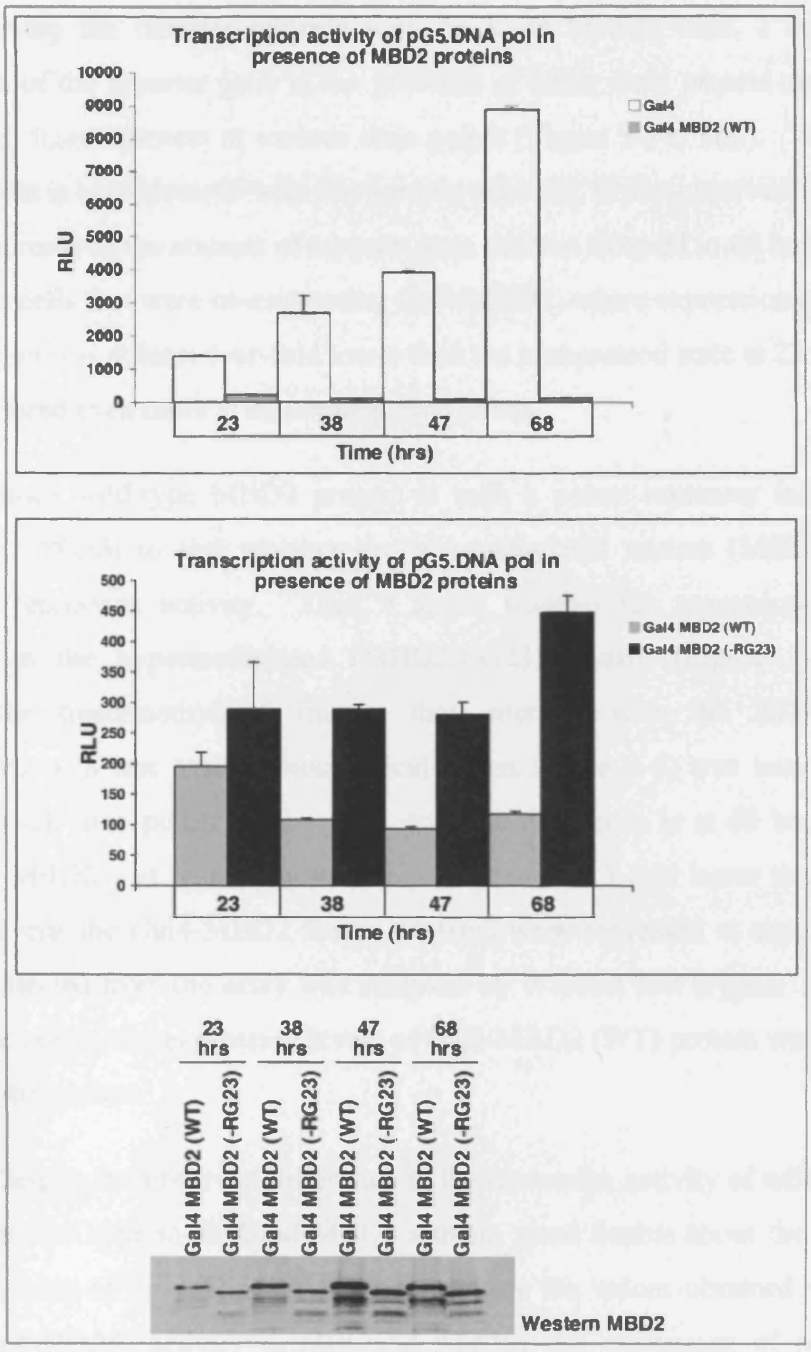

Figure 3-18 pG5 DNA polymerase β luciferase transcription assay.

(Top) 293T cells were transfected with pG5 DNA polymerase β luciferase reporter plasmid, pRL-TK renilla reporter plasmid and the plasmids encoding the Gal4 proteins. RLU (firefly/renilla) for the indicated time points were obtained.

(Middle) The repression activity of wild-type MBD2 was compared against the hypermethylated mutant MBD2-RG23. All values obtained are an average of 3 independent transfections.

(Bottom) Expression levels of the Gal4-MBD2 proteins (in Middle) were determined by Western blot using total protein collected from the assay.

Using the reporter plasmid with 5X Gal4 binding sites, I quantitated the expression of the reporter gene in the presence of either Gal4 protein or Gal4-MBD2 (wild-type) fusion protein at various time points (Figure 3-18, Top). When only the Gal4 protein is co-expressed with the reporter plasmid, it was observed that there was a linear increase in the amount of reporter gene product from 23 to 68 hours. This is in contrast to cells that were co-expressing Gal4-MBD2, where expression of the reporter gene product was at least four-fold lower than the unrepressed state at 23 hour, and the values differed even more at subsequent time points.

Since wild-type MBD2 protein is such a potent repressor in this assay, it would be difficult to test whether the non-methylated mutant (MBD2-RG41) has enhanced repression activity. Thus, I tested whether the repression activity was impaired in the hypermethylated (MBD2-RG23) mutant (Figure 3-18, Middle). Indeed, the hypermethylated mutant that interacts with the HDAC silencing components with less avidity biochemically (see Figure 3-6) was less competent in repression (all time points). The most dramatic difference is at 68 hours where the wild-type MBD2 was repressing the reporter gene to 4.5 fold lower than the mutant. To ensure that the Gal4-MBD2 fusion proteins were expressed at equal levels, total protein collected from the assay was analyzed by Western blot (Figure 3-18, Bottom). At all time points, the expression levels of Gal4-MBD2 (WT) protein were comparable to that of the mutant.

Despite the observed difference in the repression activity of wild-type MBD2 protein and the hypermethylated MBD2 mutant, some doubts about the experimental setup still have to be addressed, namely whether the values obtained were a direct measure of HDAC activity in cells and whether the expression of reporter genes responded to the Gal4 fusion proteins in a dosage dependent manner.

3.4.4.2 Generation of MBD2 TRD mutants

In various cell-based studies, HDAC inhibitors (237) were administered to transfected cells and relief of the repression of the reporter was correlated to the HDAC-dependent silencing of the Gal4-fusion protein (166, 170). However, in many cases, it was noted that treating cells with the inhibitors did not fully relieve the repression (105, 166). Therefore, I removed the transcriptional repression domain of

wild-type MBD2 protein to show that the mutation removes the repression function of the protein.

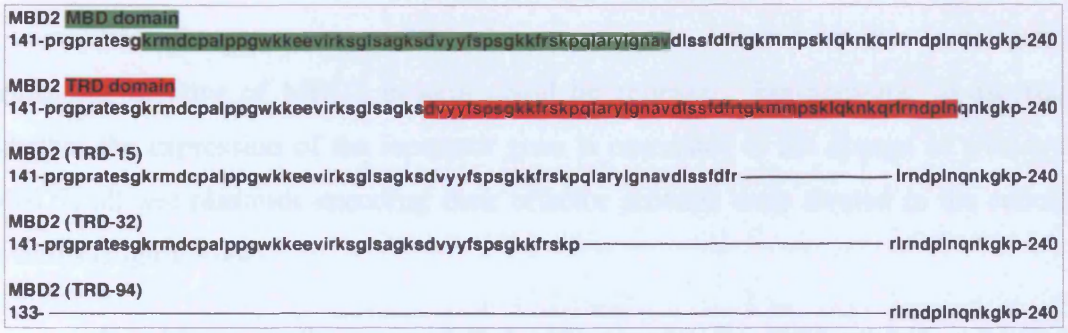

Figure 3-19 MBD2 TRD mutants.

The MBD (Green) and TRD (Red) of MBD2. Three deletion mutants of the MBD2 protein were created in attempt to disrupt the repression function of the protein.

Within the amino acid sequence of MBD2, the MBD was identified by bioinformatics (85), and the TRD was defined biochemically (23). As the two domains of the protein overlap (Figure 3-19), deletion of the TRD should be kept at a minimum to avoid disruption of the MBD. Therefore, a few TRD deletion mutants were generated and the most subtle deletion mutant that illustrates the lost in repressive function in cells was chosen.

3.4.4.3 Redefining the TRD of MBD2 protein

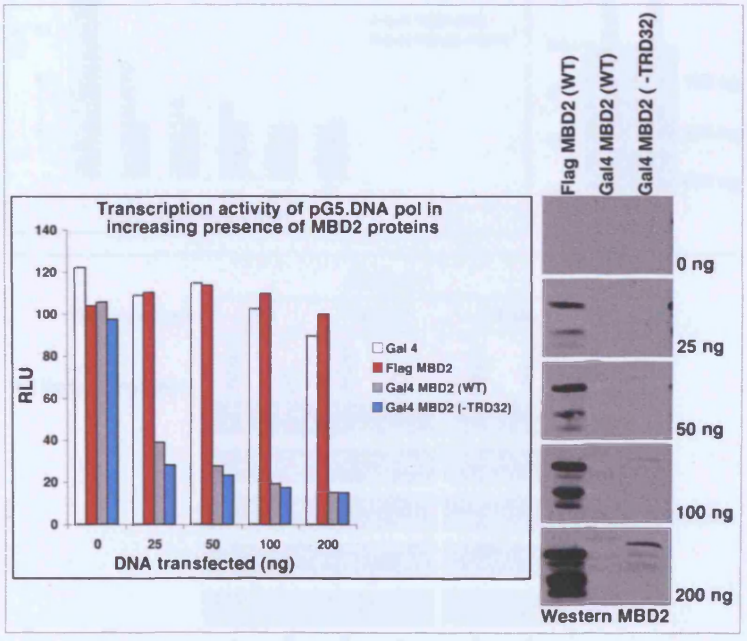

Figure 3-20 Characterization of MBD2-TRD mutants.

293T cells were transfect with the luciferase/renilla reporter plasmid as for Figure 3-18 and the indicated ng of test plasmids. RLU was obtained 48 hr post transfection and MBD2 proteins expression was quantified as for Figure 3-18. Each value obtained is a result of one transfection.

With the generation of TRD deletion mutants, I tested whether transcription repression activity of MBD2 protein could be relieved. Furthermore, to ascertain whether the expression of the repressor gene is correlated to the dosage of wild-type MBD2, all test plasmids encoding their effector proteins were titrated in the cellular systems (Figure 3-20).

In addition to the plasmid that only encodes the Gal4 protein, a plasmid encoding the FLAG-MBD2 (WT) was also included as the negative control. Both plasmids did not repress the expression of the reporter gene because the Gal4 protein lacks a repression domain and the FLAG-MBD2 (WT) was not tethered to the 5X Gal4-DNA binding site on the reporter plasmid. However, the TRD deletion mutants (MBD2-TRD15 and MBD2-TRD32, see Figure 3-19) did not show any significant loss of repression activity compared to Gal4-MBD2 (WT) protein (Data not shown for MBD2-TRD15).

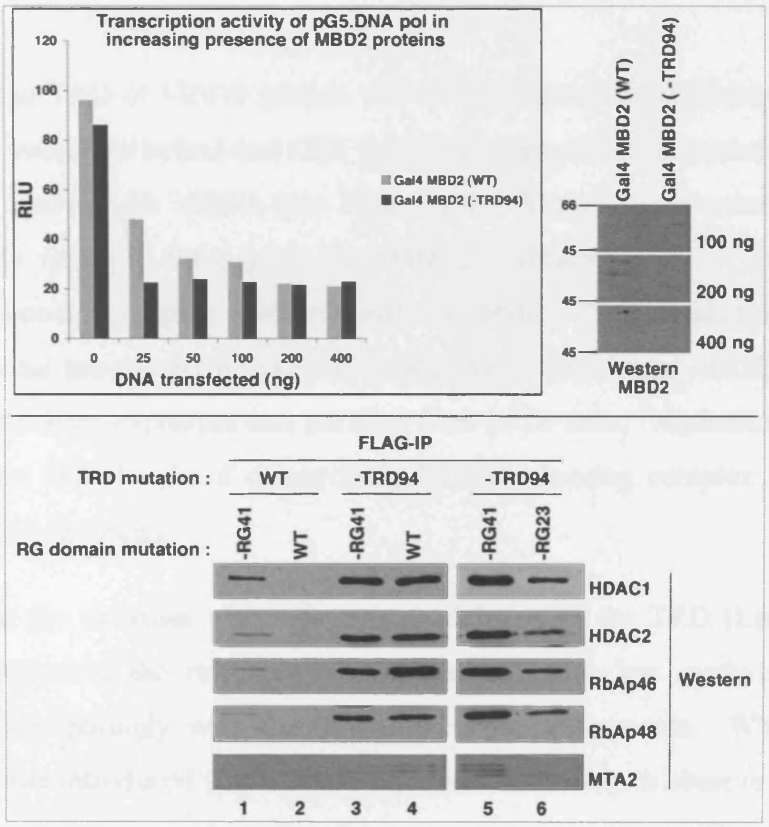

Figure 3-21 Characterization of MBD2-TRD mutants.

(Top) 293T cells were transfected with the luciferase/renilla reporter plasmid as for Figure 3-18 and the indicated amount (ng) of test plasmids. RLU and expression levels of the Gal4-MBD2 proteins were analyzed as for Figure 3-20.

(Bottom) Various RG deletion mutants in Figure 3-5 were combined with the -TRD94 mutation in Figure 3-19 and were expressed as FLAG proteins in 293T cells. The proteins were purified and normalized as for Figure 2-15 **and were analyzed for co-purifying HDAC silencing complex as for Figure 3-1.**

As the deletion of 15 to 32 residues within the TRD of MBD2 did not eliminate the repression function of the protein, a much larger deletion mutant (MBD2-TRD94, see Figure 3-19) was used (Figure 3-21, Top). From the graph, it was observed that increase transfection of Gal4-MBD2 (WT) resulted in a correlated decrease in RLU of the reporter plasmid. Beyond 200 ng of Gal4-MBD2 (WT), the RLU did not decrease any further as the maximum limit of the repression was reached (RLU $\approx$ 20). For Gal4-MBD2 (-TRD94), it was unexpected that the mutant protein possessed higher repression activity than wild-type MBD2. At 25 ng of transfection, Gal4-MBD2 (-TRD94) was able to fully repress the expression of the reporter plasmid. Judging from the Western blot of the two Gal4-MBD2 proteins (Figure 3-21, Top), the –TRD94 mutant protein was produced at a much lower level compared to Gal4-MBD2 (WT).

The TRD of MBD2 protein was defined based on its interaction with Sin3A protein in yeast two hybrid and GST pull down assay (23). However, I was unable to co-purify Sin3A with MBD2 (see Figure 3-3). Therefore, to ascertain whether the TRD really exists in the region described (23) (Figure 3-19), I tested whether the HDAC-silencing complex interacts with the MBD2-TRD94 mutant. The –TRD94 mutation was introduced into FLAG-MBD2 WT, -RG41 and –RG23 constructs and the proteins were expressed and purified from 293T cells. Normalized proteins were checked for their levels of co-purifying HDAC silencing complex by Western blot (Figure 3-21, Bottom).

