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Marie-Eve Pelletier
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)
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Department of Cellular and Molecular Medicine
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

PRMT8: Characterization of a Novel Neuron-Specific Protein Arginine Methyltransferase
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Jocelyn Côté
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

Laura Trinkle Mulcahy

Jean-François Couture

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

PRMT8: Characterization of a Novel Neuron-Specific Protein Arginine Methyltransferase

This thesis is submitted as a partial fulfillment of the M.Sc. program in Cellular and Molecular Medicine

Marie-Eve Pelletier

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Faculty of Medicine, University of Ottawa

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ABSTRACT

Methylation of arginine residues is a post-translational modification mediated by specific enzymes known as the Protein Arginine Methyltransferase (PRMT) family. In this thesis, we present our research on PRMT8, an enzyme catalyzing the formation of asymmetric dimethylarginine (aDMA), which displays a unique distribution at the plasma membrane of neuronal cells of the central nervous system (CNS). An ontogenic analysis of PRMT8 in mouse tissues revealed that its expression in the brain is induced during the perinatal stage and is maintained in adulthood. The P19 cell line was identified as a valid model for endogenous PRMT8 and showed the induction and requirement of PRMT8 to achieve neuronal processes. A P19 PRMT8 knock-down cell line does not survive neuronal differentiation while, in contrast, no cell death is observed when the muscle lineage is induced. Taken together, these findings suggest an important role for PRMT8 in neuronal differentiation and/or function in the CNS.

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LIST OF ABBREVIATIONS

aDMA	: Asymmetric dimethylarginine (ω-N^G,N^G-dimethylarginine)
AdoMet	: S-adenosyl-L-methionine
AdOx	: Adenosine dialdehyde
BSA	: Bovine serum albumin
BTG1	: B-cell translocation gene 1, anti-proliferative
CARM1	: Coactivator-associated arginine methyltransferase 1 (also referred to as PRMT4)
CNS	: Central nervous system
DDX3	: Dead-box RNA helicase
DMSO	: Dimethylsulfoxide
DNA	: Deoxyribonucleic acid
EFIα	: Elongation factor 1 alpha
ES	: Embryonic stem cells
EWS	: Ewing sarcoma protein
FBS	: Fetal bovine serum
FCS	: Fetal calf serum
FUS	: FUS (fused in sarcoma) / TLS (translocated in liposarcoma)
Fyn	: Protein tyrosine kinase p59
GAP	: GTPase-activating protein
GAPDH	: Glyceraldehyde-3-phosphate dehydrogenase
GAR	: Glycine/arginine-rich motif of fibrillarin
GFP	: Green fluorescent protein
GST	: Glutathione-S-transferase tag
HDACs	: Histone deacetylases
His	: Polyhistidine residues (generally 6 or more)

HIV-1	: Human immunodeficiency virus type 1
hnRNP K	: Heterogeneous nuclear ribonucleoprotein K
IF	: Immunofluorescence
IHC	: Immunohistochemistry
IPTG	: Isopropyl-β-D-thiogalactopyranoside
ISH	: *In situ* hybridization
JMJD6	: Jumonji domain-containing 6 protein
kDa	: Kilodalton
LCK	: Lymphocyte specific protein tyrosine kinase p56
MBP	: Myelin Basic Protein
MHC	: Myosin heavy chain
MMA	: Monomethylarginine (ω-N^G-monomethylarginine)
mRNA	: Messenger ribonucleic acid
NC	: HIV-1 nucleocapsid protein
NES	: Nuclear export signal
PAD	: Peptidylarginine deiminase
PCR	: Polymerase chain reaction
PI3K	: Phosphatidylinositol-3-OH kinase
PKCε	: Protein kinase C, isoform epsilon
PLCγ	: Phospholipase-C, isoform gamma
PRMT	: Protein Arginine Methyltransferase
RA	: Retinoic acid
Rev	: HIV-1 regulator of viral protein expression
RIP140	: Receptor-interacting protein 140
RNA	: Ribonucleic acid
RNAi	: RNA interference
RT°	: Room temperature

RT-PCR	: Reverse transcriptase-polymerase chain reaction
sDMA	: Symmetric dimethylarginine (ω-N^G,N'^G-dimethylarginine)
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFM	: Serum free media
SH3	: SRC homology 3 domain
shRNAmir	: microRNA-adapted short hairpin RNA
SMN	: Survival of motor neurons protein
snRNP	: small nuclear ribonucleoprotein particle
Tat	: HIV-1 transactivator protein
TIS21/BTG2	: Nerve growth factor-inducible anti-proliferative, B-cell translocation gene 2

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1. INTRODUCTION

1.1. ARGININE METHYLATION

1.1.1. THE EMERGENCE OF ARGININE METHYLATION

Arginine is a positively charged amino acid that often mediates hydrogen bonding and amino aromatic interaction with proteins and nucleic acids (Bedford, Richard 2005). Arginines can be post-translationally modified by methylation and the number of proteins thought to harbour this modification has recently increased drastically (Bedford, Richard 2005, Pahlich, Zakaryan & Gehring 2006). However, the emergence of arginine methylation dates back to the late 1960s. Methylation of arginine residues was first reported by Paik and Kim in 1968. Paik had, in fact, been working on lysine methylation for over a decade (Paik et al. 1957) when he and Kim, observed methylation of both lysine and arginine residues on histone proteins that were purified from calf thymus (Paik, Kim 1968). These results led them to suggest the existence of at least two enzyme systems in calf thymus, one that methylates the ε-NH_2 group of the lysine residue, and another that methylates arginines (Paik, Kim 1968, Paik, Paik & Kim 2007). Again, in their study on lysine methylation occurring in calf thymus, they were among the first to purify a methyltransferase; an enzyme they termed Protein Methylase I (Paik, Kim 1968). Besides, they surprisingly realized later on that this enzyme was, in fact, a protein arginine methyltransferase rather than a lysine methyltransferase as expected (Paik, Paik & Kim 2007). Consequently, a whole new field of research was opened up, giving rise to many questions about the mechanism, the cellular function and the importance of arginine methylation in living organisms (Paik et al. 1957).

1.1.2. METHYLATED ARGININES

As stated earlier, arginine is a positively charged residue, but more than that, it is a unique amino acid as its guanidine group contains five potential hydrogen bond donors that are advantageously organized to interact with hydrogen bond acceptors (Bedford, Clarke 2009). More specifically, the combination of two H-bonds between arginine and aspartate is known to be very stable in proteins (Bedford, Clarke 2009, Mitchell et al. 1992). In the case of interactions between proteins and DNA, arginine residues are the most frequent hydrogen bond donors to backbone phosphate groups as well as to thymine, adenine and guanine bases (Luscombe, Laskowski & Thornton 2001, Bedford, Clarke 2009). Moreover, hydrogen bonds also join arginine residues and adjacent phosphate groups in RNA loops (Calnan et al. 1991, Bedford, Clarke 2009). These examples highlight the importance of arginine residues in the network of biological interactions. Therefore, a modification or an alteration of this amino acid could have major impacts, and that is what happens when arginine is post-translationally modified by methylation. This modification does not alter the positive charge of the arginine, rather it increases its bulkiness, blocks hydrogen bonding, and increases its hydrophobicity (Boisvert, Chenard & Richard 2005). So far, the best characterized effect of arginine methylation is its influence (both negative and positive) on protein-protein interactions (Boisvert, Chenard & Richard 2005) but, in a broader view, it involves the regulation of various cellular processes (further discussed in section 1.3). Nonetheless, arginine methylation seems to have a milder effect on proteins than other post-translational modifications, modulating certain processes rather than acting as an on/off switch like phosphorylation, for example (Pahlich, Zakaryan & Gehring 2006). Three forms of

methylated arginines have been identified in mammalian cells. The addition of a single methyl group on the terminal nitrogen atom generates ω-N^G-monomethylarginine (MMA). But it is also possible to add two methyl groups on arginine residues and this is how the two other forms of methylated arginines are obtained. First, the most prevalent structure of dimethylarginine, ω-N^G,N^G-dimethylarginine, occurs when both methyl groups are placed on one of the terminal nitrogen atoms of the guanidino group, and this is designated as asymmetric dimethylarginine (aDMA) (Bedford, Clarke 2009). Second, ω-N^G,N'^G-dimethylarginine or symmetric dimethylated arginine (sDMA) is the result of the addition of one methyl group on each of the terminal guanidino nitrogens (Bedford, Clarke 2009) (Figure 1A). These three distinct derivatives have been detected on a wide range of proteins expressed either in the nucleus, cytoplasm or organelles of eukaryotes (Bedford, Richard 2005). There is also evidence that in some cases, the same arginine residue can be either symmetrically or asymmetrically dimethylated, enabling regulation of distinct protein-protein interactions leading to opposing biological consequences (Pahlich, Zakaryan & Gehring 2006). The discovery of arginine methylation not only raised many questions about the structure of altered residues and the identity of methylated proteins, but it also challenged the particularity of the enzymes responsible for this modification.

1.2. PROTEIN ARGININE METHYLTRANSFERASES (PRMTS)

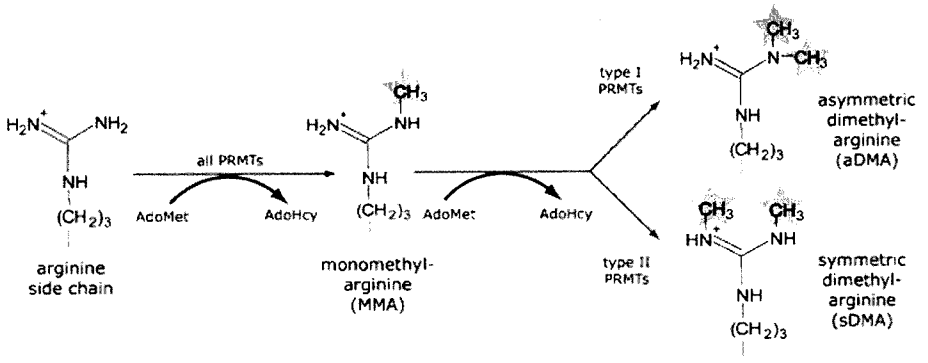
1.2.1. PRMTS, AN INTRIGUING FAMILY OF ENZYMES

From a general point of view, the addition of methyl groups to nitrogen, carbon, sulfur and oxygen atoms of small molecules, lipids, proteins and nucleic acids is accomplished by enzymes denominated as methyltransferases (Bedford, Richard 2005). More specifically, the methyltransferase family encompasses various subtypes of enzymes that methylate particular substrates. In the case of arginine methylation, the reaction is accomplished by specific enzymes, members of the Protein Arginine Methyltransferase family (PRMT) that transfer the methyl group of S-adenosyl-L-methionine (AdoMet) to this basic amino acid via N-methylation of the side-chain nitrogens (Bedford, Richard 2005, Pahlich, Zakaryan & Gehring 2006) (Figure 1A). PRMTs can be divided into two major classes, based on the type of dimethylarginine they generate: type I catalyze the formation of aDMA, whereas type II lead to the production of sDMA (Bedford, Clarke 2009). Both types of PRMTs induce monomethylation of arginine residues (MMA) as a reaction intermediate (Pahlich, Zakaryan & Gehring 2006). However, no enzyme has, to date, demonstrated the potential of forming both aDMA and sDMA (Bedford, Clarke 2009). So far, nine members of the PRMT family have been identified in mammalian cells (Figure 1B). These enzymes all harbor the set of four conserved amino acid sequences of seven-strand twisted β-sheet (motifs I, post I, II and III), that are common among AdoMet-dependent methyltransferases for binding to the AdoMet cofactor(Katz, Dlakic & Clarke 2003). In addition to these four motifs, the presence of THW loops is also particular to the PRMT subfamily (Bedford, Clarke 2009). The core conserved region of PRMTs is approximately 310 amino acids long, but each

enzyme exhibits distinct protein substrate specificities, which are ascribed to the unique N-terminal region of each PRMT that greatly varies in length (Goulet et al. 2007). As mentioned before, there are nine PRMTs, although more variants, resulting from alternative splicing, have also been described. For example, the analysis of the genomic organization of the 5'-end of the human PRMT1 gene unveiled seven protein isoforms (PRMT1v1 → v7), all differing in their N-terminal region and displaying diverse biochemical characteristics (Goulet et al. 2007). Nevertheless, PRMTs can be classified according to their enzymatic activity: PRMT1, -2, -3, -4, -6 and -8 are type I PRMTs; while PRMT5, -7 and -9 are type II enzymes (Pahlich, Zakaryan & Gehring 2006, Bedford, Clarke 2009). PRMT2 was recognized because of its homology to known arginine methyltransferases and its ability to bind AdoMet (Qi et al. 2002), but its methylation activity was only recently reported to allow its classification in the subtype of PRMTs generating aDMA (Lakowski, Frankel 2009). PRMTs are generally ubiquitously expressed enzymes across the different cell types and tissues (Bedford, Richard 2005). However, they can achieve a certain degree of specificity by varying level of expression or having distinct subcellular localizations depending on the PRMT, the isoform and the cell type. For example, the analysis of rat tissues revealed opposite PRMT1:PRMT3 ratio in heart versus small intestine (Tang et al. 1998). In addition, immunofluorescence experiments on RAT1 cells (fibroblasts) showed PRMT1 as a mostly nuclear protein, compared to PRMT3 which was more abundant in the cytosol (Tang et al. 1998). Today, these results could be revised according to the research of Herrman and colleagues who reported the high mobility of PRMT1 both in the cytoplasm and the nucleus. They also mentioned reversible accumulation and immobility of PRMT1

in the nucleus, following the inhibition of methylation (Herrmann et al. 2005). They suggested a regulation model where PRMT1 would be trapped in the nucleus by unmethylated substrates such as core histones and heterogenous ribonucleoprotein particles (hnRNPs) upon inhibition of methylation. PRMT1 would then be released when re-methylation is allowed and the reaction has been executed (Herrmann et al. 2005). Subcellular localization can also greatly vary depending on the PRMT1 alternative splicing variants analyzed, since a leucine-rich nuclear export signal (NES) that can be recognized by the CRM1 nuclear export receptor was identified on PRMT1v2 and thus, mostly restricts the expression of this isoform to the cytosol (Goulet et al. 2007). Moreover, PRMT1v4-v7 represent the four minor isoforms and their expression is more restricted to specific tissues since v4 is only found in the heart, v5 in the pancreas and v7 mainly in the heart and skeletal muscles (Goulet et al. 2007). However, the foremost exception in the arginine methyltransferase family is PRMT8. This PRMT is the only one to display tissue specificity as being restricted to the brain, as well as a particular localization as being targeted to the plasma membrane (Lee et al. 2005) (further discussed in section 1.4).