In the situation where there is no deletion in the TRD (Lane 1 and 2), the proteins followed the rule that MBD2 proteins with less methylarginine content interact more strongly with the HDAC-silencing components. When the –TRD94 mutation was introduced (Lane 3 and 4), it was surprising to observe that the proteins

interacted even more strongly with the HDAC-silencing components than those containing an intact TRD (Lane 1 and 2).

To ensure that the effect of arginine methylation still holds true, I compared the -TRD94 versions of non-methylated MBD2 (-RG41) and hypermethylated MBD2 (-RG23) for their association with HDAC interacting components (Lane 5 and 6). Arginine methylation still controls the levels of co-purifying HDAC-silencing complex, but it was observed that the –TRD94 mutation introduced into MBD2-RG23 also increased its affinity for HDAC silencing components. I did not further characterize this unexpected effect of –TRD94 mutation but I speculate that it could be caused by the removal of the MBD domain from the MBD2 protein (Figure 3-19).

Applying the data from the biochemical analysis to the cell based assay, (Lane 2 and 4 are FLAG version of Gal4-MBD2 (WT) and Gal4-MBD2-TRD94 respectively), I can appreciate why deletion of the 94 residues from the presumed TRD resulted in the increase in repression activity of the MBD2 protein.

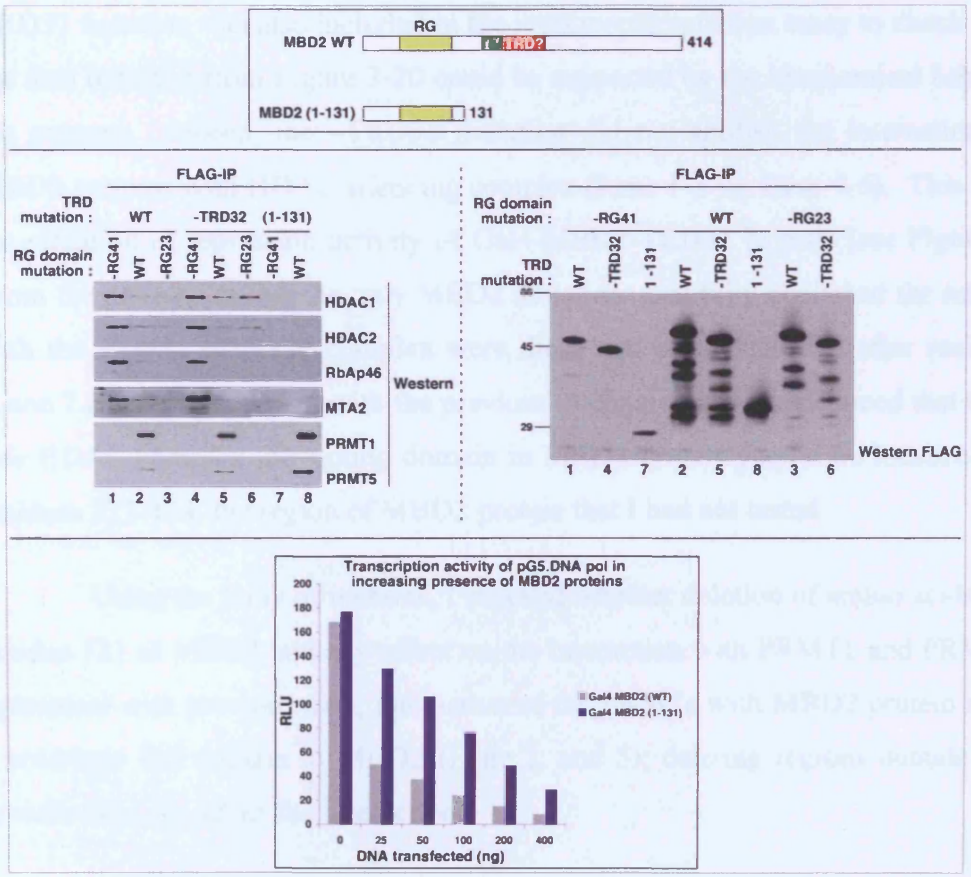

Figure 3-22 Repression activity of the MBD2 truncation mutant.

(Top) Map of MBD2 truncation mutant (MBD2 1-131) cloned to abolish the ability of the protein to interact with the HDAC silencing complex.

(Middle) Various RG deletion mutants in Figure 3-5 were combined with the –TRD32 deletion in Figure 3-19 and the MBD2 truncation mutant (Above). These constructs were expressed as FLAG proteins in 293T cells. The proteins were purified and normalized as for Figure 2-15 **and were analyzed for the amount of co-purifying HDAC silencing complex as for Figure 3-1 (Left). The normalization of the FLAG proteins was illustrated by Western blot (Right, similar lane number indicate similar sample).**

(Bottom) To test the repression activity of the MBD2 truncation mutant, 293T cells were transfected with the luciferase/renilla reporter plasmid as for Figure 3-18 and the indicated amount (ng) of test plasmids. RLU were analyzed as for Figure 3-20.

Since my data suggest that the TRD of MBD2 is not located at the region previously reported (see Figure 3-19) (23), the most straightforward approach to generate a Gal4-MBD2 transcription repression mutant was to create a C-terminus truncation mutant (Figure 3-22, Top).

Before using the protein in the cell based repression assay, the MBD2 1-131 mutation was expressed as a FLAG-tagged protein to verify its ability to recruit the HDAC-silencing complex (Figure 3-22, Middle). MBD2 proteins containing the –TRD32 mutation was also included in the immunoprecipitation assay to check whether the data obtained from Figure 3-20 could be supported by the biochemical behavior of the mutants. Indeed, the –TRD32 mutation did not abolish the interaction of the MBD2 proteins with HDAC silencing complex (Lane 1-3 vs. Lane 4-6). This explains the retention of repression activity of Gal4-MBD2-TRD32 in cells (see Figure 3-20). From the proteins tested, the only MBD2 mutations that fully abolished the interaction with the HDAC silencing complex were those that were truncated after residue 131 (Lane 7 and 8). Combined with the previous interaction assay, I deduced that the *bona fide* HDAC complex interacting domain in MBD2 protein should be localized within residues 227-414, the region of MBD2 protein that I had not tested.

Using the array of mutants, I checked whether deletion of amino acids beyond residue 131 of MBD2 had any effect on the interaction with PRMT1 and PRMT5. In agreement with previous data, interaction of the PRMTs with MBD2 protein relies on a wild-type RG domain in MBD2 (Lane 2, and 5); deleting regions outside the RG domain does not affect the interaction.

With the biochemical properties of the MBD2 1-131 protein characterized, I tested whether the Gal4-MBD2 (1-131) displayed any loss in repression function in the cell based repression assay (Figure 3-22, Bottom). From the graph, it was observed that that the truncation mutant was less competent than wild-type MBD2 in repressing the expression of the reporter plasmid. Therefore, MBD2 1-131 serves as a suitable negative control for the assay. However, the expression levels of the Gal4-MBD2 proteins could not be checked in this experiment because the epitope of the MBD2 antibody was deleted by the truncation.

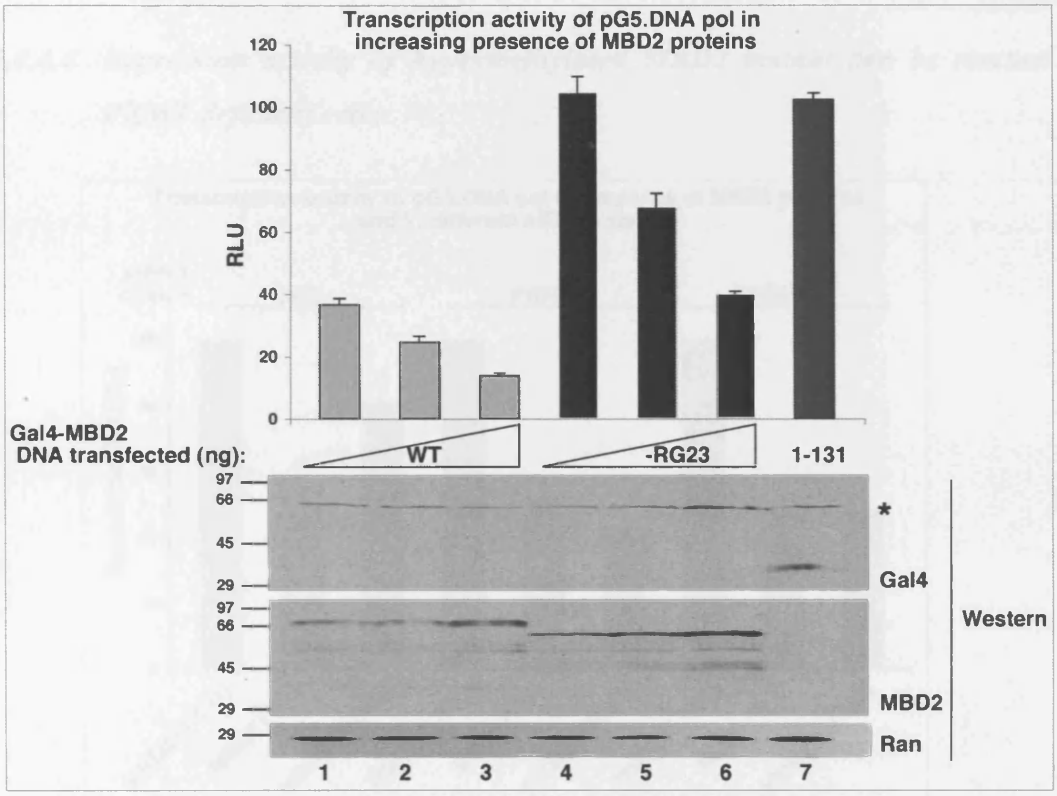

Figure 3-23 Hypermethylated MBD2 mutant have impaired repression activity.

(Top) 293T cells were transfect with the luciferase/renilla reporter plasmid as for Figure 3-18 and the indicated Gal4-MBD2 plasmids. RLU and expression levels of the Gal4-MBD2 proteins were analyzed as for Figure 3-18.

(Bottom) Gal4-MBD2 proteins were quantified by Western blot with the indicated antibodies. (*) Denotes the position of non-specific band and Ran indicate normalized protein loading.

After testing commercially available antibodies against the Gal4-MBD2 1-131 mutant, the best antibody that detects the presence of the protein was used to repeat the cell-based assay (Figure 3-23). As the Gal4 antibody was still quite insensitive, it detected only the highly expressed Gal4-MBD2 1-131 (Bottom, lane 7),

and a ~60kDa nonspecific protein that migrates very similarly to Gal4-MBD2-RG23. Therefore, the MBD2 antibody, which does not recognize the truncation mutant, was used to determine the expression levels of Gal4-MBD2 (WT) and Gal4-MBD2-RG23.

While the Western blot revealed that the expression level of the Gal4-MBD2 proteins were in the order of 1-131 > -RG23 > WT, it was observed that the wild-type MBD2 protein had more repression activity than the hypermethylated mutant MBD2–RG23. The HDAC-interacting mutant (MBD2 1-131) had no (or basal) repression activity.

3.4.4.4 Repression activity of hypermethylated MBD2 mutant can be rescued in PRMT deficient cells

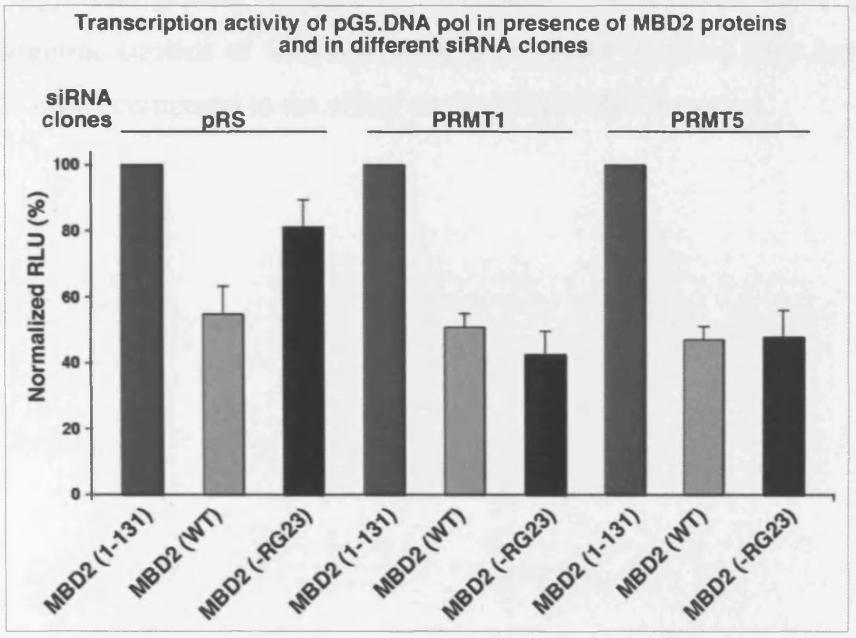

Figure 3-24 Rescue of MBD2-RG23 repression activity in siRNA clones.

The siRNA clones in Figure 2-22 **were transiently co-transfected with reporter plasmids as for Figure 3-23. As these cells are more sensitive to repression by Gal4-MBD2, the Gal4 plasmids are co-transfected at a tenth of the amount used in Figure 3-23 and at the same ratio corresponding to Lane 1 (WT), Lane 4 (-RG23) and Lane 7 (MBD2 1-131). RLU were obtained as for Figure 3-18 and the variation in expression of firefly luciferase in different cells are compensated by calculating the percentage expression against the MBD2 (1-131) repression mutant.**

To test whether the loss of repression function of Gal4-MBD2-RG23 is a true reflection of hypermethylation at arginine residues, I repeated the assay in the PRMT

siRNA clones to test whether the transcriptional repression activity of the mutant proteins could be rescued in the PRMT deficient cells (Figure 3-24).

While the repression activity of Gal4-MBD2-RG23 was still impaired in the control siRNA clone (pRS), depletion of both PRMT1 and PRMT5 in cells resulted in the ability of the hypermethylated mutant to repress the reporter to levels comparable that of the wild-type MBD2.

Wild-type MBD2 was also expected to repress transcription more efficiently in these cells. I find that the difference in repression activity of wild-type MBD2 in control cells versus PRMT-deficient cells was slight. This is because the methylarginine content of the wild-type protein in control cells is much lower than that of the hypermethylated MBD2-RG23 mutant. Therefore, further lowering the methylarginine content of wild-type MBD2 in PRMT-depleted cells has a much less dramatic effect compared to the effect on the MBD2-RG23 mutant.

Chapter 4 Discussion

4.1 MBD2 and MeCP2 are methylated in vitro and in vivo

Using the commonly employed *in vitro* and *in vivo* methylation assay (141), I deduced that MBD2 and MeCP2 are post-translationally modified by protein methyltransferases. *In vitro*, these modifications are localized towards the N-terminal portion of both proteins (Figure 2-2), the region that contains the highest concentration of arginine in the context of RG or GR (14 in MBD2, 5 in MeCP2, Figure 4-1). Therefore, the modifications are possibly catalyzed by protein arginine methyltransferases.

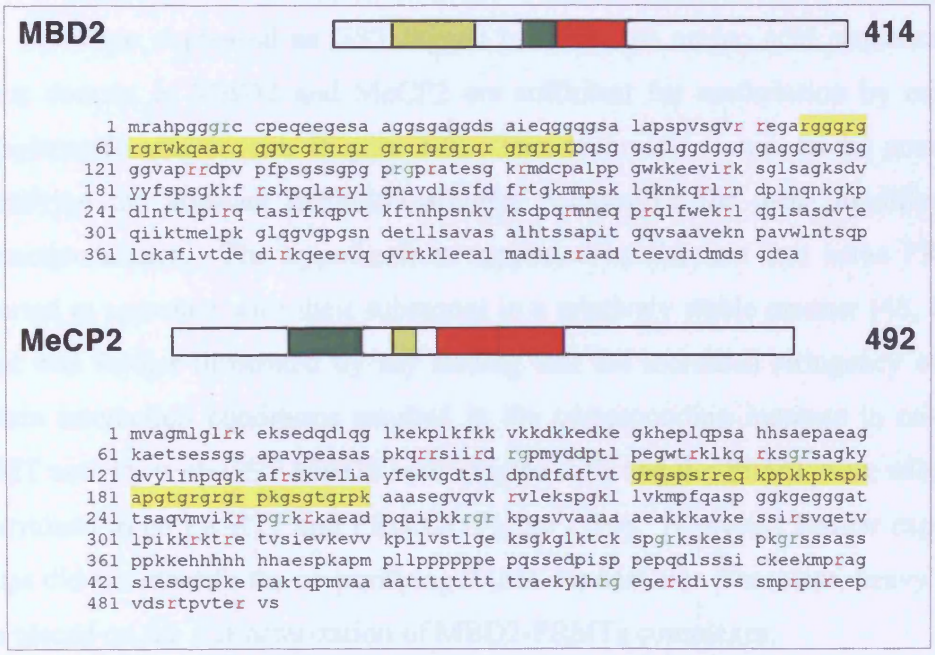

Figure 4-1 The RG repeat domain.

The RG repeat domain of MBD2 (Top) and MeCP2 are shown (Yellow box). Outside the RG repeat domain, there are several other arginines (Red) and some are positioned next to a glycine (Green).

As arginine residues positioned beside glycine are the preferred substrates for most PRMTs (16, 126, 154), I removed the RG repeat domain of both proteins and subjected them to metabolic labeling in cells (Figure 2-5). Only full-length MBD2 and

MeCP2 are acceptors of the post-translationally added ^{3}H-methyl groups. Outside the RG repeat domain, additional RG motifs exist in both proteins (2 in MBD2, 8 in MeCP2, Figure 4-1). Although, these arginines are possible targets of PRMT, I did not observe any incorporation of ^{3}H-methyl groups after long-term exposure of the Fluorograph in Figure 2-5. Judging by the distribution of RG repeats in MeCP2 and the corresponding ^{3}H-labelling signals, I established that the RG repeat domain is the major site of PRMT modification in this protein. For MBD2, it was more straightforward as far fewer RG repeats exist outside the domain.

Based on the methylation assays and my laboratory's interest in the RNA binding function (mediated by RG repeat domain) of both proteins (102), I focused on characterizing the post-translational modifications within the RG domains.

4.2 MBD2 interacts with PRMT

When expressed as GST-tagged proteins, the amino acid sequences of RG repeat domain in MBD2 and MeCP2 are sufficient for methylation by mammalian methyltransferases *in vitro* (Figure 2-6). This observation suggests the possibility of identifying the arginine methyltransferases responsible for these modifications by interaction assays. The hypothesis is supported by the fact that some PRMTs are reported to associate with their substrates in a relatively stable manner (48, 77). This point was further illustrated by my finding that the increased stringency of protein-protein interaction conditions resulted in the corresponding increase in co-purifying PRMT activity with GST-MBD2 baits (Figure 2-9) and the PRMT were subsequently determined to be PRMT1 and PRMT5 (Figure 2-10). However, similar experimental setups did not identify the co-purifying PRMT for MeCP2. Therefore, heavy emphasis was placed on the characterization of MBD2-PRMTs complexes.

While the data from binding assays consistently and convincingly show that the RG domain of MBD2 interacts with PRMT1 (Figure 2-10) and PRMT5 (Figure 2-11), various other experiments were also employed to substantiate this finding. This is because the highly basic RG domain of MBD2 (pI = 13.31) might interact non-specifically with the acidic PRMT1 (pI = 5.29) and PRMT5 (pI = 6.24).

4.2.1 PRMT5

A more physiological immunoprecipitation system revealed that endogenous MBD2 interacts stably with PRMT5 and MEP50 but exhibited weak or insignificant interaction with pICln (Figure 2-13). These three proteins form the 20 S methylosome, a complex that controls the proper biogenesis of pre-mRNA splicing machinery through methylation of Sm proteins. In the case of Sm proteins, MEP50 is required for the methyltransferase activity of PRMT5 whereas pICln is postulated to direct substrate specificity (71, 72). The region on Sm protein that binds pICln is different from the region that mediates the Sm-PRMT5 interaction. Unlike the endogenous MBD2, the recombinant RG domain of MBD2 interacts strongly with pICln. This observation is possibly an artifact due arising from the huge amount of PRMT5 that is attracted to the peptide (Figure 2-11).

It may be argued that the association between PRMT5 and MBD2 is be due to the existence of both proteins in the Sin3A containing repression complex (23, 178). However, using the purification scheme for MBD2 protein complexes, I was unable to co-purify Sin3A protein (Figure 3-3). It was demonstrated that the MBD2-PRMT5 interaction is mediated by the RG domain and removal of the domain from MBD2 results in the loss of interaction between PRMTs and MBD2. (Figure 2-15)

By thorough characterization of antibodies against dimethylarginines, I confirmed the presence of asymmetric and symmetric dimethylarginines within the RG domain of MBD2 (Figure 2-18 to Figure 2-20). This observation correlates with the finding that MBD2 arginine methylation is mediated by a type I and a type II PRMT (PRMT1 and PRMT5 respectively).

MBD2 is also reported to be a putative type II PRMT substrate in a large-scale proteomics study that used the Sym11 antibody to immunoprecipitate candidate proteins that contain symmetric dimethylarginines (25). When probing the Sym11 antibody against MBD2 in Western blot, I was unable to identify the symmetric dimethylarginine modification present (Figure 2-18). This might be due to the structural difference between native MBD2 used for immunoprecipitation compared to the denatured MBD2 protein in Western blots.

4.2.2 PRMT1

In comparison with MBD2-PRMT5, the endogenous MBD2-PRMT1 complex seemed to be less stable (Figure 2-14). The physical association between MBD2 and PRMT1 can only be illustrated with the recombinant RG domain protein (Figure 2-10) or the overexpressed MBD2, which binds PRMT1 and PRMT5 but not PRMT 3 or PRMT4 (Figure 2-15). In both instances, the dimethylarginine contents on MBD2 are either very low or not present. Endogenous MBD2 (Figure 2-28) or MBD2 expressed at endogenous levels (Figure 2-20) displayed higher asymmetric dimethylarginine content and did not co-purify stably with PRMT1.

Most *bona fide* PRMT1 substrates reported do not form endogenous complexes with the arginine methyltransferase and the molecular interactions are usually demonstrated by less physiological experiments (48, 90, 211). This is further illustrated by the inability of endogenous hnRNP A1 protein to co-purify with the enzyme (Figure 2-14). Therefore, to ascertain the relationship between PRMT1 and MBD2, I partially depleted PRMT1 from cells to demonstrate the corresponding reduction in asymmetric dimethylarginine content of the protein (Figure 2-25). Further characterization in PRMT1 null ES cells also revealed the total elimination of asymmetric dimethylarginine in the endogenous MBD2 proteins (Figure 2-28). These data are in agreement with another report, which states that PRMT1 is indispensable for the asymmetric arginine methylation of its substrate proteins (179).

The weak interaction between the highly methylated species of MBD2 and PRMT1 most probably reflects on the dynamics of the methyltransferase. In a study by Herrmann *et al.*, the accumulation of hypomethylated substrates caused the predominantly cytoplasmic PRMT1 proteins to be immobilized in the nucleus. When methylation was resumed, mobility of PRMT1 increased and the protein equilibrated back to the cytoplasm (91). As the majority of PRMT1 substrates such as histones and hnRNPs are nuclear proteins, Herrmann *et al.* speculate that PRMT1 only stably associate with unmethylated substrates and release them after the methylation event. This could explain the observable interaction of PRMT1 with the recombinant MBD2 RG domain or the overexpressed MBD2 protein.

4.3 Complexity of MBD2 arginine methylation

4.3.1 Asymmetric versus symmetric dimethylation

Similar to the MBD2, some PRMT substrates are reported to contain both asymmetric and symmetric dimethylarginine modifications, examples include SmD1, SmD3 and SmB, B′ from the splicesosome protein family (71, 160) and the high mobility group A1a protein (244). Notably, two proteins that are involved in transcription regulation, the histone H4 (177, 226) and the SPT5 (121), are reported to be substrates of PRMT1 and PRMT5. Mass spectrometry analysis of these two proteins revealed that asymmetric or symmetric dimethyl groups can be targeted onto the same arginine residue and this led to speculations on the possible antagonistic actions between PRMT1 and PRMT5 (16).

As the mass spectrometry analysis was unable to resolve the RG domain peptide digested from full-length MBD2 (Figure 2-4), I do not have information regarding the position of asymmetric dimethylarginine versus symmetric dimethylarginine or even the *bona fide* sites of arginine methylation in the mammalian MBD2 protein. I can only speculate on how these two species of dimethylarginines may distribute along the RG domain of MBD2 based on the common observation that RGG motifs can only contain asymmetric dimethylarginine while RG motif may contain both (154) (Figure 4-2, Top).

Nevertheless, with the highly specific Asym24 and Sym10 antibody, I was able to perform semi-quantitative assays to determine the nature of arginine methylation on MBD2 purified from different cellular systems. In siRNA cells with partially depleted PRMT1 activity, the total population of MBD2 protein purified had reduced asymmetric and symmetric dimethylarginine content (Figure 2-25). On the other hand, in prmt1 ES cells, the asymmetric dimethylarginine in endogenous MBD2 are totally abolished while the symmetric dimethylarginines are significantly increased (Figure 2-28 and Figure 2-29).

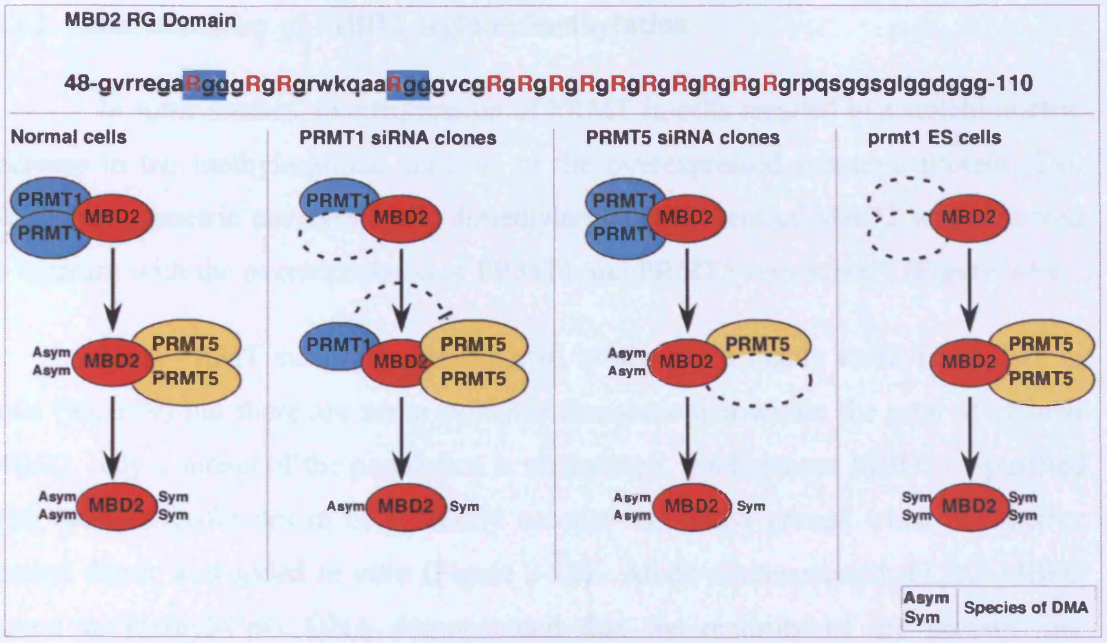

Figure 4-2 Species of dimethylarginine present on MBD2 protein.

(Top) The amino acid sequence of MBD2 RG domain. The most likely arginine substrates for PRMT are in red. Possible sites for asymmetric dimethylarginine modification are shown (Blue box).

(Bottom) Predicted mechanisms of MBD2 arginine methylation in different mammalian cell systems. Models are based on the methylarginine content of MBD2 observed in the cells and the knowledge of deficiency in PRMT1 or PRMT5.

The difference can be explained by the remaining PRMT1 in siRNA cells binding to MBD2 protein but not methylating them. It must be noted that normal level of endogenous PRMT1 may be crucial for its dimerization in cells, a feature that is essential for the methyltransferase activity (239, 240). Since unmethylated substrates are known to have tighter association with PRMT1 (91), the unmethylated MBD2-PRMT1 complex might deny the efficient methylation of MBD2 by PRMT5. This resulted in the decrease in both species of dimethylarginines on the MBD2 protein.

For the case of prmt1 ES cells, symmetric methylation of MBD2 by PRMT5 is not interfered by PRMT1. Therefore, the type II methyltransferase may modify substrate proteins more efficiently, resulting in an MBD2 protein with high symmetric dimethylarginine content. To my knowledge, this is the first example of the widely speculated antagonism between type I and type II PRMT (16) (Figure 4-2).

4.3.2 Stoichiometry of MBD2 arginine methylation

In some studies, overexpression of PRMT in cells resulted in a stoichiometric increase in the methylarginine contents of the overexpressed substrate protein (26). Neither asymmetric nor symmetric dimethylarginine content of MBD2 was observed to increase with the overexpression of PRMT1 and PRMT5 respectively (Figure 6-6).

Most PRMT substrates are reported to achieve a highly methylated state in cells (90, 179) but there are some evidence that show that within the pool of cellular MBD2, only a subset of the population is methylated. Endogenous MBD2 co-purified with methyltransferases in cells readily accepts ^{3}H-methyl groups when radioactive methyl donor was added *in vitro* (Figure 2-12). Albeit overexpressed, FLAG-MBD2 bound to methyl-CpG DNA demonstrated that the majority of the proteins are unmethylated and have a better affinity for the DNA (Figure 3-12 and Figure 3-13). Partially disrupting the RG domain of MBD2 results in hypermethylation, and this is illustrated by probing the proteins with methylarginine antibodies and post-translationally labeling *in vivo* (Figure 3-6). Reassuringly, the hypermethylated mutants exhibit a higher degree of protein expression compared to wild-type MBD2. This indicates that the low methylarginine content observed in wild-type MBD2 is not because there was insufficient PRMT available in cells to execute the modification. The hypermethylated mutants do not interact stably with PRMT and this supports the hypothesis regarding the dynamics of PRMT1-MBD2 interaction (see Chapter 4.2.2). This is the first demonstration that a PRMT substrate with extensive RG repeats can have an increase in methylarginine contents by removal of some arginine residues that are the possible sites of this modification.