A


all PRMTs
AdoMet
AdoHcy
AdoMet
AdoHcy
type I PRMTs
type II PRMTs
arginine side chain
monomethyl-arginine (MMA)
asymmetric dimethyl-arginine (aDMA)
symmetric dimethyl-arginine (sDMA)

B

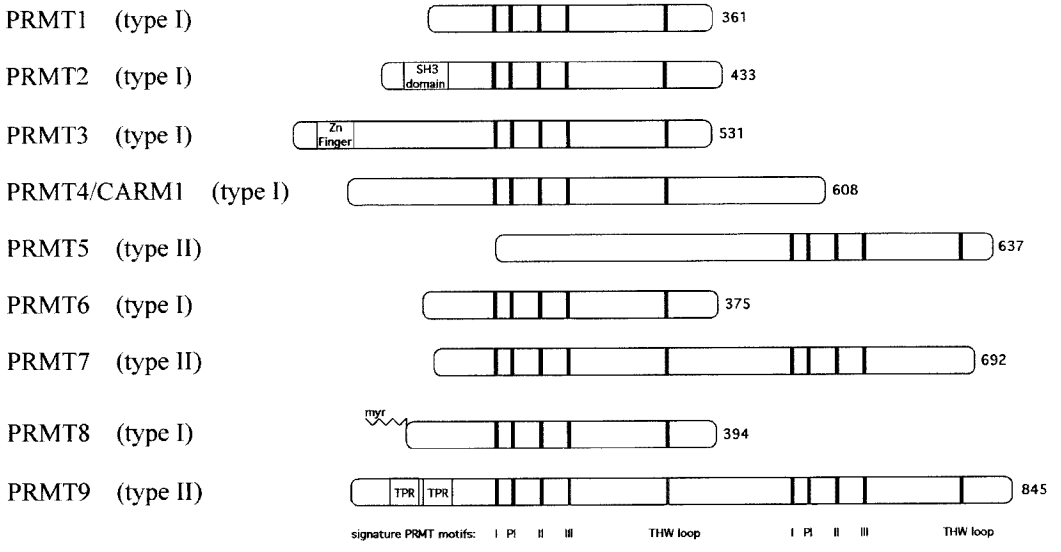

PRMT1 (type I)
PRMT2 (type I)
PRMT3 (type I)
PRMT4/CARM1 (type I)
PRMT5 (type II)
PRMT6 (type I)
PRMT7 (type II)
PRMT8 (type I)
PRMT9 (type II)
SH3 domain
Zn Finger
myr
TPR
TPR
361
433
531
608
637
375
692
394
845
signature PRMT motifs: I PI II III THW loop I PI II III THW loop

Figure 1: Arginine methylation by PRMTs. A) All PRMTs catalyze the formation of monomethylarginine (MMA) where the methyl donor S-adenosyl-L-methionine (AdoMet) is converted to S-adenosyl-L-homocysteine (AdoHcy). In a second step, the type I PRMTs transfer a second methyl group to the same guanidino nitrogen resulting in an asymmetric dimethylarginine (aDMA), whereas the type II PRMTs catalyze the formation of symmetric dimethylarginine (sDMA). B) Overview of the human PRMT family. The mammalian PRMT family contains nine highly related members. They all harbor the signature AdoMet binding motifs I, post I, II and III and the conserved THW loop. Protein domains that might assist in substrate recognition are indicated in gray boxes. (Modified from Pahlich, Zakaryan & Gehring 2006 and Bedford, Clarke 2009)

1.2.2. REGULATION OF PRMTS

Even though the field of arginine methylation arose over 40 years ago, many questions still need to be elucidated. Not only do numerous substrates of each PRMT remain unidentified, but the mechanisms of regulation are also unclear. The regulation of arginine methylation is partly achieved by the modulation of the enzymatic activity of PRMTs, but unfortunately, little is still known. It has been reported that homo-oligomerization of PRMTs is sometimes required for enzymatic activity. For example, dimerization of PRMT1 is essential for AdoMet binding (Zhang, Cheng 2003). But more than that, both PRMT1 and PRMT5 are catalytically active only in multimer complexes and not as dimers or tetramers (Lim et al. 2005). There are also examples where PRMTs can be regulated by other binding proteins that are not necessarily substrates. It has been shown that BTG1 and TIS21/BTG2 proteins can associate with PRMT1 via their boxC domain and stimulate methylation activity toward selected substrates (Lin et al. 1996, Berthet et al. 2002). The opposite situation can also be observed when the orphan receptor TR3 binds the catalytic domain of PRMT1 and causes an inhibition of its enzymatic activity (Lei et al. 2009). Other PRMT-binding proteins sometimes modulate more than one enzyme. For instance, DAL-1/4.1B tumor suppressor interacts with PRMT3 and inhibits its ability to methylate substrates (Singh et al. 2004); while the binding of DAL-1/4.1B to PRMT5 either inhibits or enhances the methylation activity of this enzyme in a substrate-specific manner (Jiang, Roemer & Newsham 2005). However, most of these experiments were performed *in vitro* and the relevance of these potential regulators, as well as precise mechanisms of action, remain largely unknown. Another interesting fact is that post-translational modifications can alter the efficiency of a PRMT.

It has been reported that the phosphorylation of CARM1 (PRMT4) at a conserved serine residue mediates inactivation of its enzymatic activity, most likely by diminishing its ability to bind AdoMet (Higashimoto et al. 2007). These examples remain a mere glimpse of what is occurring in cells to regulate PRMTs' catalytic activity. The full regulation mechanism of PRMTs still needs to be elucidated, but we can hypothesize that a great network of binding proteins, which varies from one PRMT member to another, would be involved in the process.

1.2.3. ANTAGONISTS OF PRMTS?

For the longest time, arginine methylation was believed to be an irreversible post-translational modification, since no arginine demethylase could be identified. In 2004, two groups reported arginine deimination as a way to antagonize arginine methylation. The human peptidylarginine deiminase 4 (PAD4) can convert arginine or monomethylarginine to citrulline, which can prevent methylation of particular arginine residues on specific histone sites, leading to changes in gene expression (Cuthbert et al. 2004, Wang et al. 2004). However, this demethylimination reaction does not occurr on dimethylated arginine, due to steric occlusion that prevents its entry in the active site of PAD (Pahlich, Zakaryan & Gehring 2006, Holbert, Marmorstein 2005). Moreover, this reaction is not a way to truly reverse arginine methylation since the unmethylated arginine residue is not regenerated. Nonetheless, the search for arginine demethylases led to the identification of the first arginine demethylase: Jumonji domain-containing 6 protein (JMJD6) that could demethylate both mono- and dimethylarginine residues (Chang et al. 2007). This enzyme seemed to be active on specific residues, since they

reported activity on histone H3 at arginine 2 and histone H4 at arginine 3, while other methylated sites studied were not altered in the presence of JMJD6 (Chang et al. 2007). However, there is controversy around these results since another group challenged the demethylase activity of JMJD6 on the arginine-serine (RS) domain sequences of many of the JMJD6-interacting proteins, but did not observe dimethylarginine-demethylation (Webby et al. 2009). They also tested histone H3 and H4 fragment peptides and again, recombinant JMJD6 did not produce demethylated arginine. Rather, they reported a lysyl-hydroxylation enzymatic activity of JMJD6 toward U2AF65, a protein associated with RNA splicing (Webby et al. 2009). If we admit that arginine methylation is a transient alteration and that, similarly to PRMTs, demethylases are substrate-specific, then there is a high potential for identifying many arginine demethylases and gaining understanding of methylation turnover.

1.3. Roles of PRMTs

1.3.1. Cellular functions of PRMTs

It has been established that arginine methylation is implicated in many cellular processes, including: DNA repair; RNA transcription, transport and splicing; protein translation and compartmentalization; as well as signal transduction (Pahlich, Zakaryan & Gehring 2006). As stated earlier, methylated arginine residues were first observed in the late 1960s on histone proteins (Paik, Kim 1968). These proteins interact to form a scaffolding unit to compact DNA and structure chromatin (Kornberg, Lorch 1999, Ng et al. 2009). In more detail, DNA is wrapped around a globular histone octamer composed

of a tetramer of histone H3/H4 and two dimers of H2A/H2B (Ng et al. 2009). The subunit composition and post-translational modifications of histones can influence chromatin function by impacting on the availability of regulatory elements in the genome (Agalioti et al. 2000). The N-terminal histone tails protrude from the globular core and thus, are often the targets of modifying enzymes (Ng et al. 2009). The PRMTs are among the active enzymes on histone proteins, with PRMT1 catalyzing the methylation of Arg 3 of H4, CARM1 being active on Arg 2, 17 and 26 of H3 as well as PRMT5 methylating Arg 8 of H3 and Arg 3 of H4 (Pahlich, Zakaryan & Gehring 2006) (Figure 2). The methylation of these specific arginine residues can lead to opposite consequences, since the formation of aDMA in histones by PRMT1 or CARM1 results in gene activation, compared to the generation of sDMA by PRMT5 which culminates in gene repression (Pahlich, Zakaryan & Gehring 2006). The methylation of arginine residues can also impact on the recruitment of binding interactors via a specific protein domain.

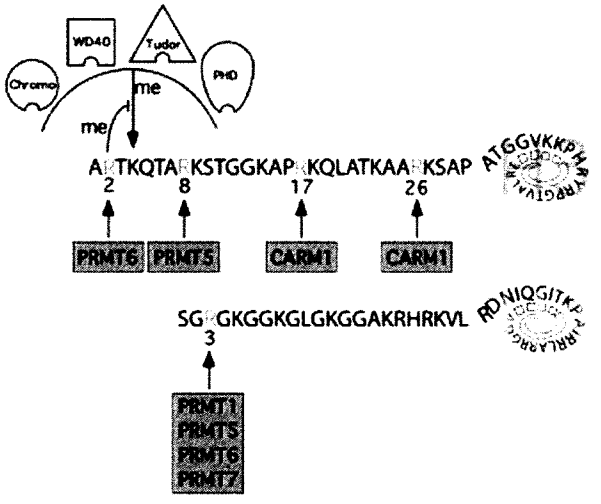

WD40
Tudor
Chromo
PHD
me
me
2
8
17
26
PRMT6
PRMT5
CARM1
CARM1
3
PRMT1
PRMT5
PRMT6
PRMT7

Figure 2: Arginine methylation of histones H3 and H4 N-terminal tails. The sites of PRMT-mediated methylation on the histone tails are identified. H3R2 methylation antagonizes the docking of a number of effector proteins, including those containing chromo, PHD, Tudor and WD40 domains. H4 is also methylated at Arg3 by various PRMTs. (Adapted from Bedford, Clarke 2009)

The Tudor domain is a small conserved ~60 amino acid motif that serves to mediate intermolecular protein interactions (Goulet et al. 2008). This module has demonstrated the ability to interact with methylarginine-containing protein partners (Cote, Richard 2005). A number of proteins sharing that motif exert varying functions, but are all members of the Tudor domain family (Adams-Cioaba, Min 2009). For example, the Tudor domain of the survival of motor neurons (SMN) protein recognizes sDMA on Sm proteins and leads to the proper assembly and localization of spliceosomal small nuclear ribonucleoprotein particles (snRNP) (Cote, Richard 2005). This large snRNP complex is implicated in the processing of nuclear pre-mRNA to remove introns and ligate exons, an essential mechanism for accurate protein expression (Pellizzoni, Yong & Dreyfuss 2002).

Arginine methylation can also act as a localization signal. The receptor-interacting protein 140 (RIP140) is a corepressor for nuclear receptors and many transcription factors. In differentiating adipocytes, activated PKC_{ε} stimulates phosphorylation on two specific serine residues of nuclear RIP140 (Ser102 and Ser1003) (Gupta et al. 2008). This leads to the recruitment of chaperone 14-3-3 which is associated with PRMT1 (Gupta et al. 2008). This PRMT then methylates RIP140 at Arg240, 650 and 948, which results in a reduction of its interaction with a corepressive enzyme machinery containing HDACs and triggers its export to the cytoplasm via the exportin (CRM1)-containing complex (Gupta et al. 2008). Interestingly, it has been proposed that PRMT1 increases the affinity of RIP140 for CRM1, which could provide a potential mechanism to explain the often-observed influence of arginine methylation on intra-cellular localization of a number of proteins.