4.3.3 Can MBD2 arginine methylation be reversed?

The apparent complexity of MBD2 arginine methylation suggests that factors other than PRMT1 and PRMT5 might participate in the overall regulation of arginine residues within the RG domain. The most obvious candidate would be an arginine deiminase.

It is generally believed that arginine methylation does not alter the appearance of proteins in one or even two dimensional gel electrophoresis (77, 97). To my

knowledge, there is only one report that briefly describes the anomalously slow migrating properties of RGG motif containing proteins (231). However, for arginine deimination, two independent groups reported on a more believable alteration in electrophoretic mobility of citrullinated histone due to the loss of one positive charge on the modified end product (54, 227).

Although no direct evidence is available at the moment, the low stoichiometry of methylarginine contents on MBD2 and the change in electrophoretic mobility of the protein that is dependent on genetic background or Adox treatment of cells (Figure 6-5) suggest that this PRMT substrate might be subjected to arginine deimination as well.

4.4 The consequences of MBD2 arginine methylation

Although the numbers of newly identified PRMT substrates are increasing at an exponential rate, little is known about the cellular events that trigger arginine methylation of the proteins (16, 126). A few pieces of evidence suggested that PRMT are possibly required for cellular development (111) and treatment of cells with growth factors increase arginine methylation of cellular proteins (44, 45).

As the nature of this study is more directed towards protein biochemistry, I did not investigate the possible cellular cascades that might increase the arginine methylation of MBD2 protein. In addition, pharmacological intervention of MBD2 arginine methylation is useful only to a certain extent. This is because decreasing the methylarginine content of MBD2 by Adox has the undesirable effect of disrupting the integrity of the NuRD complex (Figure 3-4), the 1 MDa co-repressor partner of MBD2 (65, 242). However, with various deletion mutants that produce an assortment of MBD2 with different amount of methylarginines (Figure 3-5 and Figure 3-6), I was able to probe the molecular effects of arginine methylation on this protein.

4.4.1 Arginine methylation decreases MBD2-NuRD interaction

Deleting the RG domain, or expressing the wild-type MBD2 protein in PRMT deficient cells revealed that MBD2 with a reduced methylarginine content have a higher affinity for components of the NuRD complex. The increase of co-purifying

HDAC silencing complex is demonstrated by Western blot (Figure 3-1) and *in vitro* HDAC assay (Figure 3-2).

When MBD2 hypermethylation mutants were tested for the quantity of co-purifying HDAC silencing complex, the correlation between increasing arginine methylation and decreasing affinity for the corepressor complex become evident (Figure 3-6). Among seven hypermethylated mutants, six were incapable of efficient interaction with the components of NuRD complex compared to the wild-type MBD2 (Figure 3-7). The significance of how one hypermethylated MBD2 mutant (MBD2-RG18) avoids the effect of methylation and interacts stably with the NuRD complex is not known and will be best explored by structural analysis.

With regard to protein-protein interactions, asymmetric dimethylation of arginines are illustrated to be detrimental for the interaction between proline rich and SH3 domain (15) whereas symmetric dimethylation facilitates Tudor domain interaction (30, 70) (see Chapter 1.7.1). In all cases, the effects were determined empirically. While the MBD2/NuRD interaction may serve as an ideal case study for such molecular dissection, certain issues must be addressed before putting arginine methylation into the equation. At least ten proteins exist in the NuRD complex (65) and which protein comes into direct contact with MBD2 remains unknown. Within the MBD2 protein, the region that is reported to recruit the Sin3A corepressor complex (23) is not the region required for association with the NuRD. After the deletion of Sin3A binding region, the TRD mutants were still capable of co-purifying MTA2 and core HDAC components (Figure 3-21). Subsequent experiments localized the NuRD interacting domain to residues 227-414 on the MBD2 protein (Figure 3-22). Thus, more characterization is required to pinpoint the specific domain.

4.4.2 Arginine methylation decreases MBD2-methyl-CpG DNA interaction

MBD2 proteins assembled on methyl-CpG DNA and eluted in a stepwise manner with increasing concentrations of NaCl revealed that arginine methylated species of MBD2 have less affinity for the DNA and eluted earlier than the majority of the proteins that are unmethylated (Figure 3-12 and Figure 3-13). This control is found to be exclusive to wild-type MBD2 proteins as arginine methylated species of RG mutants exhibited similar elution pattern with the total population of the protein.

Such differences in affinity between arginine methylated proteins and nucleic acids are seldom demonstrated in the characterization of nucleic acids binding PRMT substrates, the major class of proteins that are subjected to this modification (154). In 1994, Rajpurohit *et al.* used a continuous gradient elution to remove hnRNP A1 proteins that were non-specifically binding to single stranded DNA and RNA. They found that arginine methylated species of hnRNP A1 required slightly lesser concentration of NaCl to be eluted from DNA (40 mM) and RNA (35 mM) compared to their unmethylated counterpart (185). In a subsequent report by Valentini *et al.*, the specific RNA binding partners of yeast hnRNP were identified, and measuring the dissociation constant between the hnRNP and the RNA showed that arginine methylation does not alter the affinity of the protein towards the RNA (222).

While Rajpurohit *et al.* demonstrated the difference in affinity between hnRNP A1 protein and nucleic acids using non-specific targets (185), my experiments used the specific DNA partner of MBD2. Furthermore, I illustrated that my assay system was highly stringent and only allowed binding of MBD2 to methylated CpG DNA but not the unmethylated DNA comprising the same sequence (Figure 3-10 to Figure 3-11). However, my experiment used a step salt elution gradient while Rajpurohit *et al.* used a continuous gradient, which may more accurately measure the difference in affinity between each species of protein. This is because, at 200 mM increments of NaCl, methylated MBD2 proteins eluted at 400 mM and the majority of proteins eluted at 600 mM (Figure 3-12). When the salt increments were reduced to 100 mM, methylated MBD2 proteins eluted at 500 mM while unmethylated MBD2 remains unchanged (Figure 3-13). Thus, only the continuous salt gradient elution is able to pinpoint the exact elution requirement of arginine methylated MBD2. If performed, the elution difference between modified and unmodified species of MBD2 is predicted to be as subtle as the values reported by Rajpurohit *et al.*

The subtle difference in methyl-CpG DNA binding affinity of arginine methylated MBD2 probably does not reflect on the total elimination of MBD2 repressive functions. In cells, MBD2 was demonstrated to have a wider substrate specificity than MeCP2. It could occupy genomic loci of MeCP2 when the latter protein was depleted (117). While the methyl-CpG DNA probe used in my study contains a high proportion of methyl-cytosine (Figure 3-10), the presence of one

methylated CpG is sufficient for MBD2 binding (85). Therefore, proper investigation of arginine methylated MBD2 binding sites in the genome might reveal that this modification influences the sequence specificity of MBD2 protein.

Another important point to consider with regard to the conclusion drawn from the methyl-CpG DNA binding experiment (Figure 3-12) is that the MBD2 proteins used were overexpressed in cells and therefore had low methylarginine content. As it is unfeasible to purify a large quantity of endogenous MBD2 in native form, I can only predict the outcome of such experiment. In the process of characterizing MeCP1 (MBD2/NuRD), Meehan *et al.* pointed out the negligible methyl-CpG DNA binding activity of embryonic stem cells extracts compared to HeLa and various primary cells (157). I did not perform parallel comparisons but my attempts to detect the methylarginine contents of endogenous MBD2 purified from HeLa[13] cells did not yield positive signal in Western blot compared to the MBD2 from the ES cells (Figure 2-28 and Figure 2-29). Therefore, I speculate that the lack of MeCP1 activity observed in ES cells might be due to by the higher methylarginine contents of the MBD2 proteins from these cells.

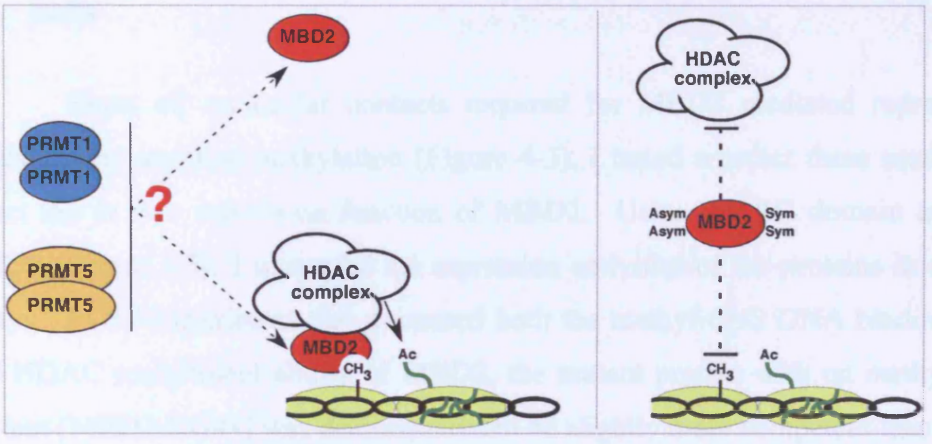

Figure 4-3 The overall molecular effects of MBD2 arginine methylation.

(Left) The exact cellular location for the PRMTs to methylate MBD2 is not known; the preferred forms of MBD2 proteins for recognition by PRMTs also need to be investigated.

(Right) At molecular level, MBD2 arginine methylation decreases the affinity of the repressor to interact with the HDAC co-repressor complex (NuRD) and methyl-CpG DNA.

[13] Data not shown for determination of methylarginine contents of MBD2 from HeLa cells.

4.4.3 Independent regulation of MBD2-NuRD and MBD2-methyl-CpG DNA interaction by PRMT

While MBD2 arginine methylation has inhibitory effect on the affinity of this protein towards NuRD complex and methyl-CpG DNA, I believe that the control of MBD2 interaction with these factors by PRMT activity is two independent events.

For MBD2-methyl-CpG DNA interaction, the inhibitory effect of arginine methylation is exclusive for wild-type MBD2 proteins (Figure 3-12). Hypermethylated MBD2 mutants that display compromise affinity for NuRD complex (Figure 3-6 and Figure 3-7) do not elute faster than the total population of protein in methyl-CpG DNA interacting column (Figure 3-13). Removal of the MBD domain from the MBD2 protein (MBD2-TRD94) produces mutants that have high affinity for the NuRD complex (Figure 3-21). This mutation, when combined with various RG domain mutations, show that arginine methylation still controls the affinity of MBD2 for the NuRD complex when a functional MBD domain is not present.

4.5 Hypermethylated MBD2 has impaired repression activities in cells

Since all molecular contacts required for MBD2 mediated repression are weakened by arginine methylation (Figure 4-3), I tested whether these modifications affect the *in vivo* repression function of MBD2. Using the RG domain mutants of MBD2 (Figure 3-5), I measured the repression activities of the proteins in cell-based assays. In the experiment that measured both the methyl-CpG DNA binding affinity and HDAC recruitment ability of MBD2, the mutant protein with on methylarginine content (MBD2-RG41) was demonstrated to be slightly more competent than the wild-type MBD2 in mediating the repression of CpG-methylated reporter plasmid (Figure 3-15 and Figure 3-17). The subtle difference, and the irresponsiveness of the reporter plasmid expression towards the dosage of MBD2 transfected led me to refine the experiment.

Taking the DNA binding ability of MBD2 out of the picture, I measured repression capability of Gal4-MBD2 fusion protein. I found that hypermethylated MBD2 mutant (MBD2-RG23) is at least 2.5 times less competent compared to wild-

type MBD2 in mediating HDAC dependent transcription repression (Figure 3-18 and Figure 3-23). The loss of function in the MBD2-RG23 mutant was rescued when the experiment was repeated in PRMT deficient siRNA cells that were less capable of executing arginine methylation on MBD2 proteins (Figure 3-24).

Though consistent with the biochemical effects MBD2 arginine methylation, the cell-based repression assays are not the best methods to measure the repression activity of MBD2. This is because transfected reporter plasmids in the cells assemble into chromatin with structures and histones compositions that are dissimilar to cellular chromatin (209). This is relevant to repressor proteins such as MBD2, which mediates transcription silencing by chromatin remodeling.

A more representative reporter system may be produced by integration of the reporter plasmids into the genome. This was not done due to the time constraints and the fact that the plasmid expression would be tightly controlled by the position of the genome at which it integrated, and becomes unresponsive to MBD2 mediated repression. Alternatively, genes controlled by MBD2 in cells can be quantified with respect to MBD2 arginine methylation. However, GSTP1 (an MBD2 controlled gene in MCF-7 cells (140)) was demonstrated to maintain normal protein levels in cells cultured from mice with homozygous deletion of MBD2 gene (Figure 3-17)[14].

4.6 Can MBD2 be responsible for histone arginine methylation?

Almost all reported transcription-regulating PRMT substrates are classified as transcription activators or transcription factors, and repressor proteins are seldom identified as PRMT substrates (126). Nucleolin, a possible transcription repressor (234), is reported as a PRMT substrate (163) but the significance of this modification has not been investigated.

Nevertheless, PRMTs are known to interact with transcription repression complexes. However, the usual targets for methylation are not within the complexes (6, 177, 178). PRMT5 interacts with the SWI/SNF (177, 178), and with the Blimp1 repressor, both of which are implicated with HDAC containing chromatin remodeling

[14] Data not shown for GSTP1 expression level in mbd2 and WT mtf.

complexes (6). In both instances, histone arginines (H4R3 and H2AR3 for (6) only) are the reported substrates of these interactions and the evidence was provided by *in vitro* methylation assays using the co-purified methyltransferase activities. Activities of PRMT5 in these complexes are speculated to contribute to transcription repression.

When this thesis was in preparation, Le Guezennec *et al.* reported on the association between PRMT5 and MBD2 proteins expressed in stable cell lines (125). While their investigations were mainly focused on the mutual exclusiveness of MBD2/NuRD and MBD3/NuRD complexes (see Chapter 1.4.2), they had also co-purified PRMT5 with MBD2 and illustrated the *in vitro* methylation of MBD2. In a further experiment, they used ChIP to illustrate that MBD2 and PRMT5 co-localized on the p16/INK4A and p14/ARF (INK4a/ARF) locus and the specific genomic region contains dimethylated histone H4 arginine 3. The H4R3 modification could be significantly reduced when cells were treated with DNA methylation inhibitors

It must be noted that besides associating with the MBD2 (146), the INK4a/ARF locus is subjected to transcription regulation by many other chromatin remodeling complexes (46). Well-characterized examples of regulatory factors binding to the INK4a/ARF locus include protein members from the E2F family (59) and the SWI/SNF complex (35). Both E2F and SWI/SNF are capable of synergistic interaction with PRMT5, and the resultant H4R3 methylation was observed (62, 177, 178).

Based on my observation on the stable association between MBD2 and PRMT5 (Figure 2-13), I do not exclude the possibility that the INK4a/ARF-MBD2-PRMT5 association identified by ChIP is a real physiological complex, and not an artifact arising from the independent binding of both proteins on the locus. However, with regard to H4R3 methylation in the INK4a/ARF locus, Le Guezennec *et al.* proved the presence of dimethylated H4R3 by ChIP assay using an antibody that was generated against asymmetrically methylated H4R3 peptide (125). The asymmetrically methylated H4R3 is highly unlikely to be the end product of PRMT5 catalysis.

Since this study shows that MBD2 also associates with PRMT1, it may be tempting to speculate that the methyl-CpG DNA dependent asymmetric methylation of

H4R3 observed by Le Guezennec *et al.* could be a result of this interaction. If proven, this could support the findings that speculate the H4R3 asymmetric methylation by PRMT1 to be an early transcription activation event that leads to the acetylation of histone tail by the p300 acetyltransferase (5, 226). Such conclusions would be consistent with my overall observation of how arginine methylation of MBD2 relieves transcription repression activity.

4.7 Future perspectives

While the possibilities that MBD2 mediates the PRMT modification of histone arginine may be appealing, there are unanswered questions regarding the PRMT modification of MBD2.

I need to know the specific cellular localization of MBD2 protein when it is modified by PRMT1 or PRMT5. Both PRMT are reported to be predominantly cytoplasmic (48, 91, 188) but evidence exists to show that they can be found in the nucleus (62, 91, 121). Newly translated MBD2 may be methylated as it emerges from the ribosome, but it is also possible that the modification can occur in the nucleus. With such knowledge, I would be able to predict and test for the preferred forms of MBD2 substrate for each PRMTs, whether it is monomeric MBD2, MBD2 associating with NuRD complex or methyl-CpG DNA, or both (Figure 4-3).

The MBD2-7SK RNA complex is another form of MBD2 that is most likely to have altered preference for PRMT methylation. As 7SK RNA binds MBD2 directly through the RG domain (102, 114), whether the RNA-protein interaction can affect, or be affected by, MBD2 arginine methylation can be addressed by preexisting techniques acquired for this study. The significance of MBD2 arginine methylation in the context of its interaction with 7SK RNA will be a completely new topic that may answer many speculations and probably can open up many perspectives in the study of PRMTs.

Unmethylated MBD2 binding to PRMTs may also serve important functions. Blimp1 repressor and PRMT5 translocate from the nucleus to the cytoplasm of mice embryonic stem cells when the cells are undergoing extensive epigenetic reprogramming. This causes the expression of Blimp1 controlled genes (6). As

MBD2 with low methylarginine content binds both PRMTs stably, functions of these MBD2-PRMT complexes should be characterized.

For methylated species of MBD2, I have not yet addressed whether an individual MBD2 molecule contains both asymmetric and symmetric dimethylated arginine, or whether asymmetric and symmetric modifications are mutually exclusive. If MBD2 containing only asymmetric or symmetric dimethylarginine can be separated, and purified with their interacting partners, I may be able to identify novel protein-protein interactions mediated by the modifications. This is supported by recent evidence, which indicates that dimethylated arginine is also involved in protein binding (30, 49, 70).

These results may provide us with clues to the mysteries surrounding the importance of MBD family members. Here, I find that arginine methylation of MBD2 serves one purpose of relieving its transcription repression activity, and currently, the significance of having two different PRMTs that interact with, and produce different end products on MBD2, remains to be elucidated. Can this be related to the control of cellular development process? Recent reports have already show that MBD2 (98, 99), the NuRD complex (108), PRMT1 (180) and PRMT5 (6) may be involved in gene expressions which are critical during development. Can these factors work together to control developmental gene expression?

If so, I would need to check at which developmental stages are the MBD2 proteins specifically methylated at the arginines. If MBD2 protein generally contains methylated arginine throughout all stages of cellular differentiation, maybe the down regulation of MBD2 repressive functions by arginine methylation can explain the apparent lack of obvious disease phenotype in the initial characterization of the MBD2 -/- mice (86). To test such hypothesis, generation and characterization of mice that express only the MBD2b protein (85), which can escape regulation by arginine methylation will be most appropriate for addressing this question (Figure 4-4).

Figure 4-4 The MBD2b.

The MBD2b protein is an isoform of MBD2 that arise from the alternative translation of the MBD2 mRNA using the internal methionine located at the N-terminus of the MBD domain (Green). As it also contains the TRD (Red) that is required for repression, MBD2b is predicted to be functionally active. MBD2b lacks the RG domain (Yellow).

Chapter 5 Materials and Methods

5.1 General techniques, solution and buffers

Most media and commonly used solutions were provided by Cancer Research UK central services.

Media/solution	Composition
L-Broth (LB)	170 mM NaCl, 0.5% w/v yeast extract, 1% w/v bactotryptone and antibiotics
PBSA	170 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4
TE	10 mM Tris-HCl pH 7.0 and 1 mM EDTA
10 X DNA loading	60% w/v sucrose, 0.1% w/v bromophenol blue and 0.1% w/v xylene cyanole FF
10 X TBE (Ambion)	0.89 M Tris, 0.89 M Borate and 20 mM EDTA
50 X TAE	2 M Tris-Acetate and 50 mM Na_2EDTA
DEPC water	0.1% diethyl pyrocarbonate in water, autoclave after mixing

Table 5-1 List of common solutions provided by Cancer Research UK central services.

5.1.1 SDS polyacrylamide gel electrophoresis (PAGE)

Solution	Composition
Separating gel acrylamide	33.5% acrylamide and 0.3% bis
Separating gel buffer	380 mM Tris pH 9.1, 0.1% SDS 0.075% APS and 0.05% TEMED
Stacking acrylamide	30% acrylamide and 0.44% bis
Stacking acrylamide buffer	120 mM Tris pH 6.8, 0.1% SDS, 0.03% APS and 0.2% TEMED
2x SDS sample buffer	125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue and 10% β-mercaptoethanol
MW markers (100 μg/ml each)	lysozyme (14.3 kDa), carbonic anydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorlyase (97.4 kDa) and β-galactosidase (116 kDa)
4x Running buffer	12 g/L Tris-base, 57.6 g/L glycine and 4% SDS

Table 5-2 Components for SDS-PAGE in this study.

Separating acrylamide was prepared at final concentrations of 7.5 - 15% and stacking acrylamide was always at 4%.

5.1.2 SDS PAGE staining

5.1.2.1 Coomassie staining

Solution	Composition
Fixing solution	50% v/v methanol and 10% glacial acetic acid
Coomassie stain	2.2 g/L Coomassie blue R250 in fixing solution
Destaing solution	20% methanol and 10% glacial acetic acid

Table 5-3 List of general solutions required for Coomassie staining

SDS-PAGE gels were stained with Coomassie for 25 min and were destained until appropriate.

5.1.2.2 Silver staining

SDS-PAGE gels were stained in Silver Quest (Invitrogen LC6070) silver staining kit according to manufacturer's instruction.

5.1.3 Western blotting

SDS-PAGE gel and Protran (Schleicher and Schuell) nitrocellulose transfer membrane were immersed in 50 mM Tris, 14.5 mM glycine and 20% methanol (transfer buffer) for 15 min. Proteins in the gel were transferred onto the membrane by semi-dry transfer unit (Hoefer) according to manufacturer's instructions. Transferred membranes were stained with Ponceau S to reveal MW markers and were then washed briefly with 0.1% Tween in PBS (washing buffer) and blocked with 5% non fat dried milk (w/v) in washing buffer for 1 hr. Primary and secondary antibodies were diluted in washing buffer and were incubated with the membrane according to the antibody protocol. Membranes were washed for 30 min with 5 changes of washing buffer after each antibody staining. For visualization, the membranes were incubated in enzymatic chemiluminescent (ECL, Amersham) reagents and exposed to Kodak BioMax MR film (Sigma) according to manufacturer's instruction.

5.1.4 Fluorography

Fluorography was used for visualization of proteins with ^{35}S or ^{3}H-labels in this study. After Coomassie staining, SDS-PAGE gels were immersed in Amplify Fluorographic reagent (Amersham) for 15 min and were dried in a gel dryer (Bio-Rad) for 1 hr. Gels were exposed to Kodak BioMax MS film (Sigma) for at least 24 hr before developing the film.

5.2 Cloning

5.2.1 DNA preparation

Most plasmid DNA used for this study were raised in Max Efficiency (Invitrogen) *E. coli* DH5α cells. Gateway vector plasmids containing the lethal *ccdB* gene were raised in *E. coli* DB3.1 (Invitrogen). All plasmid DNA were isolated using Qiagen Mini or Maxi kit according to manufacturer's instruction and were resuspended in TE buffer.

5.2.2 Bacterial transformation

Plasmid DNA (1- 10 ng) were incubated with competent cells on ice for 30 min and were subjected to heat shock at 42°C for 90 sec. Cells were returned on ice for 3 min and warm LB was added to the cells for incubation on a shaker at 37°C for 40 min. After incubation, the cells were centrifuged at 1000 g for 30 sec to remove the LB and were plated onto LB agarose with appropriate antibiotic.

5.2.3 Annealing of oligos for cloning

5′-phosphorylated oligos (sense and antisense strands) were ordered from Sigma Genosys and 10 ng of each strand was resuspended in a buffer containing 30 mM HEPES-KOH pH 7.4, 100 mM K-acetate and 2 mM Mg-acetate. The reactions were incubated at 95°C for 4 min and then 75°C for 10 min after which they were equilibrated to room temperature by standing on the bench for 1 hr. Reactions were ready for ligation thereafter.

5.2.4 Ligation and sequencing

Ligation reactions for cloning purpose were carried out using Rapid DNA ligation kit (Roche Applied Science) according to manufacturer's instruction.

Clones were verified by sequencing. Purified DNA was incubated with BigDye Terminator v3.1 (Applied Biosystems) and appropriate primer. Sequences were obtained from 3730 DNA Analyzer (Applied Biosystems) operated by CRUK equipment park.

5.2.5 pRetro.Super plasmid for siRNA expression

To create insert encoding siRNA against the PRMT genes, mRNA sequences of human PRMT1 (NM_001536) and PRMT5 (NM_006109) were analyzed by Extractor 5 software. Four sets of sequences were selected for each PRMT gene and were synthesized and annealed as described in Chapter 5.2.3.

The pRetro.Super plasmid was digested with BglII and HindIII (New England Biolabs) and was ligated with the annealed oilgos to obtain the desired siRNA expression plasmids.

5.2.6 Gateway cloning

All FLAG and Gal4 fusions of MBD2 genes were generated using the Gateway (Invitrogen) cloning system.

pGal (1-94) Gateway destination plasmid was created by digesting the original plasmid with BamHI (New England Biolabs). T4 DNA polymerase (New England Biolabs) was used to blunt the ends of the linearized vector. To prevent recircularization, plasmid DNA was treated with calf intestinal phosphatase (New England Biolabs) and reading frame B from the Gateway vector conversion system (Invitrogen) was cloned into the vector. Ligated plasmids were raised and maintained in *E. coli* DB3.1 cells.

The gene of interest in the Gateway pDONR vector (see Table 5-7) can be easily transferred into any gateway destination vector by recombination reactions

carried out using gateway LR clonase (Invitrogen) and according to manufacturer instruction.

5.2.7 MBD2 deletion mutants

5.2.7.1 RG deletion mutants

Plasmids encoding MBD2 RG mutants were obtained by digestion of full-length MBD2 in pDNOR221 with FspI and SacII (New England Biolabs) to delete nucleic acids corresponding to residues 50-94 of the MBD2 protein. Double stranded mutagenic oligos were annealed and ligated into the remaining plasmid fragment to obtain the desired mutation.

5.2.7.2 TRD deletion mutants

To create MBD2 with a deletion of 15 residues in the TRD, full-length MBD2 in pDONR221 was digested with BbvCI and BspEI (New England Biolabs) to delete nucleic acid corresponding to residues 195-226 of the MBD2 protein. For larger deletion mutants, the plasmid was digested with BamHI and BspEI (New England Biolabs) to delete nucleic acids corresponding to residues 126-226. For MBD2 1-131, pDONR221-MBD2 was digested with BamHI and a mutagenic oligo was cloned into the plasmid to introduce a stop codon after residue 131 of the MBD2 protein

5.3 Production of proteins from bacteria cells

All recombinant proteins from bacteria were prepared and purified as described in Jeffery and Nakielny (102).

5.4 Mammalian cell culture

5.4.1 Cell types and media

Cell type	Medium
293T, HeLa, Ramos, Neuronal 2A, PC1 and all mouse tail fibroblast	Dulbecco's modified Eagle's medium (DMEM), supplemented with 4500 mg/L glucose, 2 mM L-glutamine, 0.1 units/ml penicillin, 0.1 μg/ml streptomycin and 10% fetal calf serum (Normal DMEM).

All siRNA stables	Normal DMEM with 2 μg/ml puromycin (Sigma)
All Flp-In T-Rex stables	Normal DMEM with 2.5 μg/ml blasticidin, 50 μg/ml hygromycin and induce with 1 - 25 ng/ml tetracycline for protein expression. All antibiotics mention were purchased from Invitrogen.
All embryonic stem cells	DMEM supplemented with 4500 mg/L glucose, 4 mM L-glutamine, 0.12 units/ml penicillin, 0.12 μg/ml streptomycin, 15% ES screened fetal bovine serum (Hyclone), 50 μM β-mercaptoethanol and 50 units/ml ESGRO (Chemicon International).

Table 5-4 List of mammalian cells used in this study and the media used for their maintenance.

All mammalian cells were cultured in Costar 100 mm tissue culture dish or 6 well plates unless otherwise stated. For ES cells, culture dish was pretreated with 0.1% gelatin (Sigma) for 30 min before addition of trypsinized ES cells. Incubation conditions were set at 37°C in 5% CO_2 incubator throughout.

5.4.2 Transfection

Effectene transfection reagent (Qiagen) was used for all transfection assays in this study. Cells in 100 mm plates were typically transfected when they were at 30 – 40% confluence. Unless otherwise stated, 6 μg of plasmid DNA, 48 μl EC buffer and 60 μl effectene reagent was used for each plate and cells were harvested 48 hr after transfection.

5.4.3 Generation of stable clones

5.4.3.1 Stable siRNA clones

To generate stable siRNA clones, 293T cells in 100 mm plates were transfected with pRetro.Super plasmids (1 μg DNA, 8 μl EC and 10 μl Effectene) encoding siRNA. Two days after transfection, normal medium was removed and replaced with selective medium (see Table 5-4). The cells were maintained in the selective medium for one week and replaced with fresh selective medium every two days until colonies were formed. Colonies were picked from each plate and were cultured in one well of a 6 well plate until confluence. Stable cells with significant knockdown of target protein in Western blot were subcultured. Clones displaying

highest level of knockdown for each protein after 2 months were kept for further analysis.