In recent years, it has been hypothesized that the regulation of protein activity by arginine methylation participates in a form of innate cellular immunity against the human immunodeficiency virus type 1 (HIV-1) (Xie et al. 2007). In more details, HIV-1 transactivator Tat protein plays a key role in HIV replication by drastically increasing viral gene transcription efficiency (Boulanger et al. 2005). However, arginine methylation of Tat at Arg52 and Arg53 by PRMT6 negatively regulates its transactivation activity which results in decreased viral replication and production (Xie et al. 2007, Boulanger et al. 2005). HIV-1 nucleocapsid protein (NC) can also be methylated by PRMT6 at Arg10 and 32, which leads to the reduction of RNA annealing and initiation of reverse transcription (Invernizzi et al. 2007). In addition, HIV-1 Rev protein, the regulator of viral protein expression, is another substrate of PRMT6 that can alter viral production (Invernizzi et al. 2006). In fact, the methylation of a single arginine residue, located in the N-terminal portion, diminishes Rev-mediated viral RNA export from the nucleus to the cytoplasm, which is likely detrimental for the virus (Invernizzi et al. 2006).

All of these examples reflect only a fraction of the molecular mechanisms guided by PRMTs in regulating various cellular processes.

1.3.2. NEURONAL DIFFERENTIATION AND ARGININE METHYLATION

As described previously, the molecular function of PRMTs can lead to the alteration of gene transcription, protein localization or signal transduction. But most importantly, what are the physiological consequences associated with the involvement of one or more PRMTs in a biological system? A great example of that would be the coordination of neuronal differentiation. Previous studies on neuronal differentiation have shown changes in the pattern of methylated proteins upon induction of differentiation. More specifically, an increase in protein methylation involving predominantly PRMT1-like activity was noted and the inhibition of methylation interfered in neurite outgrowth (Cimato et al. 1997, Cimato et al. 2002). In a more recent study, the P19 embryonal carcinoma cells have been used to demonstrate that the methyltransferase-inhibition interferes with neuronal differentiation (Hong et al. 2008). The use of a methyltransferase inhibitor, adenosine dialdehyde (AdOx), resulted in a decreased number of neurites and a flattened morphology. In more details, the transcription of developmental-regulatory genes, such as the neuron-positive markers Wnt-1, Brn-2, NeuroD and Mash-1 were attenuated during neuronal differentiation; while the undifferentiated cell-marker Oct-3 mRNA was up-regulated (Hong et al. 2008). The mechanism of action of selected genes during neuronal differentiation of P19 cells has previously been studied. Implicated genes have been classified as positive regulator of neurogenesis, necessary for neuronal differentiation or not critical for neurogenesis (reviewed in Soprano, Teets & Soprano 2007), but the orchestration of all these processes is not clearly understood. It has been reported that CARM1 regulates neuronal differentiation by maintaining the neuronal progenitors in a proliferative state. This was initially shown to involve post-translational

mechanisms with the arginine methylation of HuD by CARM1 that maintains PC12 cells in a proliferative state until nerve growth factors down-regulate HuD methylation which induces differentiation of these cells (Fujiwara et al. 2006). The effect of CARM1 on the maintenance of cell pluripotency was very recently shown to involve transcriptional regulation as well. Pluripotent ES cells have CARM1 associated with the *Oct4* and *Sox2* promoters which display arginine methylation of histone H3 at Arg17 and Arg26 (Wu et al. 2009). Depletion of CARM1 results in the reduction of Oct4 and Sox2, leading to the induction of trophectoderm differentiation (Wu et al. 2009). These results suggest that arginine methylation contributes to the regulation of cell differentiation at many levels and potentially, but not limited to, transcriptional regulation. Since PRMT8 is the only tissue-specific PRMT and is restricted to the brain, it is tempting to speculate that it should be an important player in some of these regulatory phenomena of neuronal differentiation.

1.4. Protein Arginine Methyltransferase 8 (PRMT8)

1.4.1. The discovery of PRMT8

Most of the known methylated proteins are substrates of PRMT1, which is the predominant type I PRMT in mammalian cells. It has been estimated to account for 85% of cellular PRMT activity (Tang et al. 2000, Pahlich, Zakaryan & Gehring 2006), but we should consider the fact that other methyltransferases are less well characterized. For now, very little is known about the PRMTs that were more recently discovered and their study opens up a wide range of possibilities. Thus, we decided to concentrate our

research on a particular member of the PRMT family: PRMT8. PRMT8 was first described by Lee and colleagues in 2005. Its primary amino acid sequence is very similar to ubiquitously expressed PRMT1, with over 80% identity, but unique to PRMT8, is an N-terminal extension of 76 amino acids (Lee et al. 2005) (Figure 3). This N-terminal sequence harbours a myristoylation concensus motif (MGXXX(S/T)), which leads to the covalent addition of myristate, a 14-carbon saturated fatty acyl chain, to the glycine residue adjacent to the initiation methionine (Waksman & Gordon 2001, Utsumi et al. 2004). This post-translational modification is thought to target PRMT8 to the plasma membrane (Lee et al. 2005). There is also a patch of basic residues, close to the myristoylation motif, that could facilitate electrostatic interactions with the membrane lipids (Lee et al. 2005).

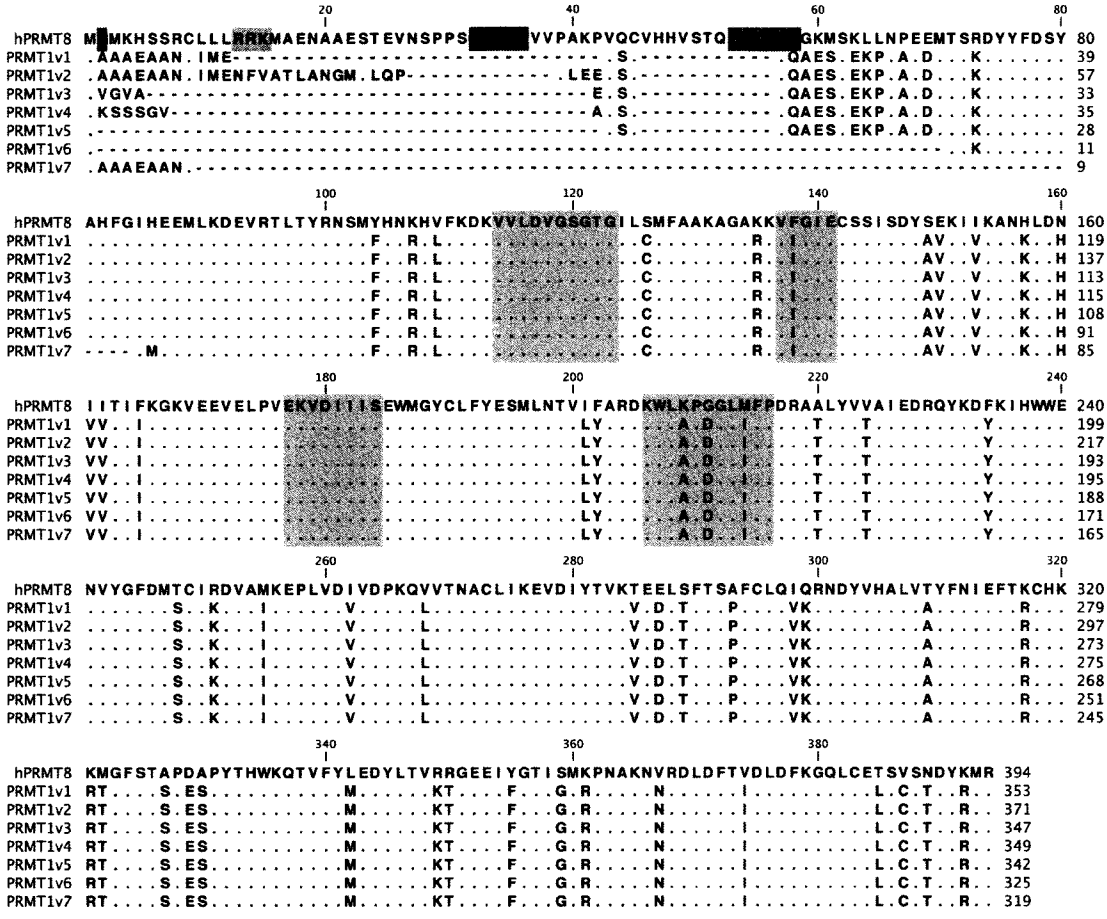


Figure 3: Amino acid alignment of human PRMT8 and the seven isoforms of PRMT1. The signature methyltransferase motifs are boxed in green, which appear in the following order: I, post I, II and III. The N-terminal glycine residue that functions as the site of myristoylation is highlighted in red and the cluster of basic amino acids, which facilitate the membrane targeting of PRMT8, in pink. The two SH3 domains present within the N-terminal region are boxed in blue.

A Northern analysis of RNA from a number of different human tissues (brain, heart, kidney, liver, spleen, testis, etc) revealed the singular expression pattern of PRMT8. PRMT8 was only detected in the brain as a major transcript of 2,6 kb and two minor transcripts of 2,2 and 2,9 kb. It is important to note that this Northern analysis had been previously probed with PRMT1 to reduce cross reactivity with the PRMT8 probe since they share over 420 bp at the RNA level (Lee et al. 2005).

1.4.2. PRMT8 FUNCTION

The catalytic activity of PRMT8 is also very similar to PRMT1 as being a type I enzyme (generates aDMA) and by methylating the same panel of substrates *in vitro*, which include the glycine/arginine-rich motif of fibrillarin (GAR domain), Npl3 and histone H4 (Lee et al. 2005). Some arginine methyltransferases, such as PRMT1 and CARM1 (PRMT4), can homodimerize through a hydrophobic face called the antenna region (Zhang, Cheng 2003, Xu et al. 2004). Using recombinant proteins, they found that PRMT8 could coimmunoprecipitate not only itself, but also PRMT1 (Lee et al. 2005). This is not surprising given the high degree of homology between PRMT8 and PRMT1, but it implied that PRMT1 activity could be recruited to the plasma membrane. PRMT8 is the only PRMT known to be localized at the membrane, as well as the only member of this family of enzymes displaying a tissue-specific expression pattern.

1.5. HYPOTHESIS

We hypothesize that PRMT8 methylation activity will be implicated in the signaling pathways leading to neuronal differentiation.

1.6. PROJECT OUTLINE

A. To determine the tissue distribution and developmental expression profile of PRMT8

B. To establish a good mammalian cellular system for the study of endogenous PRMT8

C. To initiate studies to determine the function and regulation of PRMT8 in neuronal cells

2. MATERIAL AND METHODS

2.1. DNA CONSTRUCTS

PRMT8 N-terminal region (from glycine 2 to asparagine 65) was amplified from human EST (#MGC-26069, ATCC) and introduced in-frame with a GST tag in the EcoR1/SalI restriction sites of the pGEX-4T2 vector (GE Healthcare) (Primers: forward 5'-CCGGAATTCTGGGCATGAAACACTCCT-3' and reverse 5'-GACGTCGACAGG-TTCAGCAGCTTGGACATCTTG. Full-length PRMT8 was amplified from the same EST and subcloned in N-terminus of the GFP tag using EcoR1/SalI restriction sites of the pEGFP-N2 vector (Clontech) (Primers: forward 5'-CCGGAATTCACCATGGGCATG-AAACACTCCT-3' and reverse 5'-TCAGTCGACCACGCATTTTGTAGTCATT-3'). His-tagged PRMT8 was obtained by the amplification of full-length PRMT8 and subcloning in N-terminus of the His tag (6X His) using EcoR1/SalI restriction enzymes(Primers: forward 5'-CCGGAATTCGGGCATGAAACACTCCTCCCG-3' and reverse 5'-TCAGTCGACACGCATTTTGTAGTCATT-3'). T7-tagged PRMT8 results from the amplification of full-length PRMT8 and insertion in C-terminus of the T7 epitope in the XbaI restriction site of a modified pCG-T7 vector kindly provided by Dr. Jeremy Sanford (University of California Santa Cruz, CA, USA) (Primers: forward 5'-CTAGTCTAGAGCAATGGGCATGAAACACTCCTCCCG-3' and reverse 5'-CTCGTCTAGAGGCTTACTAACGCATTTTGTAGTCATTAG-3'). The DNA sequences of all constructs were verified by sequencing (StemCore Laboratories, Ottawa, ON, Canada). The expression vectors encoding GST-fused SH3 domains of LCK, PLCγ, PI3K, Fyn, GAP and Spectin as well as GST-SMN-Tdr were a kind gift from Dr. Stéphane Richard (McGill University, Montréal, QC, Canada)

2.2. RNA SILENCING

The RNA interference (RNAi) method was used to achieve knock-down of PRMT8. Plasmids combining the design advatanges of microRNA-adapted short hairpin RNA (shRNAmir) with the lentiviral vector were designed and obtained from Open Biosystems. Two different short hairpin RNA expression plasmids (pGIPZ-shPRMT8 B5 & D10) as well as control empty vector (pGIPZ) were respectively stably transfected in P19 cells using linear 25 kDa polyethylenimine (PEI) (Sigma) solution at 1mg/mL pH 6.8 in a ratio of 4:1 (μg / μg) with the amount of plasmid transfected.