5.4.3.2 Stable Flp-In TREx clones

Expression plasmids (1 μg) encoding FLAG-MBD2 proteins were co-transfected with 9 μg of pOG44 into Flp-In TREx 293T (Invitrogen) cells using Effectene (Qiagen) transfection reagent (10 μg total DNA / 80 μl EC / 100 μl Effectene). 48 hr after transfection, the normal medium was replaced with selective medium (see Table 5-4) and the cells were maintained for 1 to 2 weeks until the appearance of colonies. Selected cells were checked for their tetracycline-induced expression of FLAG-MBD2 protein by Western blot (as described in Chapter 5.4.3.1).

5.4.4 Cell lysis buffers

All mammalian cell lysate and protein assay buffers were supplemented with 10 μg/ml of aprotinin, leupeptin and pepstatin, 0.5 mM PMSF and 2 mM DTT and were prepared at 4 °C.

Buffer	Contents
RIPA	10 mM Na_2HPO_4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% sodium deoxycholate and 1% NP40
TCE	50 mM HEPES-KOH pH 7.5, 150 mM K-acetate, 5 mM Mg-acetate and 0.1% digitonin
RSB	10 mM Tris pH 7.4 and 2.5 mM $MgCl_2$ (NaCl concentration can be variable)
Detergents for RSB	0.5% Triton X (for protein complex) or 0.5% Empigen (for pure proteins)
NaCl for RSB	100 mM to 1.6 M NaCl with 1 X RSB. Usually at 800 mM NaCl.
PRMT	20 mM Tris-HCl pH 8.0, 200 mM NaCl and 0.4 mM EDTA

Table 5-5 List of cell lysis buffers used in this study.

5.4.5 Preparation of cell lysate

Cells (~1 X 10^6) were washed once in ice cold PBSA and were lysed in 1 ml of the appropriate buffer (see Table 5-5). The lysates were sonicated with 5 x 10 sec blast with 5 min interval on ice between each blast, lysates were left on nutator for at least 30 min. Insoluble proteins were removed by centrifuging at 14,000 g for 10 min.

For Western blots, the lysates were normalized by Bradford assay to enable equal loading of proteins. For purification of endogenous proteins or GST pull down assay, lysates were subjected to a further centrifugation step of 100,000 g for 1 hr and the supernatant was passed through 0.22 μM filter to obtain a clear colorless lysate. Otherwise, cell lysates were used directly for immunoprecipitation or other biochemical assays. Unused lysates were flash frozen in liquid nitrogen and stored at -80 °C.

5.4.6 Purification of FLAG-tagged proteins from 293T cells

Cells lysates were prepared from transfected 293T cells using RSB-800 with 0.5% Triton X-100 lysis buffer (see Table 5-5). To purify FLAG-proteins without any interacting partners, the same lysis buffer was used with 0.5% Empigen BB (Calbiochem) in place of Triton X-100.

Anti-FLAG antibody M2-agarose beads (Sigma; 25 μl bead volume/plate of cells, washed with lysis buffer) were added to the cell lysate and the slurry was rotated for 2 -16 hr. After that, FLAG-tagged proteins bound to the beads were washed 4 times with RSB800-0.5% Triton X-100 buffer and once with PRMT buffer, each wash involved a centrifuging step of < 500 g for 30 sec and rotation at 4°C for > 2 min after addition of fresh buffer. Washed beads were eluted with 2 X SDS sample buffer for Western analysis or resuspended in PRMT buffer for biochemical assays.

To obtain unbound FLAG-tagged proteins in native form, 80 μg of 3X FLAG peptide (Sigma) in 20 mM Tris-HCl pH 7.4, 100 mM NaCl and 2.5 mM $MgCl_2$ was added to the beads and the slurry was rotated at 4°C for 1 hr. The proteins were eluted by passing the slurry through a Micro Bio-Spin column (Bio-Rad). FLAG-proteins purified in this way were quantified by comparing against BSA standards in SDS-PAGE/Coomassie. All FLAG- proteins were > 90% pure as judged by SDS-PAGE and Coomassie staining.

5.4.7 Purification of endogenous proteins/enzymatic activity from cells

Antibodies (2 – 5 μg) for immunoprecipitation were incubated with 1 ml cell lysate at 4°C for 3 to 16 hr. 50 μl of protein G sepharose (Amersham) beads washed

once in cold PBS was added to capture the antibody-protein complex for at least 1 hr. The beads were washed 4 times with the lysis buffer and twice with PRMT assay buffer. For Western blot analysis, the protein complexes were eluted from the protein G beads by boiling in SDS-sample buffer, and then separated by SDS-PAGE. To assay for co-purifying PRMT activity, the protein complexes on the beads were resuspended in 30 µl of PRMT assay buffer containing 2 µCi of ^{3}H-S-adenosyl-methionine. The labeling was carried out at 37°C for 90 min and the proteins were separated by SDS-PAGE and visualized by means of Western blot and Fluorograph.

5.4.8 Immunofluorescence

Cells (HeLa) on glass coverslips in a 6 well plate were transfected with plasmids (1 µg DNA/ 8 µl EC /10 µl Effectene per well) and were harvested after 18 hr. The cells were fixed with 2% formaldehyde in PBS at room temperature for 30 min and were permeabilized with acetone for 3 min at -20 °C. Blocking was carried out in 3% BSA in PBSA for 1 to 16 hr and the coverslip was covered with primary antibody diluted in blocking buffer for 1 hr. After primary antibody staining, the coverslips were washed 3 times in PBSA with 5 min incubation at room temperature between each wash. Secondary antibody diluted in blocking buffer was applied to the coverslips for 30 min and the coverslips were washed as previously. Stained coverslips were mounted onto glass slides with Vectashield mounting medium containing DAPI (Vector laboratories) and edges of the coverslip were sealed with nail varnish.

5.5 Specialized techniques used in this study

5.5.1 In vitro methylation assay

For all *in vitro* methylation assay, 0.5 µg of GST-tagged protein was used as substrate, 2 µCi of ^{3}H-S-adenosyl-methionine as the methyl donor, and 15 µg of cell lysate or rabbit reticulocyte lysate (Promega) as the source of methyltransferases. Cell lysate was pretreated with 0.1 mM S-Adenoysl-homocysteine (Sigma) at 37°C for 20 min when inhibition of methyltransferase activity was required. All methylation reactions were carried out in PRMT assay buffer described in Table 5-5 for 1.5 hr.

Methylated substrates were separated on SDS-PAGE, stained with Coomassie and analyzed by Fluorography.

5.5.2 In vivo labeling assay

Transfected 293T cells expressing FLAG proteins were starved in methionine deficient DMEM supplemented with 10% fetal calf serum, 100 μg/ml cyclohexamide (Sigma) and 40 μg/ml chloramphenicol (Sigma) for 30 min. The medium was removed and replaced with the same medium with 100 μCi of ^{3}H-L-methyl-methoinone (Amersham) or 10 μCi of ^{35}S-methionoine (Amersham) and in the presence or absence of protein synthesis inhibitor. The labeling reaction was allowed to proceed for 3 hr and FLAG proteins were purified by immunoprecipitation as described in 5.4.6. Proteins were eluted by boiling in SDS-PAGE sample buffer and were separated by SDS-PAGE, analyzed by Coomassie staining and Fluorography.

The *in vivo* labeling experiments were sometimes performed with cells treated with methylation inhibitors. Methylation inhibitors, Adox (Sigma) and MTA (Sigma) were used at a final concentration of 20 μM and 300 mM respectively and were added to the cells 12-36 hr prior to or during the labeling assay.

5.5.3 Precipitation of proteins

Proteins in solution were precipitated by addition of 6 volumes of a mixture of 50% ethanol, 25% methanol and 25% acetone. The mixture was vortexed vigorously and was incubated at – 20°C overnight or -80°C for 30 min. The mixture was centrifuged at 14,000 g for 20 min at 4 °C. The supernatant was removed and the pellet containing the precipitated protein was dissolved in 2 X SDS sample buffer.

5.5.4 GST pull down

To remove cellular proteins bound unspecifically to the sepharose beads, 2 ml of cell lysates described in Chapter 5.4.5 was added to 100 μl of washed glutathione sepharose beads (Amersham) and was rotated at 4°C for 1 hr. The GST-beads were removed by centrifuging at < 500 g for 30 sec. The supernatant was added with 2 – 5 μg of GST-tagged proteins and was left on the rotator at 4°C for 4 – 16 hr. After that, 50 μl of GST beads were added and the mixture was incubated for a further 1 hr to

capture the GST-tagged proteins. Proteins bound to the beads were then washed with 4 changes of lysis buffer or buffer containing higher salt content, see Table 5-5. Each wash consisting of centrifuging at 500 g for 30 sec and incubation on rotator at 4°C for > 2 min with addition of fresh buffer. In the last wash, the proteins were resuspended in PRMT buffer. To visualize the proteins by Coomassie/silver staining, PRMT buffer was removed from the beads by centrifugation and the proteins were eluted from the beads by addition of 2 X SDS sample buffer. Distinct bands on the SDS-PAGE gel were excised and were sent to CRUK protein analysis service for mass spectrometry identification.

5.5.5 HDAC assay

Biotinylated histone H4 peptide was acetylated with ^{3}H-Acetyl-coenzyme A (Amersham) using reagents and instructions provided in the Histone Deacetylase Kit (Upstate). After labeling and coupling to strepavidin agarose, the ^{3}H-histone H4 peptide was quantified by scintillation counting in Ultima Gold LLT (Perkin Elmer) scintillation fluid. Labeled peptides (30,000 to 40,000 CPM) were used for each assay to detect for HDAC activity in normalized FLAG-MBD2 protein complexes. The reaction was incubated, stopped and assayed for ^{3}H-Acetate release according to manufacturer's instruction. Sodium Butyrate (250 mM) was included as a control for unspecific release of ^{3}H-Acetate molecules.

5.5.6 Gel shift assay

Methylated DNA probe was labeled at the 5′ end using polynucleotide kinase (New England Biolabs) and γ^{32}P-ATP (Amersham) according to manufacturer's instructions. For labeling of RNA probes, plasmid DNA encoding the RNA was linearized with an appropriate restriction enzyme and transcribed *in vitro* with SP6 RNA polymerase (Promega) and in the presence of α^{32}P-UTP according to manufacturer's instruction. The reaction was treated with DNase I for 15 min at 37°C and the RNA was purified using G-50 spin column (Qiagen).

FLAG-tagged proteins purified as for Chapter 5.4.6 were incubated with the ^{32}P-labelled nucleic acids in the presence of buffer consisting of 40 mM HEPES pH 7.3, 110 mM K-acetate, 6 mM Mg-acetate, 250 mM sucrose, 1 mM DTT and 0.1%

NP40. All incubations were carried out on ice for 20 min. In RNA interaction experiments, 20 units of RNasin (Promega) were used, whereas in DNA interaction experiments, 0.1 mg/ml yeast tRNA (Ambion) was used. After incubation, the reactions were separated on a 1% agarose gel electrophoresis and the gels were resolved at 4°C using 0.5% TBE in DEPC water. The gel was dried and analyzed as for Fluorography in 5.1.4.

5.5.7 Biotin DNA interaction assay

DNA was synthesized by CRUK oligo services in the sequence of 5'-GATCJGAJGAJGAJGAJGAJGAJGAJGAJGAJGAJGAJGA-3' where J represents the cytosine base that can be methylated or unmethylated accordingly. A 5'-biotin label was coupled to the first base. Complementary strands of the sequences were synthesized and 1 μg of the sense and antisense strand was annealed as described in Chapter 5.2.3.

Blocking of the Strepavidin agarose (Sigma) beads was carried out by adding 50 μl of beads to 500 μl of 3% BSA in PBS for 1 hr. The beads were then washed 3 times with 1 ml of DNA wash buffer containing 40 mM Hepes.KOH pH 7.5, 350 mM KCl and 0.01% NP40. Beads were then added to the annealed oligos in binding buffer containing 40 mM Hepes.KOH pH 7.5, 100 mM KCl and 6 mM $MgCl_2$ to bind at 4°C on rotator for 12 – 16 hr. Non-specific binding of DNA was avoided by washing 4 times in DNA wash buffer. The strepavidin-agarose-DNA complex was added to 5 μg of FLAG-MBD2 (Empigen purified) in 250 μl buffer containing 30 mM Hepes.KOH pH 7.5, 100 mM NaCl, 5% glycerol, 0.25 mg/ml tRNA, 0.5% Triton X-100, 2 mM DTT and protease inhibitors[15]. The binding was carried out for 2 hr 30 min before the proteins were sequentially eluted from the beads. Wash buffer for the elution consisted of a base buffer containing 40 mM Hepes.KOH, 2 mM EDTA and 0.5% Triton X, the strepavidin-agarose-DNA-FLAG MBD2 complex was washed 6 times with a stepwise increase in NaCl concentration. Each wash consisted of 10 min incubation with rotation at 4°C and centrifugation at 500 g for 30 sec. The proteins were recovered as described in 5.5.3. The final fraction of proteins present on the

[15] For protease inhibitors, refer to Chapter 5.4.4.

beads was eluted with SDS-sample buffer, all fractions were loaded onto SDS-PAGE for analysis by Coomassie, and Western blots.

5.5.8 Luciferase assay using pGL2-promoter reporter plasmid

Unmethylated reporter plasmid (pGL2-promoter, Promega) was raised from *E. coli* JM109 and was subjected to mock or CpG-methylation using M.SssI (New England Biolabs). Completion of methylation was verified by digestion with FspI.

Cells of 50% confluence in a 6 well plate were transfected with 2.45 μg total plasmid and 24.5 μl Effectene Transfection reagent (Qiagen). Each transfection consisted of 2 μg of methylated or unmethylated reporter plasmid expressing the firefly luciferase gene and 200 ng of control plasmid encoding the *Renilla reniformis* luciferase (pRL-TK, Promega). 250 ng of FLAG-MBD construct was included to test their repression activity.

Luciferase levels were measured after 12-15 hr using the Dual-Luciferase Assay Kit according to manufacturer's instruction (Promega). Corrected values were obtained by calculation of firefly/renilla luciferase. Relative transcription was obtained by calculating methylated/unmethylated reporter using the corrected values. Relative transcription activity is the average of three independent experiments.

5.5.9 Luciferase assay using pG5-DNA polymerase-β reporter plasmid

Reporter plasmid (2 μg of pG5 DNA polymerase-β) encoding the firefly luciferase protein and 5 ng of control plasmid encoding the *Renilla reniformis* protein (pRL-TK, Promega) were cotransfected with 45 - 405 ng of Gal4-MBD2 fusion proteins to assay for their repression activity. The total DNA mix was topped up with empty pGal vector to 2.45 μg and transfected with 24.5 μl of Effectene Transfection reagent (Qiagen) to 293T cells at 70% confluence in a well of the 6 well plates. Firefly and renilla luciferase activities were measured 48 hr post transfection using the Dual-Luciferase Assay Kit (Promega) and according to the manufacturer's instruction. Relative luciferase unit (RLU) was expressed as a ratio of luciferase/renilla activity and each sample usually represents the average of 3 independent transfections. Proteins used for the assay were collected by precipitating the supernatant as described

in Chapter 5.5.3 and resuspending the pellet in SDS sample buffer, both fractions were pooled and analyzed for expression of Gal4 fusion proteins by Western blot.