2.3. CELL CULTURE AND DIFFERENTIATION

P19 embryonal carcinoma cells were a kind gift from Dr. Ilona S. Skerjanc (University of Ottawa, ON, Canada). Cells were maintained in Minimum Essential Medium Alpha (MEM-α) with 2mM L-glutamine (Gibco), supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin and 10% fetal bovine serum (FBS) (Hyclone). Differentiation of P19 cells necessitates that they form aggregates, thus either bacterial-grade Petri dishes coated with 0.15% agarose or ultra-low attachment tissue culture dishes (Corning) were used when required. Neuronal differentiation of P19 cells was induced by the formation of embryoid bodies and addition of 1 μM of *all-trans*-retinoic acid (RA) (Sigma) resuspended in DMSO to 1mM. After 4 days of RA-treatment, aggregates were partially dissociated by 0.25% trypsin, re-plated in tissue culture dishes using serum free MEM-α and allowed to differentiate for 4-5 days. The P19 muscle lineage required 4 days of treatment with 1% DMSO combined with the

formation of embryoid bodies. Intact cell aggregates were then transferred to tissue culture plates using MEM-α, 10% FBS. For P19-pGIPZ and P19-shPRMT8 stable cell lines grown as monolayers, 1.5 μg of puromycin per mL of culture media was added. During RA or DMSO treatment, puromycin was reduced to 1 μg/mL for these two cell lines and completely withdrawn after re-plating. The HEK293T cell line, derived from the human embryonic kidney line 293, was kindly provided by Dr. Stéphane Richard (McGill University, Montréal, QC, Canada) and grown as a monolayer in Dulbecco's modified Eagle's medium (Gibco) supplemented with 1mM sodium pyruvate, 100 units/mL penicillin, 100 mg/mL streptomycin and 10% fetal calf serum (FCS) (Hyclone). All cells were grown at 37°C and 5% CO_2 in a humidified incubation chamber.

2.4. SEMI-QUANTITATIVE RT-PCR

Total RNA was extracted from C57Bl/6 wild-type mouse tissues or P19 cells using Trizol reagent (Invitrogen, Life Technologies) according to manufacturer's instructions. RNA was quantified using a spectrophotometer. First strand cDNA was synthesized using 1 μg of total RNA and the avian myeloblastosis virus reverse transcriptase (AMV-RT) (Promega) with an oligo-dT_{18} primer. The expression of PRMT8 and Mash-1 were analyzed by semi-quantitative RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. cDNA was amplified using 2X GoTAQ® Green Master Mix (Promega) in a final reaction mixture of 10μL. The following pairs of forward and reverse primer sets were used: PRMT8, 5'-ATGGCGGAGAATGCAGTCGAAAG-3' and 5'-GCCCATCCACTCGCTGATGAT-GA-3' (PCR product size, 519 bp); Mash-1, 5'-CTCGTCCTCTCCGGAACTGATG-3'

and 5'-CGACAGGACGCCCGCCTGAAAG-3' (PCR product size, 301 bp); and GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTG-TA-3' (PCR product size, 452 bp). The amplification reactions were done as described: PRMT8, 95°C for 3min, 94°C for 35 cycles of 30 sec each, 65°C for 30 sec, and 72°C for 45 sec with extension at 72°C for 10 min; Mash-1, 95°C for 3min, 94°C for 30 cycles of 30 sec each, 65°C for 30 sec, and 72°C for 30 sec with extension at 72°C for 10 min; GAPDH 98°C for 2min, 95°C for 22 cycles of 30 sec each, 65°C for 30 sec, and 72°C for 1 min with extension at 72°C for 10 min. PCR products were electrophoresed on 2% agarose gels with TBE buffer (50mM Tris, 50mM Boric Acid, 0.8mM EDTA) and stained with ethidium bromide. Amplified bands were visualized under UV light and photographed.

2.5. PROTEIN PURIFICATION

GST fusion proteins were overexpressed in *Escherichia coli* BL-21 cells (Stratagene) by induction with a final concentration of 0.1mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, cells were spun down, resuspended in 10 mL of 1X PBS supplemented with Complete™ protease inhibitor cocktail (Roche Applied Science), and subsequently broken down by sonication. The GST recombinant proteins were purified using glutathione-agarose (Sigma). Following washes with 1X PBS, 1% Triton X-100, Complete™ protease inhibitor, proteins were eluted from the sepharose with 60mM glutathione in PBS pH 7.4. The purified tagged proteins were dialyzed overnight at 4°C against 1X PBS then concentrated using Centricon centrifugal devices (Millipore).

2.6. GENERATION AND PURIFICATION OF PRMT8 ANTIBODY

GST-N-terminal PRMT8 recombinant protein was overexpressed in BL-21 cells as described in section 2.5. Bound proteins on GST sepharose were directly eluted and denaturated in 2X Laemmli reducing buffer (100mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.2mM DTT with bromophenol blue). Protein samples were resolved on a 12% SDS-polyacrylamide gel electrophoresis (PAGE), gel was stained with Coomassie Brilliant Blue to visualize protein bands. The protein band corresponding to GST-N-terminal PRMT8 (~33 kDa) was excised from the gel and sent to Cedarlane Laboratories for purification and rabbit immunization. PRMT8 antibodies were then purified from rabbit antisera. Briefly, GST expressed tag and GST-N-terminal PRMT8 proteins were overexpressed as described above. Bacterial pellets were resuspended in cold NETN buffer (100mM NaCl, 1mM EDTA, 20mM Tris pH 8.0, 0.5% NP-40 and Complete™ protease inhibitor) and lysed by sonication. The GST recombinant proteins were purified using glutathione-agarose. Beads were washed three times with 10 ml NETN buffer, twice with 10 mL of 0.1M borate buffer pH 8.0, once with 10 mL of 0.1M borate buffer pH 9.0 and once with 0.2M borate buffer pH 9.0. Beads were incubated in 10 mL of 40mM dimethylpimelimidate in 0.2M borate buffer pH 9.0 on rocker for 1h at 4°C. They were washed twice with 10 mL of 0.1M borate buffer pH 8.0. Beads were then incubated in 10 mL of 40mM ethanolamine in 0.1M borate buffer pH 8.0 on rocker for 45 min at 4°C. They were washed three times with 10 mL of 1X PBS, once with 10 mL of 0.2M glycine-HCl pH 2.5, once with 1M K_2HPO_4. The glycine and K_2HPO_4 washes were repeated before washing beads twice with 1X PBS and finally once with 1X PBS, 0.2% Tween20 (PBS-T). The PRMT8 rabbit antisera was diluted 1:1 with PBS-T then passed

through a 0.45 μm filter. The filtrate was incubated with GST expressed tag fixed on beads with end-over-end mixing at 4°C for 2 hours. The beads were spun down at 1500 x g for 2 min and supernatant was incubated with GST-N-terminal PRMT8 on sepharose with end-over-end mixing at 4°C overnight. The beads were transferred into a poly-prep chromatography column (BioRad) then washed with 30 mL of PBS-T followed by 20 mL of 1X PBS. Antibodies were eluted with 7.5 mL of glycine-HCl pH 2.5 then the eluate was neutralized with 2.5 mL of 1M K_2HPO_4. The purified antibodies were dialyzed overnight at 4°C against 1X PBS. Antibodies were concentrated using Centricon centrifugal devices (Millipore) then diluted 1:1 with glycerol before adding BSA to 1%.

2.7. WESTERN BLOT ANALYSIS

Embryos and tissues were collected from C57Bl/6 wild-type mice. Protein extracts were prepared by disrupting tissues using a homogenizer in an ice-cold 1X PBS buffer containing Complete™ protease inhibitor cocktail. Samples were sonicated then diluted 1:1 with 2X lysis buffer (20mM Tris pH 7.4, 300mM NaCl, 2% Triton X-100, 0.1mg/mL PMSF and Complete™ protease inhibitor cocktail). Lysates were incubated at 4°C for 1h30 with end-over-end mixing. Whole cell extracts were lysed in 1X lysis buffer (10mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 0.1mg/mL PMSF and Complete™ protease inhibitor cocktail) for 1h at 4°C with end-over-end mixing. Samples were then sonicated and reincubated at 4°C for 30 min. For both tissue and cell samples, cellular debris were discarded through centrifugation for 10 min at 18,000 X g and transfer of the supernatants to clean tubes. Total proteins were quantified spectrophotometrically using the DC protein assay reagent (Bio-Rad) before adding Laemmli reducing buffer to a final

concentration of 50mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1mM DTT with bromophenol blue. Protein samples were resolved on a 10% SDS-PAGE then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) for immunoblotting. Following transfer, membranes were blocked with 5% nonfat milk in PBS-T (1X PBS + 0.05% Tween20) for 1h at room temperature then probed with primary antibodies diluted in 2% milk PBS-T overnight at 4°C. The blots were washed three times in PBS-T, then incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies diluted in 2% milk PBS-T. Proteins were detected by chemiluminescence (Millipore) after three final washes in PBS-T. List of primary antibodies used: α-β-actin (#ab6276, Abcam); α-GAPDH (#MMS-580S, Covance); α-PRMT1 (hybridomas, #P1620, Sigma); α-PRMT2 (#ab66763, Abcam); α-PRMT8 (rb424, Cedarlane Laboratories); α-EFIα (#05-235, Millipore); α-FUS (#A300-294A, Bethyl Laboratories); α-DDX3 (#A300-474A, Bethyl Laboratories); α-EWS (#A300-417A, Bethyl Laboratories); α-hnRNP-K (#R8903, Sigma); α-T7-tag HRP conjugate (#69048-3, Novagen); α-GST and α-Asym (Cote, Richard 2005).

2.8. IMMUNOFLUORESCENCE

P19 DMSO treated cells plated on gelatine-coated coverslips were rinsed twice in 1X PBS and fixed in cold methanol for 5 min. After removing methanol, cells were air dried for 5 min at room temperature (RT°) then rehydrated in 1X PBS for 15 min at RT°. Cells were rendered more permeable by incubation in 0.5% Triton X-100 in 1X PBS for 5 min at RT° then washed three times with 1X PBS. The cells were then incubated for 1h at RT° with MF20 specific antibody against the various isoforms of the myosin heavy

chain (MHC), a kind gift from Dr. Bernard Jasmin (University of Ottawa, ON, Canada). After washing cells once with 0.1% Triton X-100 in 1X PBS and twice with 1X PBS, coverslips were incubated for 1h at RT° with a specific secondary antibody conjugated to an Alexa Fluor® dye. Final washes with 0.1% Triton X-100 in 1X PBS and twice with 1X PBS were done before mounting coverslips onto glass slides using Vectashield mounting medium with DAPI (Vectors Laboratories). Cells were observed with a Zeiss Axio Imager Z1 instrument.

2.9. IMMUNOPRECIPITATION

P19 embryonal carcinoma cells (3x100mm plates) were transiently transfected with pCG-T7-PRMT8 using PEI linear 25 kDa polyethylenimine (PEI) (Sigma) solution at 1mg/mL pH 6.8 in a ratio of 4:1 (μg / μg) with the amount of plasmid transfected. Cells were harvested and lysed in 1X lysis buffer as described above. T7-Tag® antibody agarose (#69026, Novagen) were used to immunoprecipitate transiently expressed T7-PRMT8 recombinant proteins. 50 μL of T7-beads were added to P19 mock (negative control) and P19 T7-PRMT8 (positive control) cell lysates, Samples were incubated at 4°C for 3 hours with constant end-over-end mixing. P19 T7-PRMT8 protein extracts were replaced by P19 mock lysates and T7-beads were incubated for an additional 2 hours at 4°C with mixing. The beads were then washed twice with lysis buffer and once with 1X PBS. Laemmli reducing buffer was then added to the beads before performing western blot analysis as described above. For Asym immunoprecipitation, P19-pGIPZ (mock) or P19-shPRMT8 (k/d) cells were lysed in 1X lysis buffer as described above. Lysates were

incubated on ice with the primary antibody for 1h with occasional gentle agitation. Then 20 μL of a 50% protein A-Sepharose slurry (Sigma) was added and incubated at 4°C for 30 min with end-over-end mixing. The beads were washed twice with lysis buffer and once with 1X PBS. Laemmli reducing buffer was then added to the beads before the western blot analysis was performed as described above.

2.10. GST PULL-DOWN

GFP-tagged PRMT8 was transiently transfected into HEK293T cells according to manufacturer's protocol with Lipofectamine 2000 (Invitrogen). Cells were harvested and lysed in 1X lysis buffer as described above then incubated with 200 μg of GST-SH3 domain fusion proteins fixed to beads and mixed end-over-end for 2 hours at 4°C. The beads were spun down at 1500 rpm for 2 min and supernatant removed. The beads were washed twice in lysis buffer then once in 1X PBS. Proteins were resolved by SDS-PAGE and analyzed by western blot. For GST-SMN-Tdr pull-down, the same procedure was applied with P19-pGIPZ and P19-shPRMT8 cell lysates, but endogenous proteins bound to the Tdr columns were detected using immunoblotting with respective antibodies.