5.6 Antibodies used in this thesis

All information on antibodies used for this study is summarized in the following table.

Antibodies	Product number	Host animal	Manufacturer	Applications in this thesis
Control IgG	SC-2025	Mouse	Santa Cruz Biotechnology	control for IP
Control IgG	SC-2027	Rabbit	Santa Cruz Biotechnology	control for IP
Control IgG	SC-2717	Sheep	Santa Cruz Biotechnology	control for IP
Control IgG	SC-2028	Goat	Santa Cruz Biotechnology	control for IP
Flag M2 HRP	A8592	Mouse	Sigma	WB
Flag-M2	F3165	Mouse	Sigma	WB, IF
Flag-M2 Agarose	A2220	Mouse	Sigma	IP
Gal4	SC-577	Rabbit	Santa Cruz Biotechnology	WB
HDAC1	2E10	Mouse	Upstate	IP
HDAC1	PC-544	Rabbit	Calbiochem	IP, WB
HDAC2	H54	Rabbit	Santa Cruz Biotechnology	IP, WB
hnRNP A1	4B10	Mouse	Dreyfuss	WB
hnRNP A1	Y15	Goat	Santa Cruz Biotechnology	IP, WB
IgG Goat	Sc-2020	Donkey	Santa Cruz Biotechnology	HRP conjugate secondary
IgG Mouse	115-035-068	Goat	Jackson Immuno Research	HRP conjugate secondary
IgG Rabbit	62-6120	Goat	Zymed	HRP conjugate secondary
IgG Sheep	12-342	Rabbit	Upstate	HRP conjugate secondary
MBD2	S923	Sheep	Upstate	IP
MBD2	N18	Goat	Santa Cruz Biotechnology	IP, WB

MBD2	D15	Goat	Santa Cruz Biotechnology	IP, WB and IF
MEP50	MC1F5	Mouse	Gideon Dreyfuss	WB (1:2000)
Methylarginines	7E6	Mouse	Abcam	WB
Methylarginines	21C7	Mouse	Abcam	WB
Methylarginines	16B11	Mouse	Abcam	WB
Methylarginines	5D1	Mouse	Abcam	WB
Methylarginines	Sym10	Rabbit	Upstate	WB
Methylarginines	Asym24	Rabbit	Upstate	WB
Methylarginines	Sym11	Rabbit	Upstate	WB
Mi2	H242	Rabbit	Santa Cruz Biotechnology	WB
MTA2	C20	Goat	Santa Cruz Biotechnology	IP, WB
p53	pAB1801	Mouse	Cancer Research Technologies	WB
pICln	P88320	Mouse	BD Transduction Laboratories	WB
PRMT1	07-404	Rabbit	Upstate	IP, WB and IF
PRMT3	07-256	Rabbit	Upstate	WB
PRMT4	07-080	Rabbit	Upstate	WB
PRMT5	6G8	Mouse	Gideon Dreyfuss	WB (1:100)
PRMT5	07-405	Rabbit	Upstate	IP, WB and IF
Ran	610341	Mouse	BD Transduction Laboratories	WB and IF
RbAp46	PC-546	Rabbit	Oncogene	IP, WB
RbAp48	PC-545	Rabbit	Oncogene	IP, WB
Sin3A	AK11	Rabbit	Santa Cruz Biotechnology	WB

Table 5-6 Detail list of all antibodies used in this study.

Unless otherwise stated, all antibodies were used according to the manufacturer's instruction. IP = immunoprecipitation, WB = Western blot and IF = immunofluorescence.

5.7 Plasmids used in this thesis

All information on antibodies used for this study is summarized in the following table.

Plasmids	Tag	Provider	Gene sequence from	Usage in this study
GAR	GST	Tina Braschome	NA	Expression of proteins in bacteria cells
MBD2 C-terminus	GST	Brain Hendrich	Mouse	
MBD2 full-length	GST	Brain Hendrich	Mouse	
MBD2 N-terminus	GST	Brain Hendrich	Mouse	
MBD2 RG domain	GST	Sara Nakielny	Mouse	
MeCP2 C-terminus	GST	Xingsheng Nan	Rat	
MeCP2 full-length	GST	Xingsheng Nan	Rat	
MeCP2 N-terminus	GST	Xingsheng Nan	Rat	
MeCP2 RG domain	GST	Sara Nakielny	Rat	
pCDNA3.1-Flag-Gateway destination	-	Linda Jeffery	-	Gateway cloning
pCDNA5/FRT/TO-Flag-Gateway destinaion	Flag	Janet Cronshaw	-	
pDONR221	-	Invitrogen	-	
pGal (1-94) Gateway destination	-	This study	Mouse	
MBD2 (1-131)	Flag	This study	Mouse	Expression of proteins in mammalian cells
MBD2 (-RG14a)	Flag	This study	Mouse	
MBD2 (-RG14b)	Flag	This study	Mouse	
MBD2 (-RG18)	Flag	This study	Mouse	
MBD2 (-RG23)	Flag	This study	Mouse	
MBD2 (-RG29a)	Flag	This study	Mouse	
MBD2 (-RG29b)	Flag	This study	Mouse	
MBD2 (-RG36)	Flag	This study	Mouse	
MBD2 (-RG41)	Flag	This study	Mouse	

MBD2 (-RG9)	Flag	This study	Mouse	
MBD2 (-TRD15)	Flag	This study	Mouse	
MBD2 (-TRD32)	Flag	This study	Mouse	
MBD2 (-TRD94)	Flag	This study	Mouse	
MeCP2 (R106W)	Flag	Linda Jeffery	Rat	
PRMT1	GFP	Mark Bedford	Human	
PRMT3	GFP	Mark Bedford	Human	
PRMT4	GFP	Mark Bedford	Human	
PRMT5	Flag	Westley Friesen	Human	
MBD2 (-RG)	Flag	Linda Jeffery	Mouse	
MBD2 full-length	Flag	Saori Kitao	Mouse	
MBD3	Flag	Brain Hendrich	Mouse	
MeCP2 (-RG)	Flag	Linda Jeffery	Rat	
MeCP2 full-length	Flag	Xingsheng Nan	Rat	
SmD1	Flag	Westley Friesen	Human	
pG5 DNA polymerase Beta	-	J. Millbrandt	-	Reporter gene expression studies
pGL2-promoter	-	Promega	-	
pRL-TK	-	Promega	-	
MBD2 (1-131)	Gal	This study	Mouse	
MBD2 (-TRD15)	Gal	This study	Mouse	
MBD2 (-TRD32)	Gal	This study	Mouse	
MBD2 (-TRD94)	Gal	This study	Mouse	
pGal (1-94)	Gal	Caroline Hill	-	
pRetro.Super	-	Miguel Martins	-	siRNA stable cells generation
PRMT1A	-	This study	Human	
PRMT1B	-	This study	Human	
PRMT1C	-	This study	Human	
PRMT1D	-	This study	Human	
PRMT5A	-	This study	Human	
PRMT5B	-	This study	Human	
PRMT5C	-	This study	Human	
PRMT5D	-	This study	Human	

Table 5-7 Detail list of plasmids used in this study.

Further information can be provided upon request.

Chapter 6 Supplementary results

6.1 Determine of the optimal SAH concentration for *in vitro* methylation assay

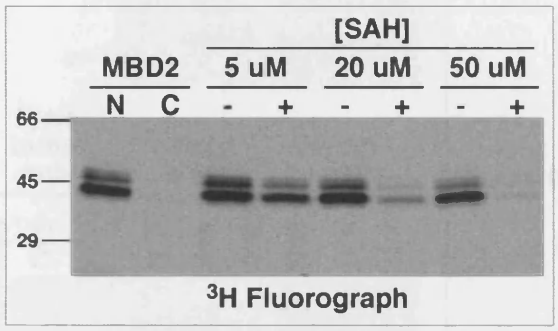

Figure 6-1 Determination of best inhibitor concentration for methylation assay.

GST-MBD2 (N and C-terminus) were subjected to methylation assay as for Figure 2-1 **and GST-MBD2 N-terminus methylation was titrated with an increasing concentration of SAH or control.**

SAH concentration was titrated from 5-50 μM (Figure 6-1) instead of the 10 μM used in previous assays. It was found that even at the presence of 50 μM SAH, the GST-MBD2 N-terminus protein was still labeled by the methyltransferases in mammalian cell lysate. Therefore, 100 μM of SAH was used in all methylation assays thereafter.

6.2 Immunopurifying endogenous MBD2 for mass spectrometry analysis

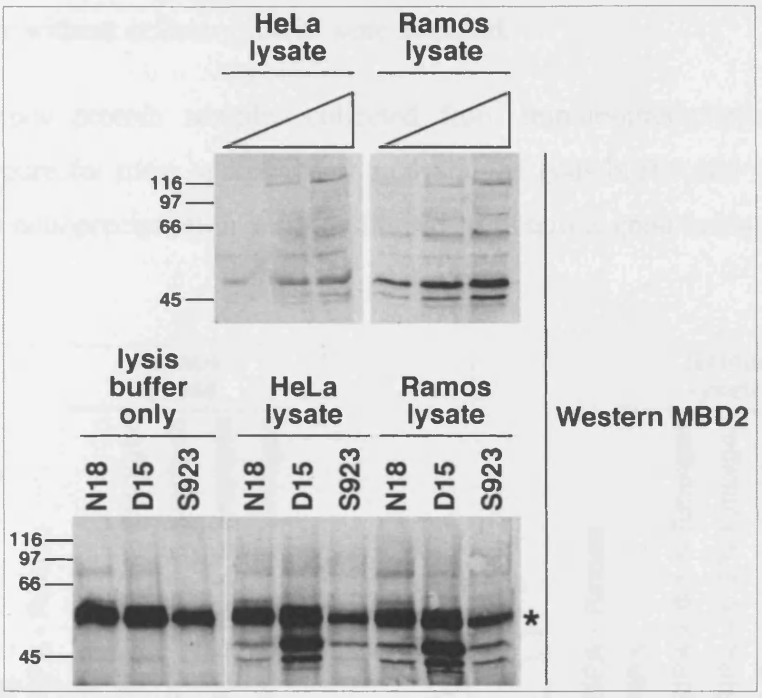

Figure 6-2 Immunopurification of endogenous MBD2 protein.

(Top) Determination of cell types that express the highest amount of MBD2 protein. Different cells types (1 X 10^6 cells each) were lysed in RIPA buffer and the Bradford normalized lysates were checked on Western blot for their endogenous levels of MBD2.

(Bottom) Determination of the suitable antibody for the immunopurification of MBD2 protein. The indicated cell lysate were used for immunoprecipitation by incubating with the indicated antibodies and the antibody-MBD2 complexes were captured by Protein G agarose. Immunopurified complex were washed 6 times with the lysis buffer, eluted with SDS-PAGE sample buffer and analyzed by Western blot. (*) Denotes antibody heavy chain.

To immunopurify endogenous MBD2 proteins, expression levels of the protein in several cell types were compared in Western blot (Figure 6-2, Top). Similar to other reports, endogenous MBD2 always resolves as a doublet band (170). It was found that MBD2 protein level was highest in Ramos cells, in other cells (eg. 293T) MBD2 proteins were not detectable (data not shown).

Various MBD2 specific antibodies were also tested for their immunoprecipitation efficiencies (Figure 6-2, Bottom), it was found that the D15 antibody immunoprecipitate MBD2 proteins most efficiently. Therefore, all

subsequent MBD2 immunoprecipitations uses this antibody. As MBD2 migrates at 48 kDa, to ensure that the immunoprecipitated bands observed were not the degradation products of the 50 kDa antibodies heavy chain, control experiments of antibodies in lysis buffer without cellular protein were included.

Since protein samples collected from immunoprecipitations need to be relatively pure for mass spectrometry analysis, the lysis buffer and wash buffer used for the immunoprecipitation were optimized to obtain a good balance between yield and purity.

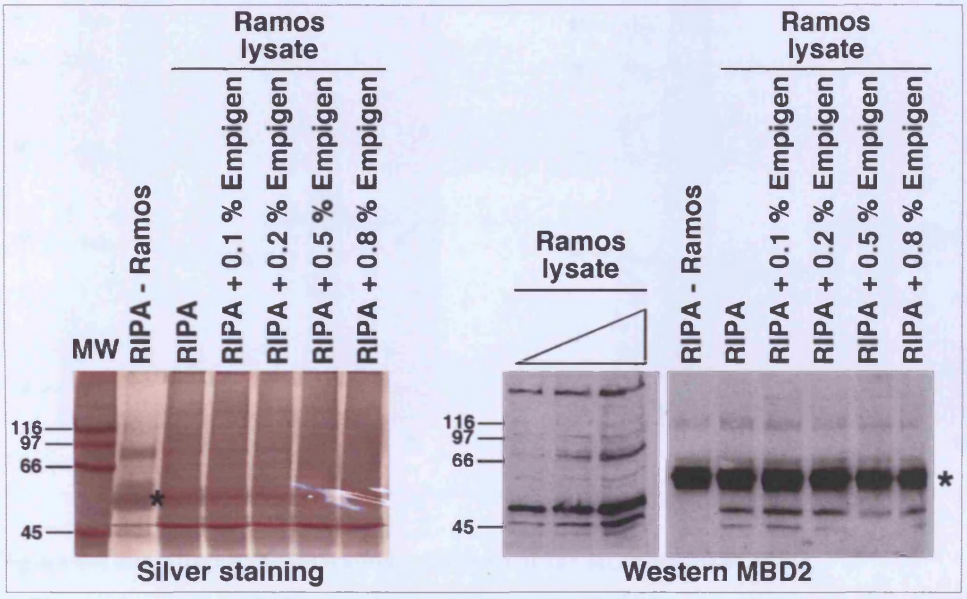

Figure 6-3 Optimization of washing condition for IP.

Endogenous MBD2 protein was immunopurified from Ramos cells lysed in RIPA buffer and the captured antibody-MBD2 complexes were washed with the indicated concentrations of Empigen BB. Half of the IP products were analyzed by silver staining (Left) and the remaining was analyzed by Western (Right). (*) Denotes antibody heavy chain.

Wash conditions for the immunoprecipitation of MBD2 were titrated with increasing amounts of detergent. Empigen BB, the zwitterionic detergent was chosen for this purpose because it is known to solublize proteins effectively but yet preserves the epitope and maintains the reactivity of antibodies (142). However, when the complexes prepared in increased percentages of Empigen BB, there were no increase the cleanliness of the samples (Figure 6-3, silver staining). Moreover, increasing the

percentage of Empigen beyond 0.2% decreases the yield of MBD2. Therefore, purification of the endogenous MBD2 protein was carried out without addition of Empigen BB.