2.11. *IN VIVO* METHYLATION ASSAY

P19-pGIPZ and P19-shPRMT8 cells were grown for 3h in methionine-free DMEM (Wisent) containing 10% fetal bovine serum and 10 μCi/mL of L-[methyl-^{3}H]-methionine (85 Ci/mmol; PerkinElmer) in the presence of translation inhibitors (100 μg/mL cycloheximide and 40 μg/mL chloramphenicol). Cells were harvested in lysis

buffer and proteins were resolved by SDS-PAGE before being transferred onto a PVDF membrane. The membrane was sprayed with EN^{3}HANCE (PerkinElmer Life Sciences) and radioactivity was visualized by fluorography (exposition at -80°C for 1 week). Membrane was then used for western blot analysis.

3. RESULTS

3.1. ASSESSMENT OF THE TISSUE DISTRIBUTION AND DEVELOPMENTAL EXPRESSION PROFILE OF PRMT8

3.1.1. PRMT8 AS A BRAIN AND SPINAL CORD-SPECIFIC TRANSCRIPT

The largely brain-specific expression of PRMT8, detected by Lee et al. in 2005 using Northern analysis, suggested neuron-specific activity. However, the specific brain structure(s) as well as the cell type(s) in which PRMT8 is expressed were not identified in that study. To obtain a morphological basis for understanding the possible function(s) of PRMT8, we performed semi-quantitative RT-PCR analysis on various adult mouse tissues. Therefore, we confirmed the presence of PRMT8 mRNA in mouse brain, brain stem and spinal cord, the latest divided in two sections: the cervical versus the lumbar segment (Figure 6A). In structures positive for the expression of the PRMT8 transcript, we wanted to assess the presence of stably translated proteins; thus, a specific PRMT8 antibody needed to be generated and characterized to perform this analysis.

3.1.2. GENERATION OF PRMT8 POLYCLONAL ANTIBODIES

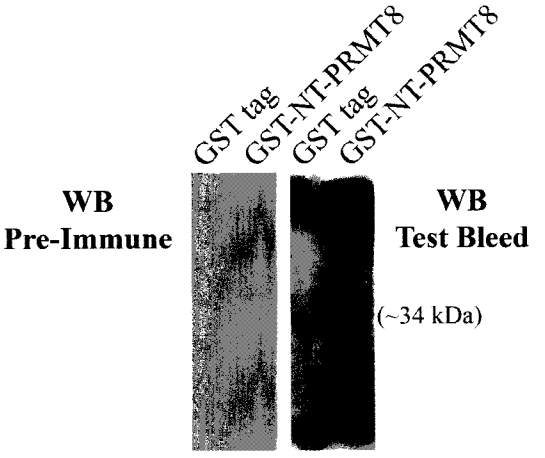
The unique N-terminal region of the human PRMT8 (from glycine 2 to asparagine 65) was subcloned in the C-terminus of the glutathione-S-transferase (GST) tag using pGEX-4T2 vector. Recombinant proteins were expressed using a bacterial system, then purified from SDS-PAGE to reduce the presence of unspecific antigen as much as possible. In association with Cedarlane Laboratories, two rabbits (424 and 728) were inoculated with GST-N-terminal PRMT8. The pre-immune and test bleed of rabbits 424

and 728 were tested by western blot. When comparing their signals on expressed GST tag versus GST-N-terminal PRMT8, rabbit 424 showed both the strongest immune response to PRMT8 and the lowest (absent) background to GST tag (Figure 4A). PRMT8 antibodies were purified from rabbit 424 using recombinant GST-N-terminal PRMT8 proteins (the antigen) fixed to GST sepharose. A western blot analysis of protein extract of wild-type adult mouse brain was performed to monitor the efficiency of antibodies purification and the specificity for the endogenous PRMT8 protein. The estimated size of the full-length PRMT8 protein is 43 kDa and a single band at ~48 kDa was detected in brain extracts (Figure 4B). Again, GST-N-terminal recombinant protein was used as a positive control and protein detection validated that specific PRMT8 antibodies had been purified. The antibodies were then tested for their capacity to immunoprecipitate native PRMT8. His-tagged PRMT8 proteins were expressed using TNT® Coupled Rabbit Reticulocyte Lysate System (Promega), an *in vitro* eukaryotic coupled transcription / translation system containing all the machinery required for protein synthesis. The plasmid used as cDNA template (pET-20b-PRMT8) contained a T7 RNA polymerase promoter and newly translated proteins were labelled by ^{35}S-methionine. An increasing amount of PRMT8 affinity purified antibodies (0.25-0.75 μg) was then used to immunoprecipitate PRMT8, in combination with Protein A-Sepharose. The proteins were resolved by SDS-PAGE, the gel was dried *in vacuo* and proteins visualized by fluorography. The antibodies seem to have only a weak affinity for His-tagged PRMT8 since an increase of antibody concentration did not result in a substantial increase in immunoprecipitated proteins (Figure 5A). After that, antibodies were used to test immunoprecipitation efficiency of endogenous PRMT8 using mouse brain lysate.

Western blot analysis did not reveal any immunoprecipitated PRMT8 protein (Figure 5B). In both experiments, rabbit IgG served as negative control for the immunoprecipitation.

A


Antigen
N-terminal PRMT8


GST tag
GST-NT-PRMT8
GST tag
GST-NT-PRMT8
WB
Pre-Immune
WB
Test Bleed
(~34 kDa)

B

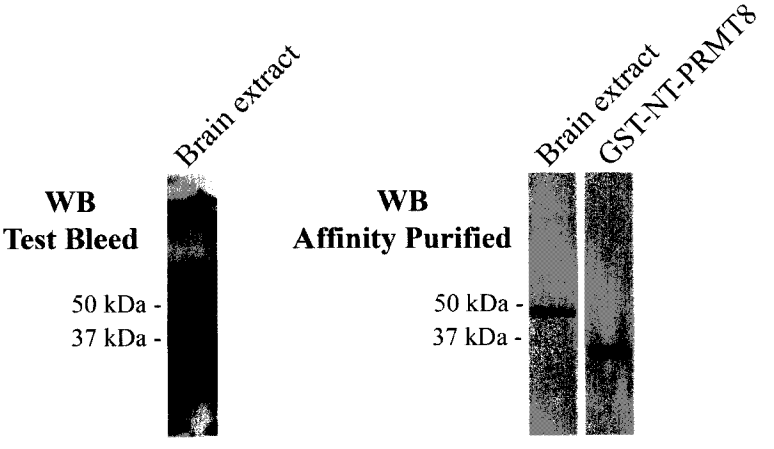
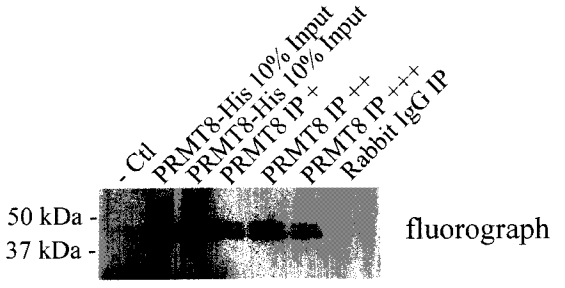

Brain extract
WB
Test Bleed
50 kDa -
37 kDa -
Brain extract
GST-NT-PRMT8
WB
Affinity Purified
50 kDa -
37 kDa -

Figure 4: Characterization of PRMT8 antibodies. A) The unique N-terminal region of PRMT8 was subcloned in the C-terminus of the glutathione-S-transferase (GST) tag. Recombinant proteins were expressed using a bacterial system and purified from SDS-PAGE. Two rabbits (424 and 728) were then immunized with GST-N-terminal PRMT8. Rabbit 424 is shown here as being positive for the production of antibodies that are specific to PRMT8 and not recognizing the GST tag. B) PRMT8 antibodies were purified from rabbit 424 using recombinant GST-N-terminal PRMT8 proteins (the antigen) fixed to GST sepharose. Protein extract of wild-type mouse brain was used to monitor the efficiency of antibody purification and the specificity for the endogenous PRMT8 protein. The estimated size of the full-length PRMT8 protein is 43 kDa. GST-N-terminal PRMT8 recombinant protein was used as a positive control for purified PRMT8 antibodies.

A


- Ctl
PRMT8-His 10% Input
PRMT8-His 10% Input
PRMT8 IP +
PRMT8 IP ++
PRMT8 IP +++
Rabbit IgG IP
50 kDa -
37 kDa -
fluorograph

B

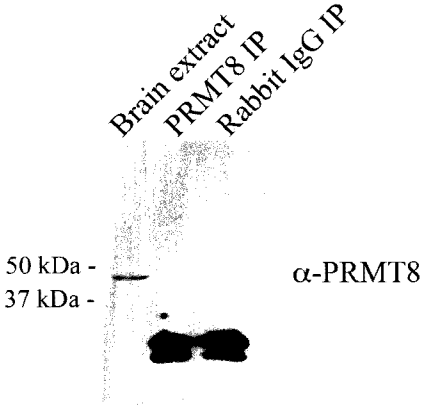

Brain extract
PRMT8 IP
Rabbit IgG IP
50 kDa -
37 kDa -
α-PRMT8

Figure 5: Verification of antibodies efficiency in immunoprecipitation. A) His-tagged PRMT8 proteins were expressed *in vitro* using a rabbit reticulocyte transcription / translation system with ^{35}S-methionine to radiolabel proteins. An increasing amount of affinity purified PRMT8 antibodies (0.25-0.75 μg) was used to immunoprecipitate recombinant proteins. Proteins were resolved by SDS-PAGE and visualized by fluorography. PRMT8 antibodies only weakly immunoprecipitate recombinant PRMT8-His. B) Protein extract of wild-type mouse brain was used to monitor the efficiency of antibodies to immunoprecipitate endogenous PRMT8 proteins. Western blot analysis did not reveal any endogenous protein pulled-down. Rabbit IgG served as negative control for the immunoprecipitation.

3.1.3. PRMT8 AS A BRAIN AND SPINAL CORD-SPECIFIC PROTEIN

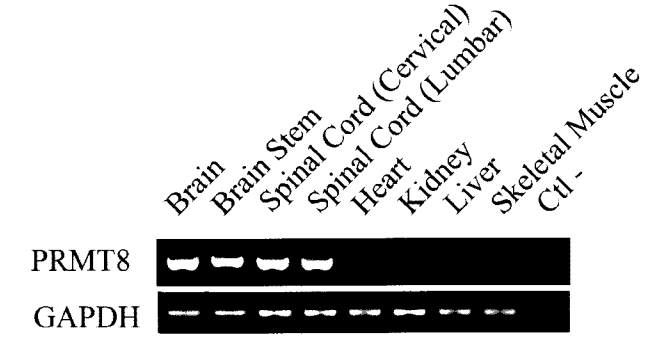
As mentioned earlier, detecting the expression of mRNA does not assess that the protein is efficiently translated and stable. Thus, we further investigated the expression of PRMT8 proteins in various adult wild-type mouse tissues by western blot. The brain, brain stem, spinal cord cervical and lumbar sections had a positive signal, whereas the heart, kidney, liver and skeletal muscle remained negative for the detection of PRMT8 (Figure 6B). GAPDH protein level was used as a loading control. These results correlate with what was observed by RT-PCR and validate PRMT8 as a protein stably expressed in the central nervous system. The tissue distribution of PRMT8 is very particular, considering that other PRMTs are expressed ubiquitously.

3.1.4. PRMT8 EXPRESSION IS INDUCED DURING MOUSE BRAIN DEVELOPMENT

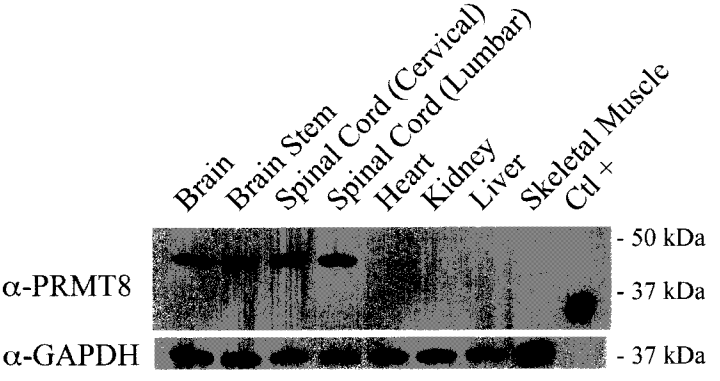
In 2006, Ikenaka et al. reported the ontogenic expression of PRMT1, which revealed the potential importance of this protein during embryonic development. More specifically, PRMT1 is strongly expressed in mouse brain at prenatal day 10.5. This level is maintained until postnatal day 7, then PRMT1 expression dramatically decreases to a very low level at postnatal day 21 (Ikenaka et al. 2006). Considering the high level of homology between PRMT1 and PRMT8, it would be interesting to determine if their ontogenic profiles follow the same pattern or not. To verify these hypotheses, proteins were extracted from mouse C57Bl/6 wt total embryos in early stages of development (embryonic day E6.5, E10.5 and E13.5) as well as from brain and heart of mice at various stages of development (embryonic day E15, E17, postnatal day P0, P7, P14, P21 and P28). A western blot analysis of these samples uncovered the precise activation switch of

PRMT8 in the mouse brain. While the signal was very weak, almost absent, in total embryos and brain sample at E15, the protein was strongly detected at E17 and increased until P21, where the high level of expression remained constant in adulthood (Figure 6C). We then performed a second western blot analysis of these samples, this time using PRMT1 as a control for the development curve. In agreement to what had been reported previously, PRMT1 protein level decreases throughout brain development. Thus, PRMT1 and PRMT8 both seem to perform important functions during brain development and this, at specific but distinct stages. In contrast, only heart samples in the early stage of development (E15) still expressed a low level of PRMT8 protein, while the signal disappeared completely after this point and remained null from E17 to P28. With these observations, we can hypothesize that a basal level of PRMT8 would be expressed in cells sustaining characteristics of pluripotent cells and when cell differentiation is engaged there would be either up-regulation or inhibition of PRMT8 expression depending on the cell lineages. The next step would be to identify which type of cells requires PRMT8.

A


Brain
Brain Stem
Spinal Cord (Cervical)
Spinal Cord (Lumbar)
Heart
Kidney
Liver
Skeletal Muscle
Ctl -
PRMT8
GAPDH

B


Brain
Brain Stem
Spinal Cord (Cervical)
Spinal Cord (Lumbar)
Heart
Kidney
Liver
Skeletal Muscle
Ctl +
α-PRMT8
α-GAPDH
- 50 kDa
- 37 kDa
- 37 kDa

C

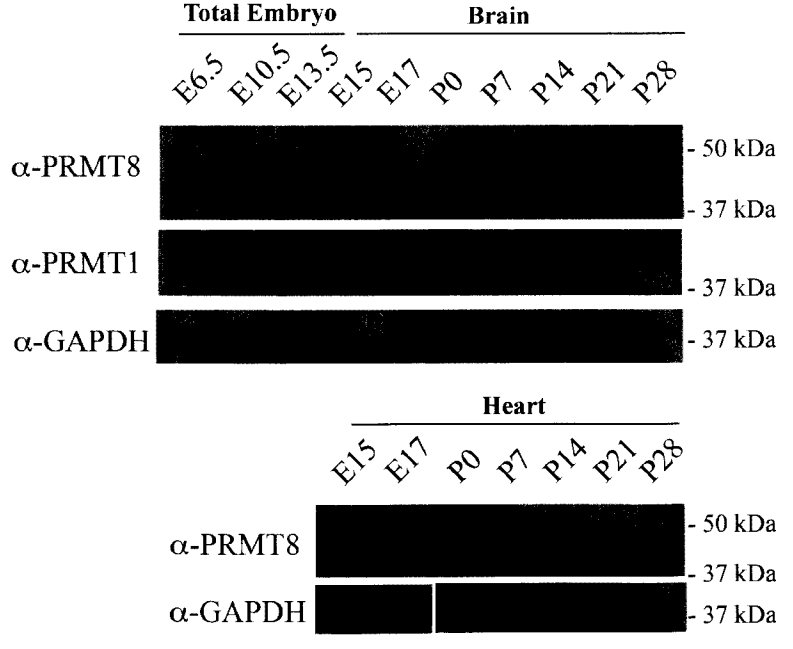

Total Embryo
Brain
E6.5
E10.5
E13.5
E15
E17
P0
P7
P14
P21
P28
α-PRMT8
α-PRMT1
α-GAPDH
- 50 kDa
- 37 kDa
- 37 kDa
- 37 kDa
Heart
E15
E17
P0
P7
P14
P21
P28
α-PRMT8
α-GAPDH
- 50 kDa
- 37 kDa
- 37 kDa

Figure 6: PRMT8 in mouse tissues. A) RT-PCR analysis identifies PRMT8 as a brain and spinal cord-specific transcript in C57Bl/6 wild-type mouse tissues. GAPDH transcript was used to confirm equal cDNA levels in all samples. B) Western blot analysis confirms the expression of stably translated PRMT8 proteins in the brain and spinal cord of wt mouse tissues. GAPDH was used as a loading control. Ctl + : GST-N-terminal PRMT8 recombinant protein. C) Protein extract of total embryos in early stages of the development (embryonic day E6.5, E10.5 and E13.5) as well as from brain and heart of mice at various stages of the development (embryonic day E15, E17, postnatal day P0, P7, P14, P21 and P28) were analyzed by western blot for the expression of PRMT8. The expression of PRMT8 is strongly increased in the brain at E17 and remains high in adulthood. In contrast, the PRMT8 signal is completely lost in the heart at E17 and remains null. GAPDH was used as a loading control. PRMT1 was used as a control for the brain development and as previously reported, it decreases throughout development.

3.1.5. PRMT8 AS A NEURON-SPECIFIC PRMT

The largely brain-specific expression of PRMT8, that Lee and colleagues detected by Northern analysis, suggested a neuron-specific activity. However, the exact brain structure(s) as well as the cell type(s) (neurons versus glial cells) in which PRMT8 is expressed were not identified in that study. One of our goals with a PRMT8 antibody was to pursue our protein expression studies by performing immunofluorescence microscopy using various cellular markers for neurons, astrocytes and oligodendrocytes. However, in April 2007, Taneda et al. published an extensive analysis of the regional distribution of PRMT8 in mouse brain using *in situ* hybridization (ISH) histochemistry. They noted an exclusive expression of PRMT8 in the brain and spinal cord (Taneda et al. 2007). The PRMT8 cRNA probe was specifically hybridized within the central nervous system (CNS) and the signals were observed only in neurons (Taneda et al. 2007). Furthermore, they reported an uneven distribution of PRMT8 mRNA expression throughout the brain, with neurons in regions related to the general somatosensory system showing the strongest signal (Taneda et al. 2007). Also, according to their study, a subset of motor neurons also expressed PRMT8 (Taneda et al. 2007). Their results not only confirmed our hypothesis that this protein is a neuron-specific PRMT, but also correlated with our observations on CNS-restricted expression.

3.1.6. PRMT8 AS A NUCLEAR PROTEIN?

While this thesis was being written, a new manuscript contradicting previous research on PRMT8 was published. Kousaka and colleagues now allege that the exact initiation methionine on PRMT8 would be further down the original in-frame sequence,

which would result in the absence of the myristoylation motif believed to target this enzyme to the plasma membrane (Kousaka et al. 2009). They raised an antiserum against PRMT8 and performed immunohistochemistry (IHC) analyses. They reported a predominantly nuclear localization, broadly distributed in the CNS neurons (Kousaka et al. 2009). In some subset of neurons, PRMT8 also seemed to be present in dendrites and axon bundles (Kousaka et al. 2009). We also tested our PRMT8 antibody by IHC on brain slices and observed a similar pattern of expression, in speckle-like distribution rather than a membrane signal (data not shown). However, we interpreted this result as a non-specific signal since our antibodies cannot immunoprecipitate endogenous PRMT8 proteins and only recognize denatured proteins on western blot.

To date, there is still little information available to fully understand the regulation and the function of the endogenous PRMT8 protein and recent publications have also raised controversy on the topic. Further investigation of the role of PRMT8 is required, but the fact that this enzyme is believed to be a neuron-specific protein and, more particularly, highly restricted to the CNS must also be taken into consideration. Thus, the identification of a cell line for the study of endogenous PRMT8 is significant and will be crucial for future functional studies on this enzyme.

3.2. ESTABLISHMENT OF THE P19 CELL LINE AS A MAMMALIAN SYSTEM FOR ENDOGENOUS PRMT8

3.2.1. THE P19 CELL LINE, A MODEL SYSTEM TO STUDY PRMT8

In order to address the role of PRMT8 in neuronal cells, we first needed to identify a cell line expressing PRMT8. After screening numerous neuronal cell lines either under differentiating or non-differentiating conditions as well as a number of non-neuronal cell lines (data not shown), P19 cells were identified as a good candidate model for our studies. The P19 line was derived from a mouse embryonic carcinoma and has the particularity of being induced to differentiate into neuronal and glial-like cells in the presence of retinoic acid (RA), while treatment with dimethylsulfoxide (DMSO) induces differentiation into cardiac and skeletal muscle-like elements (Resende et al. 2007, Bogoch, Linial 2008). The *in vitro* differentiation of P19 cells into neurons resembles stages encountered during neuronal development. Thus, this system is recognized as a model for studying the development of the central nervous system (Resende et al. 2007, Bogoch, Linial 2008). At the same time, the P19 system reflects the molecular mechanisms controlling the developmental decisions of stem cells differentiating into the cardiac or skeletal muscle lineage (Skerjanc 1999). Therefore, a parallel comparison of the role and regulation of PRMT8 can be made between the differentiations from stem cells to neurons or muscle cells. The differentiation protocols for the P19 cells are comparable for each lineage. The P19 line requires that cells form non-adherent aggregates or embryoid bodies (using ultra-low attachment culture dishes) and are treated for four days, either with 1 μM of RA (neurons) or 0.5-1% DMSO (muscles). In the case of the neuronal differentiation, aggregates are trypsinized at the end of Day 4 and plated

in regular tissue culture dishes, allowing reattachment of the cells. The use of serum free media (SFM) to avoid proliferation of glial cells, and the withdrawal of RA are applied to terminate differentiation. Numerous factors affect axonal development; the survival and maturation of neuronal P19 culture is strongly dependent on the expression of adhesion molecules, the release of cytokines, growth factors and neurotrophins as well as signalling cascade induced by cell-cell interactions (Markus, Patel & Snider 2002, Bogoch, Linial 2008). Serum in general is an ill-defined component in cell culture media, which provides a broad spectrum of macromolecules, carrier proteins, attachment and spreading factors, low molecular weight nutrients, hormones and growth factors (Gstraunthaler 2003). Thus, it has a significant influence in the differentiation process of P19 cells, but it can be difficult to control it and the yield in differentiated cells can vary from one batch of serum to another. This observation is even more striking in the case of cardiac or skeletal myocytes obtained from P19 cells. Usually, no more than 10% to 25% of total cells achieve cardiac myocyte differentiation, and the yield of skeletal myocytes is even lower, namely from 5% to 15% of the culture (Skerjanc 1999). The myogenesis protocol differs slightly from the neuronal differentiation. Cells must also form embryoid bodies in low attachment plates but are treated with DMSO instead of RA and aggregates are processed differently. Cell treatment with DMSO is interrupted at the end of Day 4, but aggregates should not be disrupted before transferring on adherent plates and the use of serum is required to achieve complete differentiation. Again, the culture conditions can have a significant influence, since unknown factors in the serum are believed to regulate the ability of P19 cells to undergo myogenesis (Wilton, Skerjanc 1999, Jamali et al. 2001).

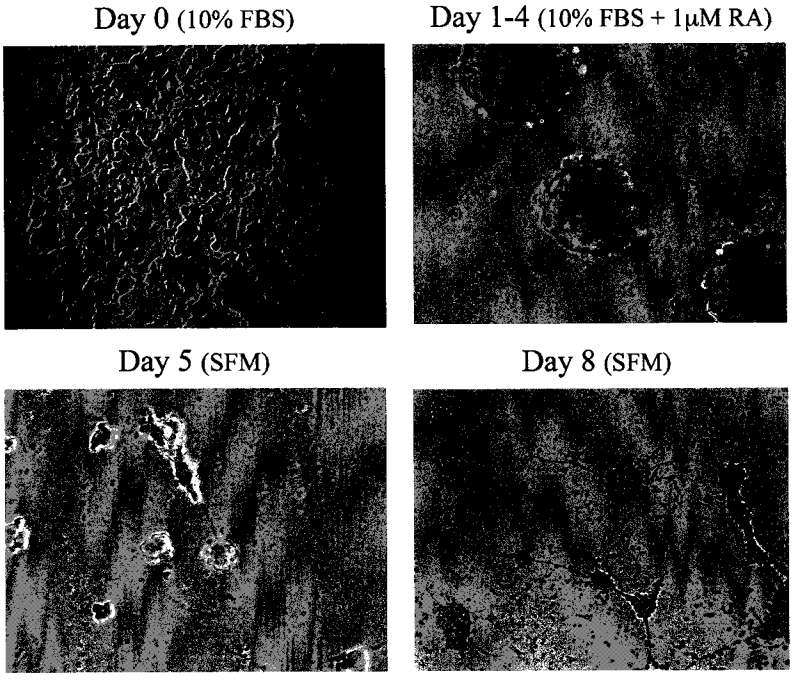
3.2.2. *PRMT8 TRANSCRIPT IS UP-REGULATED THROUGHOUT NEURONAL DIFFERENTIATION OF P19 CELLS*

We verified the expression profile of PRMT8 mRNA throughout neuronal differentiation induced by RA treatment and semi-quantitative RT-PCR analysis showed a general increase of PRMT8 transcript from Day 3 to 8, but a very high signal on Days 5 to 7 (Figure 7B). GAPDH transcript confirmed that comparable levels of cDNA were used. Mash-1 transcript was used as a positive neuronal marker for P19 cells and the expected induction of the expression at Day 1 was observed (Jing et al. 2009). The increase of PRMT8 mRNA at Day 5 of neuronal differentiation coincides with the replating of the cells in adherent culture and extension of major neuronal processes. Interestingly, the up-regulation of PRMT8 is delayed in time compared to the neuronal marker Mash-1. The latter transcript appears at a low level on Day 1 and is present at a higher but fairly constant level from Days 2 to 7, before it diminishes slightly on Days 8 and 9. These results suggest the requirement of PRMT8 at a specific time-point in the differentiation, which agrees with the marked up-regulation of PRMT8 proteins observed during mouse brain development. Brain and heart samples from adult wild-type mouse respectively serve as positive and negative controls for the expression of neuron specific Mash-1 and PRMT8 transcripts.