6.3 GST pull down assay using HeLa and Ramos cell lysates

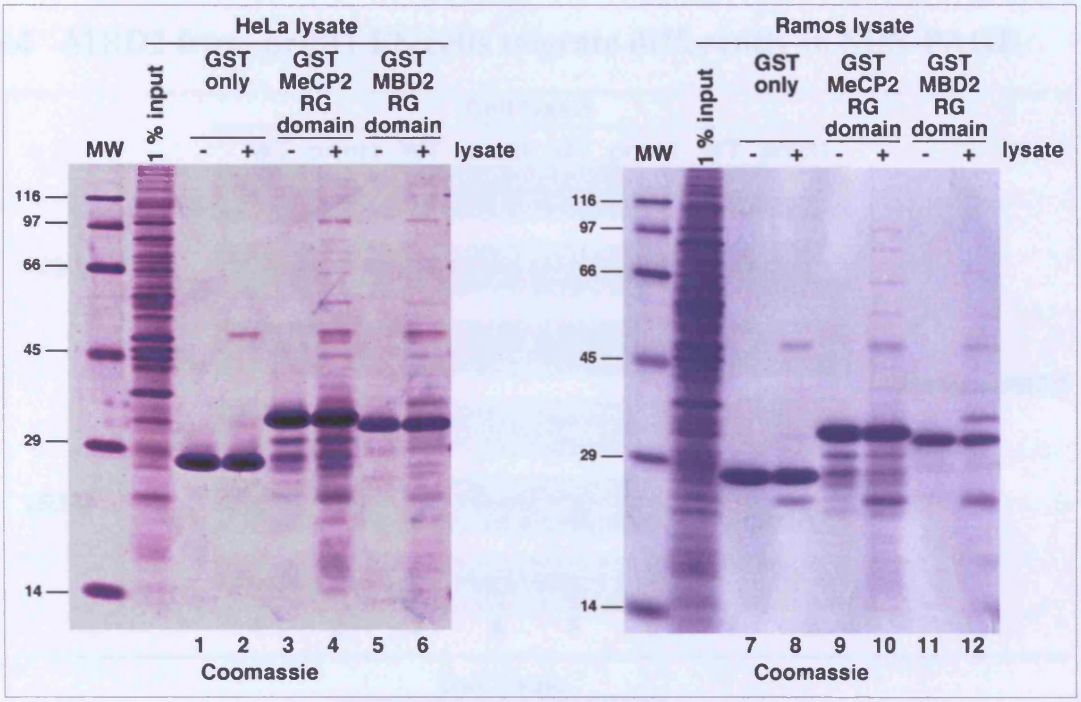

Figure 6-4 Identifying the interacting partners of the RG domain of MBD.

GST protein or the GST-tagged RG domain of MeCP2 and MBD2 proteins were incubated in the absence or presence of the indicated cell lysates at 4°C for 4 hr and the proteins were captured by Glutathione Sepharose. After extensive washing, proteins were eluted from the Sepharose beads by SDS-PAGE sample buffer, resolved by SDS-PAGE and visualized by Coomassie. Selected protein bands were excised from the gel and were sent for mass spectrometry identification.

In Figure 6-4, proteins from HeLa and Ramos cell lysates were allowed to interact with various GST proteins, the complexes and were washed under stringent conditions to enrich for proteins that were specifically interacting with the RG domains (Lane 4, 6, 10 and 12).

To ensure all protein bands on the gel were from the cell lysate were not bacterial proteins carried over from the recombinant protein, controls samples without addition of cell lysate were included (3, 5, 9 and 11). Also, to eliminate bands that

were result of the unspecific interaction between the mammalian proteins with the GST-tag or glutathione sepharose beads, the GST proteins were incubated with the lysates (Lane 1, 2, 7 and 8). From this experiment, a total of 35 protein bands were selected and excised from the gel for mass spectrometry identification (Data not shown). However, none of the bands identified was protein methyltransferase.

6.4 MBD2 from prmt1 ES cells migrate differently in SDS-PAGE

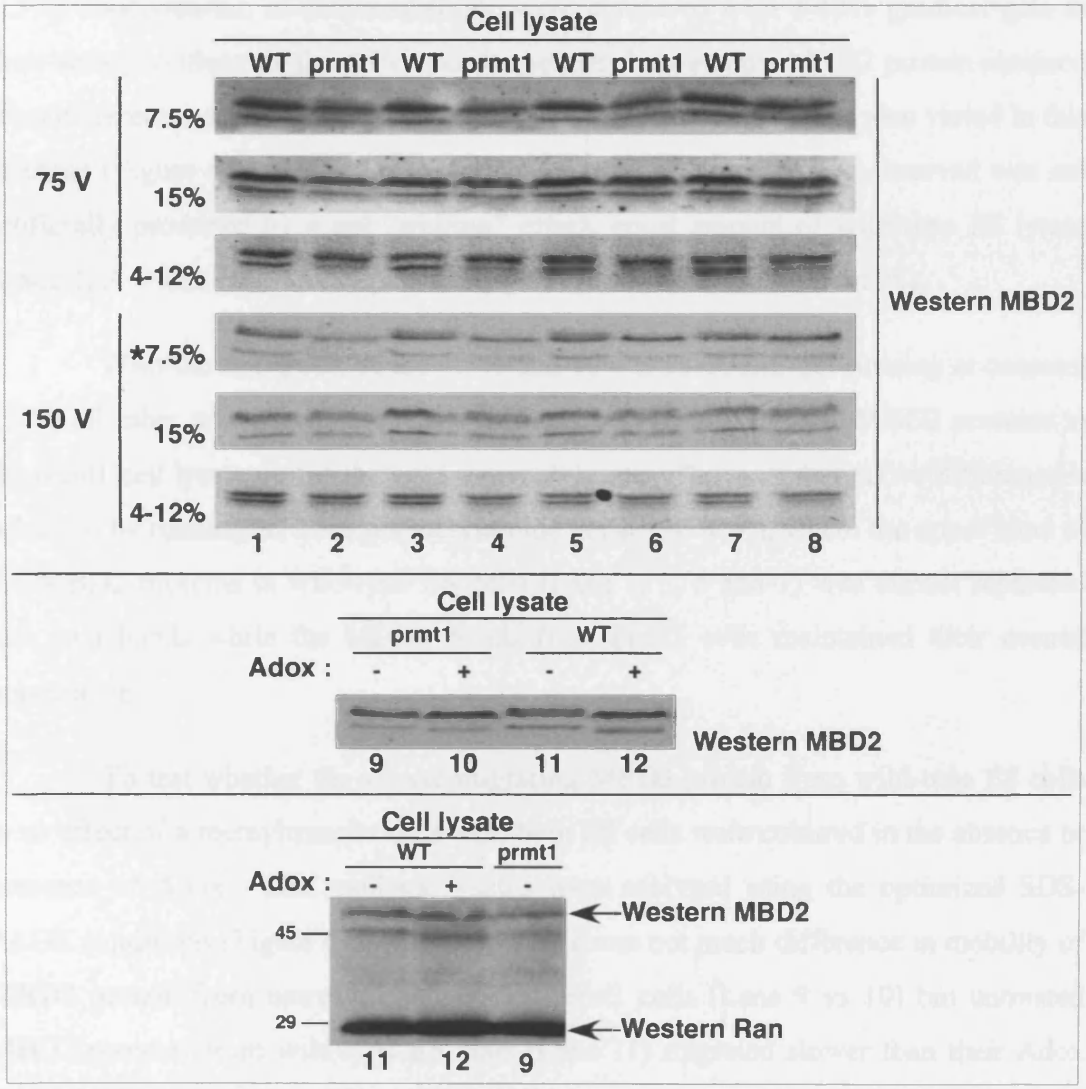

Figure 6-5 Analysis of the migration of MBD2 in SDS-PAGE.

(Top) Wild-type and prmt1 normalized cell lysate were separated on different SDS-PAGE conditions and analyzed by Western blot. The optimum condition selected is indicated with an (*).

(Middle) The indicated cells were treated in the absence or presence of Adox for 30 hr and normalized cell lysates prepared were separated with the optimum condition indicated above.

(Bottom) Samples from above were reanalyzed with MBD2 and Ran antibody probing on the same Western blot.

Various gel systems were used to separate the wild-type and prmt1 ES cell lysates to determine if there was any observable difference in gel mobility of the MBD2 proteins obtained from different cells. Gels with the lowest (7.5%) and highest (15%) concentration of polyacrylamide were compared with 4-12% gradient gels in their ability to illustrate the difference in mobility between the MBD2 protein obtained from different sources. The voltage used for the electrophoresis was also varied in this instance (Figure 6-5, Top). To ensure that any mobility difference observed was not artificially produced by a gel "smiling" effect, equal amount of wild-type ES lysate (Lane 1, 3, 5 and 7) were compared alongside prmt1 lysate (2, 4, 6 and 8).

With the exception of the 7.5% and 15% SDS-PAGE gel running at constant 75 V, all other conditions revealed a difference in mobility of the MBD2 proteins in the prmt1 cell lysate versus the wild-type cell lysate. The most distinctive difference is achieved by running in 7.5% polyacrylamide gel at 150 V (*), where the upper band of the MBD2 proteins in wild-type ES cells (Lane 1, 3, 5 and 7) was almost separated into two bands while the MBD2 bands from prmt1 cells maintained their normal appearance.

To test whether the slower migrating MBD2 protein from wild-type ES cells is an effect of a methyltransferase event, both ES cells were cultured in the absence or presence of Adox. The resultant lysates were analyzed using the optimized SDS-PAGE conditions (Figure 6-5, Middle). There was not much difference in mobility of MBD2 protein from untreated and treated prmt1 cells (Lane 9 vs 10) but untreated MBD2 proteins from wild-type ES cells (Lane 11) migrated slower than their Adox treated counterpart (Lane 12), and the MBD2 proteins from prmt1 cells (Lane 9 and 10).

The samples were reanalyzed using the same SDS-PAGE conditions and the Western blot was probed with antibodies against MBD2 and Ran (Figure 6-5, Bottom).

As observed, migration of all Ran proteins in the same gel (Lane 11, 12 and 9) was not affected by the genetic background or Adox treatment of the cells, but MBD2 from untreated wild-type ES cells (Lane 11) was migrating slower than in the rest of the samples (Lane 12 and 9).

6.5 Increasing MBD2 arginine methylation by increasing PRMT contents of the cells

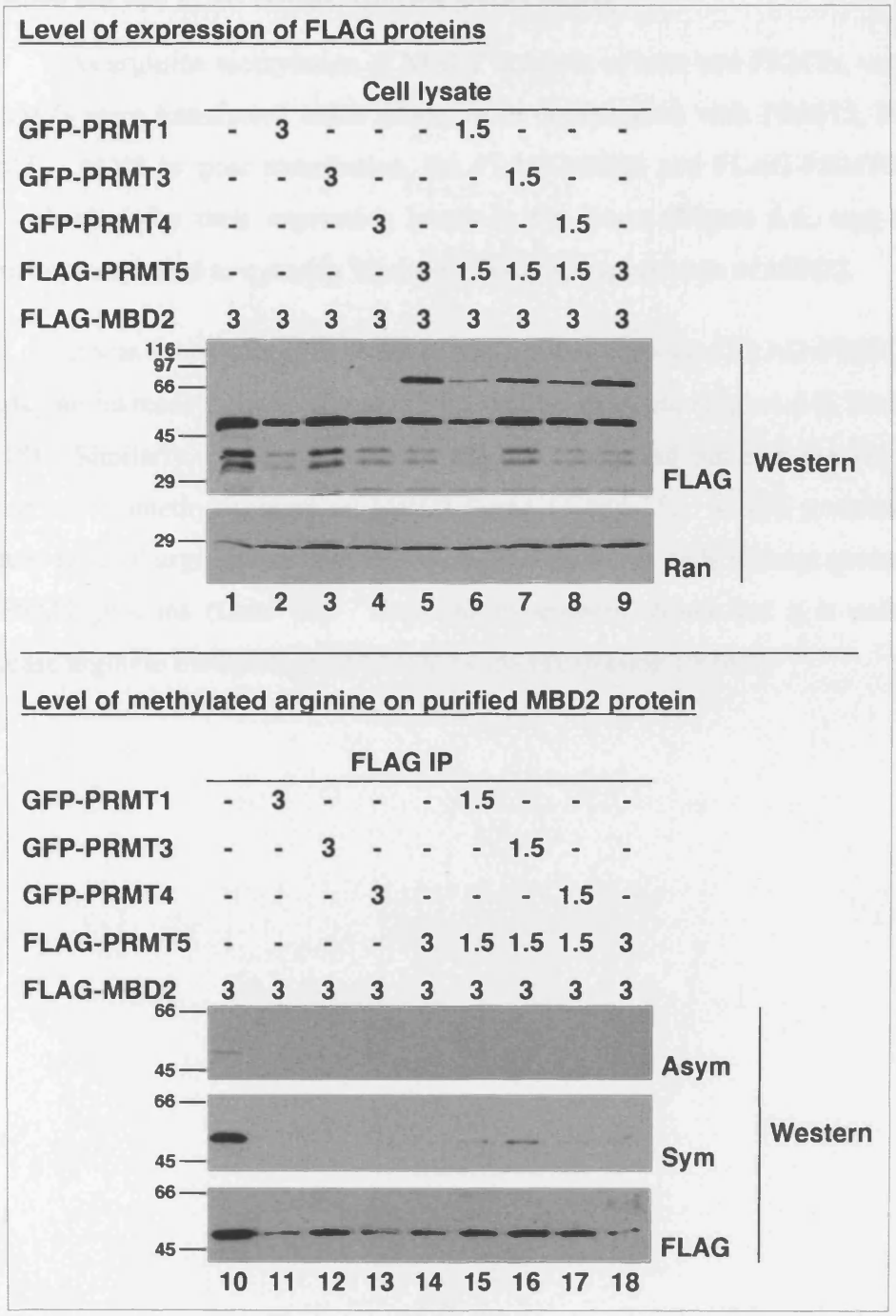

Figure 6-6 Cotransfecting PRMTs with MBD2.

(Top) 293T cells were transfected with FLAG-MBD2 and the indicated PRMT plasmids for 48hr. The cells were then lysed, Bradford normalized and Western with the

indicated antibodies to assay for the expression of the transfected plasmids, Ran levels indicates protein loading.

(Bottom) The FLAG proteins were then prepared and normalized as for Figure 2-15 **and normalized proteins were subjected to Western to detect for dimethylarginines. Western blot with FLAG antibody indicates protein loading.**

As arginine methylation of MBD2 involves at least two PRMTs, various type I PRMTs were transfected either alone, or in combination with PRMT5, the type II PRMT. At 48 hr post transfection, the FLAG-MBD2 and FLAG-PRMT5 proteins were checked for their expression levels in the lysate (Figure 6-6, top) and were immunoprecipitated to quantify for the methylarginine contents of MBD2.

It was found that MBD2 raised in cells that expressed FLAG-PRMT5 did not display an increase in level of symmetric dimethylarginine (Figure 6-6, bottom, Lane 14-18). Similarly, expression of all PRMT1 in cells did not increase the levels of asymmetric dimethylarginine on MBD2 (Lane 11 and 15). MBD2 proteins with the highest level of arginine methylation were those raised in cells without overexpression of PRMT proteins (Lane 10). Thus, the experiment shows that it is unfeasible to increase arginine methylation of MBD2 by overexpressing PRMTs.

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