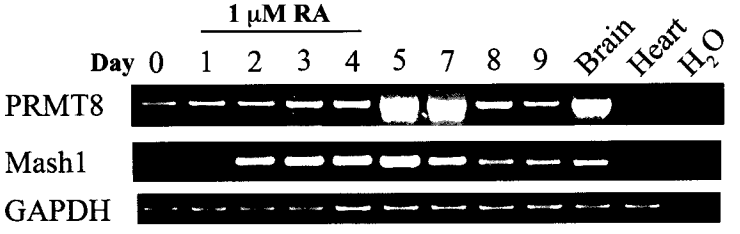
3.2.3. PRMT8 PROTEIN EXPRESSION IS SPECIFICALLY INDUCED DURING P19 NEURONAL DIFFERENTIATION

Knowing that PRMT8 transcript is up-regulated in concert with cell adhesion and elongation of P19 neuronal extensions, we verified whether these observations correlated with PRMT8 expression profile at the protein level. Thus, we further investigated the expression of PRMT8 proteins throughout neuronal differentiation of RA-treated P19 cells using western blot analysis. From Days 0 to 3, PRMT8 protein is barely detectable (Figure 7C). On Day 4, an induction of PRMT8 protein expression can be noticed followed by an increase of the signal at Day 5. The PRMT8 protein level then remains elevated at a steady intensity throughout the differentiation time course. GAPDH protein level was used as a loading control. These results correlate partially with what was observed by RT-PCR by showing the increase in PRMT8 mRNA around days when cells are replated in an adherent culture and neuronal processes are extending (Day 4 to Day 8). However, there is a discrepancy between the mRNA and protein levels observed on Days 5 to 7, since the very high signal of PRMT8 transcript does not translate into a greater amount of proteins. Consequently, the next question is to verify whether PRMT8 is required for P19 maintenance and/or differentiation.

A


Day 0 (10% FBS)
Day 1-4 (10% FBS + 1μM RA)
Day 5 (SFM)
Day 8 (SFM)

B


1 μM RA
Day 0 1 2 3 4 5 7 8 9 Brain Heart H_2O
PRMT8
Mash1
GAPDH

C

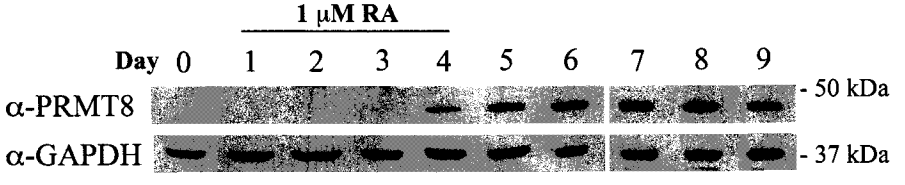

1 μM RA
Day 0 1 2 3 4 5 6 7 8 9
α-PRMT8
α-GAPDH
- 50 kDa
- 37 kDa

Figure 7: PRMT8 throughout the neuronal differentiation of the P19 cells. A) At Day 0, cells are adherent and cultured without treatment. From Day 1 to 4, cells are in ultra-low attachment tissue culture dishes where they form non-adhering aggregates or embryoid bodies and are treated with 1 μM of retinoic acid (RA). At the end of Day 4, aggregates are trypsinized and plated in regular tissues culture dishes using serum free media (SFM) without RA to terminate differentiation. By Day 8, a strong network of neuronal extensions can be observed. B) RT-PCR analysis shows an increase of PRMT8 transcript on days where cells are replated in an adherent culture and neuronal processes are extending (Day 4 to Day 8). GAPDH transcript was used to confirm integrity and to compare cDNA levels between samples. Mash1 is a neuronal marker. Mouse brain and heart samples serve as positive and negative control, respectively. C) Western blot analysis reveals an induction of PRMT8 protein expression at Day 4 and an increase of the signal at Day 5. The PRMT8 protein level then remains elevated. GAPDH protein level was used as a loading control.

3.2.4. DMSO TREATMENT OF P19 CELLS LEADS TO THE LOSS OF PRMT8

While PRMT8 transcript is present at all steps during the neuronal process, there is a loss of expression of PRMT8 upon DMSO treatment to drive P19 cells into the myocyte differentiation program as observed by RT-PCR (Figure 8A). Myosin heavy chain (MHC) expression was used as a marker of terminal differentiation into muscle as detected by immunofluorescence using the MF20 monoclonal antibody (Figure 8B). As previously reported, only a small fraction of the cell population achieved cardiac or skeletal muscle differentiation, but the cells still survived the DMSO treatment with a low or absent expression of PRMT8. These observations suggested the potential implication of PRMT8 for either neuronal differentiation or maintenance of neuronal characteristics in CNS-like systems. The following step was to determine whether PRMT8 expression in P19 cells is essential in order to achieve neuronal differentiation.

Figure 8: PRMT8 expression in P19 cells upon the induction of myocyte formation. Similarly to the neuronal differentiation protocol, at Day 0, cells are adherent and cultured without treatment. From Day 1 to 4, cells are in ultra-low attachment tissue culture dishes where they form non-adhering aggregates or embryoid bodies, but are treated with 1% DMSO. At the end of Day 4, aggregates must not be disrupted and are replated in regular tissue culture dishes using media with 10% serum to terminate differentiation. By Day 6 or 7, subpopulations of cells beating can be observed. A) RT-PCR analysis shows a decrease of PRMT8 transcript as early as Day 1 of the treatment and a complete absence of signal after Day 2. GAPDH transcript was used to confirm integrity and to compare cDNA levels between samples. Mouse brain and heart samples serve respectively as positive and negative control. B) Immunofluorescence using MF20 antibody, which recognizes the various isoforms of myosin heavy chain (MHC), allows the detection of differentiated myocytes.

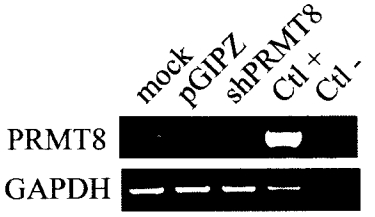
3.2.5. PRMT8 EXPRESSION IS REQUIRED FOR P19 CELLS TO DIFFERENTIATE INTO NEURONS

To investigate the requirement for PRMT8 expression in achieving neuronal differentiation in a CNS-like model, P19 cells, a PRMT8 knocked down cell line was needed. Open Biosystems' pGIPZ-shPRMT8 construct and pGIPZ empty vector were used to generate P19 cell lines with reduced PRMT8 expression (shPRMT8) or simply as control that is resistant to puromycin (pGIPZ), the selection marker. These vectors were created to combine the design advatanges of microRNA-adapted short hairpin RNA (shRNAmir) with the lentiviral vector to create a powerful RNA interference (RNAi) trigger capable of producing RNAi in most cell types[1]. Two different pGIPZ-shPRMT8 constructs (named B5 and D10) were generated according to Open Biosystems' RNAi design algorithm. These plasmids were stably transfected in P19 cells and many clones were isolated. These clones were then screened by semi-quantitative RT-PCR to identify the one showing the highest reduction of PRMT8 mRNA. The selected clone was obtained with pGIPZ-shPRMT8 D10 construct and showed no detectable level of PRMT8 mRNA (Figure 9A). The same procedure was applied with pGIPZ empty vector to confirm with the RT-PCR analysis that the plasmid and the presence of puromycin did not affect the expression level of PRMT8 transcript (Figure 9A). These two cell lines were then induced to differentiate in neurons following the same protocol as for parental cells, except for the addition of puromycin at 1 μg/mL of media on Day 0 to 4 inclusively. Puromycin was withdrawn after trypsinizing P19 RA-treated cell aggregates because it otherwise inhibited neurite outgrowth of P19 cells that were known to be resistant to this antibiotic (P19-pGIPZ). As observed with parental P19 cells, P19-pGIPZ

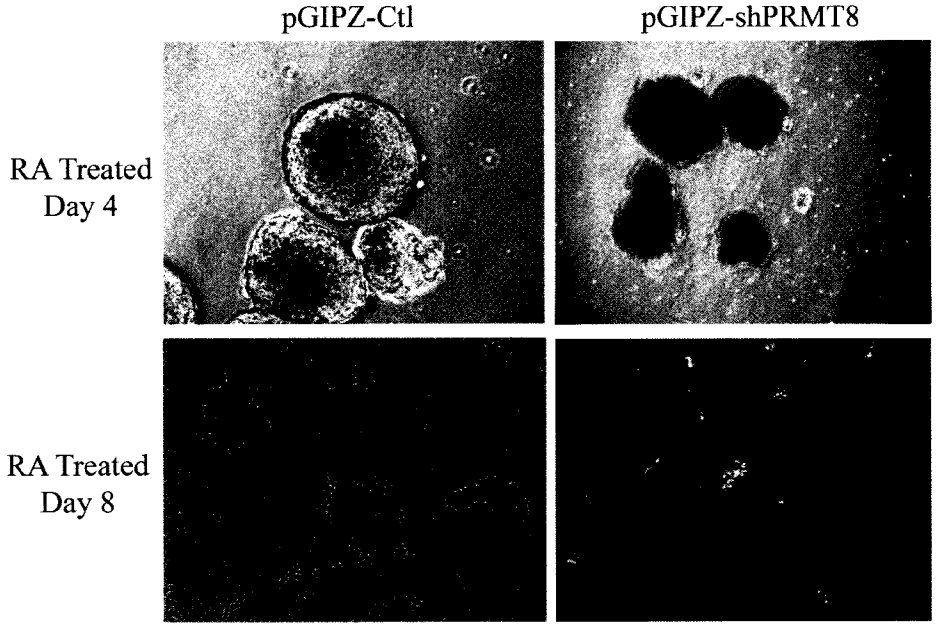
[1] Open Biosystems Product Information Booklet. Expression Arrest™ GIPZ lentiviral shRNAi

control cells extended neuronal processes after replating to adherent culture dishes (Figure 9B). In contrast, few PRMT8 knocked down cells survived the replating step and no neuronal extension could be detected. These two cell lines were then treated with DMSO to induce myocyte differentiation; once again the same protocol was applied as for P19 parental cell, except for the addition of puromycin at 1 μg/mL of media on Day 0 to 4 only. As observed with parental P19 cells, P19-pGIPZ control showed a subset of cells expressing the MHC myocyte marker after replating treated embryoid bodies to adherent culture dishes (as detected by immunofluorescence, Figure 9D). No significant cell death was observed for P19-shPRMT8 cells, neither during DMSO treatment nor at the replating step, and they displayed a very similar morphology as P19-pGIPZ cells (Figure 9C). However, myocyte differentiation could not be confirmed due to the complete absence of MHC-expressing cells in three independent experiments (data not shown). These results indicated that PRMT8 is not required for normal cell growth, but is essential for cell survival during neuronal differentiation. Even though the absence of PRMT8 did not trigger P19 cell death during myocyte formation, results suggested that PRMT8 may be required to achieve terminal differentiation.

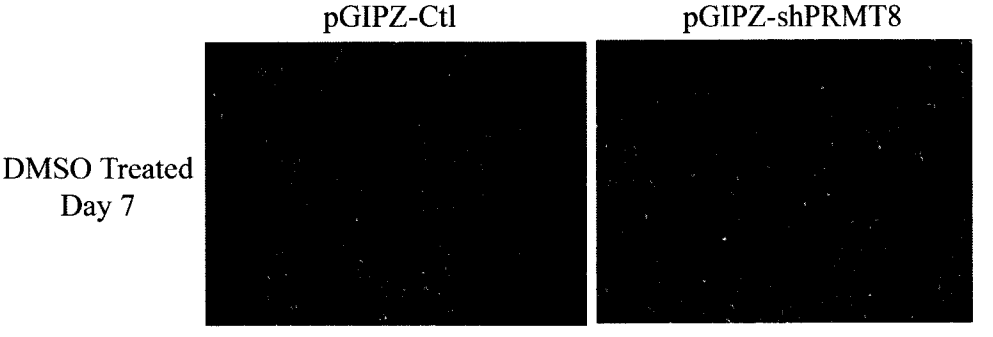
A


mock
pGIPZ
shPRMT8
Ctl+
Ctl-
PRMT8
GAPDH

B


pGIPZ-Ctl
pGIPZ-shPRMT8
RA Treated
Day 4
RA Treated
Day 8

C


pGIPZ-Ctl
pGIPZ-shPRMT8
DMSO Treated
Day 7

D

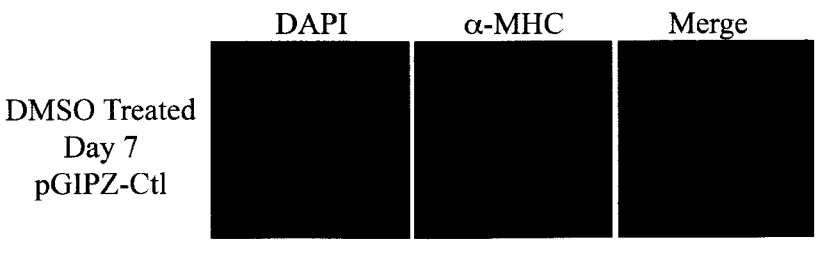

DAPI
α-MHC
Merge
DMSO Treated
Day 7
pGIPZ-Ctl

Figure 9: The effect of PRMT8 knock-down on P19 differentiation. pGIPZ-shPRMT8 construct and pGIPZ empty vector from Open Biosystems were used to generate P19 stable cell lines that are knocked down for PRMT8 (shPRMT8) or simply as control that is resistant to puromycin (pGIPZ), the selection marker. A) RT-PCR analysis indicates that pGIPZ empty vector and the presence of puromycin does not affect the expression level of PRMT8 transcript and that the shPRMT8 clone (D10 construct) used is knocked down. GAPDH transcript confirms equal cDNA levels in all samples. Ctl+: mouse brain; Ctl - : water. B) P19 stable cells lines throughout neuronal differentiation. At Day 4, P19-shPRMT8 cell aggregates are dark and contain a large proportion of dead cells compared to P19-pGIPZ embryoid bodies. At Day 8, similarly to parental P19 cells, pGIPZ-Ctl cell line has extended great neuronal processes, whereas P19 PRMT8 knocked down (shPRMT8) cells have a round shape and most likely did not survive the differentiation procedure. C) Both P19-pGIPZ and P19-shPRMT8 cell line were treated with 1% DMSO to induce muscle lineage and survived the process. Here they are shown at Day 7 of differentiation. D) Immunofluorescence using MF20 antibody (MHC), confirms the presence of myocyte differentiated P19-pGIPZ cells.

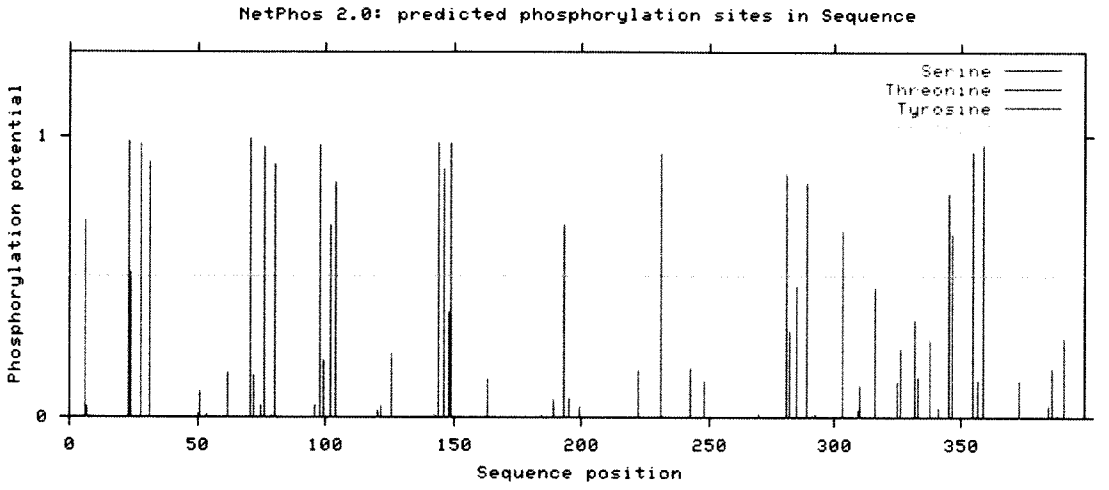
3.3. PRELIMINARY STUDIES TO DETERMINE THE FUNCTION AND REGULATION OF PRMT8 IN NEURONAL CELLS

This section of the thesis reports preliminary data of experiments performed to attempt to elucidate the role and the mechanism of PRMT8 regulation.

3.3.1. PRMT8 IS A PHOSPHOPROTEIN

Cellular signal transduction pathways involve protein kinases, protein phosphatases and phosphoprotein-interacting domain containing cellular proteins to provide multidimensional, dynamic and reversible regulation of many biological activities (Sawyer et al. 2005). Since our hypothesis is that PRMT8 methylation activity will be implicated in the signaling pathways leading to neuronal differentiation, establishing that PRMT8 is phosphorylated would indicate that it might be regulated and/or involved in signaling. NetPhos 2.0 is a server that produces neuronal network predictions for serine, threonine and tyrosine phosphorylation sites in eukaryotic protein (Blom, Gammeltoft & Brunak 1999, Jensen et al. 2002). From this prediction algorithm, five serines, two threonines and one tyrosine possible phosphorylation sites were identified with a score higher than 0.95 (Table 1 and Table 2 for complete list). As we were planning experiments to assess phosphorylation of PRMT8, we received a personal communication from Dr. Mark T. Bedford (University of Texas, USA) that confirmed that PRMT8 is a phosphoprotein. This angle will be pursued with Dr. Bedford as a joint study.

A



B

Serine predictions

Name	Pos	Context	Score	Pred
		v		
Sequence	6	GMKHSSRCL	0.700	*S*
Sequence	7	MKHSSRCLL	0.034	.
Sequence	23	NAAESTEVN	0.984	*S*
Sequence	28	TEVNSPPSQ	0.977	*S*
Sequence	31	NSPPSQPPQ	0.907	*S*
Sequence	50	VHHVSTQPS	0.015	.
Sequence	54	STQPSCPGR	0.011	.
Sequence	62	RGKMSKLLN	0.159	.
Sequence	72	EEMTSRDYY	0.148	.
Sequence	79	YYFDSYAHF	0.004	.
Sequence	102	TYRNSMYHN	0.684	*S*
Sequence	120	LDVGSGTGI	0.025	.
Sequence	126	TGILSMFAA	0.224	.
Sequence	143	GIECSSISD	0.006	.
Sequence	144	IECSSISDY	0.975	*S*
Sequence	146	CSSISDYSE	0.880	*S*
Sequence	149	ISDYSEKII	0.976	*S*
Sequence	184	DIIISEWMG	0.005	.
Sequence	195	LFYESMLNT	0.067	.
Sequence	289	TEELSFTSA	0.831	*S*
Sequence	292	LSFTSAFCL	0.010	.
Sequence	325	KMGFSTAPD	0.127	.
Sequence	359	YGTISMKPN	0.967	*S*
Sequence	386	LCETSVSND	0.171	.
Sequence	388	ETSVSNDYK	0.006	.
		^		

Table 1: Predicted serine phosphorylation sites of PRMT8. A) Graphic representation of all the predicted phosphorylation sites in human PRMT8 using NetPhos 2.0 network. B) List of predicted phosphorylated serine identifies five sites with a score higher than 0.95.

A

Threonine predictions

Name	Pos	Context	Score	Pred
		v		
Sequence	24	AAESTEVNS	0.513	*T*
Sequence	51	HHVSTQPSC	0.093	.
Sequence	71	PEEMTSRDY	0.991	*T*
Sequence	96	DEVRTLTYR	0.044	.
Sequence	98	VRTLTYRNS	0.968	*T*
Sequence	122	VGSGTGILS	0.040	.
Sequence	163	DNIITIFKG	0.137	.
Sequence	199	SMLNTVIFA	0.034	.
Sequence	248	GFDMTCIRD	0.129	.
Sequence	270	KQVVTNACL	0.010	.
Sequence	282	VDIYTVKTE	0.304	.
Sequence	285	YTVKTEELS	0.468	.
Sequence	291	ELSFTSAFC	0.006	.
Sequence	309	HALVTYFNI	0.023	.
Sequence	316	NIEFTKCHK	0.462	.
Sequence	326	MGFSTAPDA	0.246	.
Sequence	333	DAPYTHWKQ	0.142	.
Sequence	338	HWKQTVFYL	0.276	.
Sequence	347	EDYLTVRRG	0.651	*T*
Sequence	357	EIYGTISMK	0.128	.
Sequence	373	DLDFTVDLD	0.129	.
Sequence	385	QLCETSVSN	0.043	.
		^		

B

Tyrosine predictions

Name	Pos	Context	Score	Pred
		v		
Sequence	75	TSRDYYFDS	0.044	.
Sequence	76	SRDYYFDSY	0.961	*Y*
Sequence	80	YFDSYAHFG	0.903	*Y*
Sequence	99	RTLTYRNSM	0.204	.
Sequence	104	RNSMYHNKH	0.835	*Y*
Sequence	148	SISDYSEKI	0.372	.
Sequence	189	EWMGYCLFY	0.060	.
Sequence	193	YCLFYESML	0.686	*Y*
Sequence	222	RAALYVVAI	0.163	.
Sequence	231	EDRQYKDFK	0.940	*Y*
Sequence	243	WENVYGFDM	0.170	.
Sequence	281	EVDIYTVKT	0.862	*Y*
Sequence	303	QRNDYVHAL	0.664	*Y*
Sequence	310	ALVTYFNIE	0.110	.
Sequence	332	PDAPYTHWK	0.344	.
Sequence	341	QTVFYLEDY	0.033	.
Sequence	345	YLEDYLTVR	0.799	*Y*
Sequence	355	GEEIYGTIS	0.943	*Y*
Sequence	391	VSNDYKMR-	0.281	.
		^		

Table 2: Predicted threonine and tyrosine phosphorylation sites of PRMT8. List of predicted phosphorylation sites identifies two threonine and one tyrosine with a score higher than 0.95.

3.3.2. THE REGULATION OF PRMT8 BY SH3 DOMAINS?

In addition to NetPhos 2.0, the protein sequence of PRMT8 was analyzed with the NetPhosK program which lists potential kinases responsible for phosphorylating the predicted sites. Since kinases are known to often associate with their substrates, we thought that we might be able to show interaction of PRMT8 with the kinase(s) responsible for its phosphorylation. When analyzing PRMT8 sequence, one of each of the two SH3 domain consensus sequence motifs (PXXPXR and XPpXP) is present within the unique N-terminal region (refer to Figure 3). Since we already had constructs of the SH3 domains of some of the potential kinases identified by NetPhosK, GST fusion proteins of SH3 domains of LCK, PLCγ, PI3K, Fyn, GAP and Spectin were tested for their ability to interact with PRMT8 in a pull-down experiment. In more detail, GST-SH3 recombinant proteins were expressed using a bacterial system and bound on GST sepharose. GFP-tagged PRMT8 proteins were transiently transfected in 293T cells and protein lysates were incubated with GST-SH3 bound to sepharose. Pull-down proteins were analyzed by western blot, but no interactions were detected between PRMT8-GFP and the tested SH3 domains (Figure 10). Transfection efficiency as well as the use of equivalent amount of the various GST-SH3 constructs was confirmed by western blot. This was an arbitrary selection of SH3 domains and the experiment was also performed in a recent study where they showed interaction of PRMT8 with the SH3 domains of PLCγ, Fyn and a few other proteins that we did not test (Sayegh et al. 2007). However, they used a mutated form of PRMT8 (G2A) that cannot be myristoylated, which could explain the discrepancy between their results and ours.

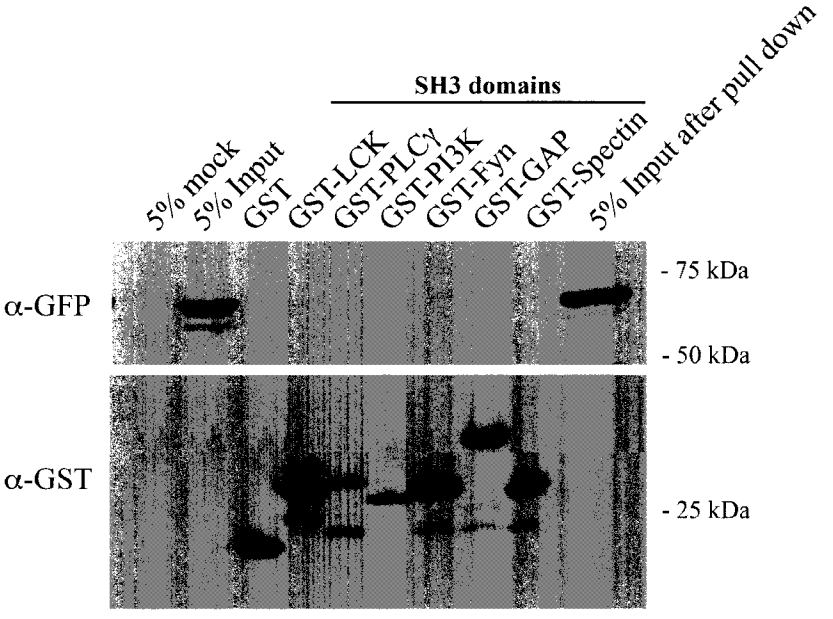

SH3 domains
5% mock
5% Input
GST
GST-LCK
GST-PLCγ
GST-PI3K
GST-Fyn
GST-GAP
GST-Spectin
5% Input after pull down
α-GFP
α-GST
- 75 kDa
- 50 kDa
- 25 kDa

Figure 10: PRMT8 SH3 domains interactions. The analysis of PRMT8 sequence identified many potential phosphorylation sites and revealed the presence of one of each of the two SH3 domain consensus sequences within the unique N-terminal region. Using NetPhosK program, we obtained a list of potential kinases involved. We performed a pull-down experiment using constructs of the SH3 domains of some of the listed kinases to see if they would interact with PRMT8. GST-SH3 domain of LCK, PLCγ, PI3K, Fyn, GAP or Spectin recombinant proteins were expressed using a bacterial system and bound to GST sepharose. PRMT8-GFP recombinant proteins were transiently expressed in 293T cells and protein lysates were incubated with GST-SH3 bound to sepharose. Transfection efficiency was confirmed by western blot using GFP antibody, but no interaction was detected. Western blot against GST confirmed the presence of GST-SH3 recombinant protein in pull-down.

3.3.3. PRMT8 POTENTIAL INTERACTORS

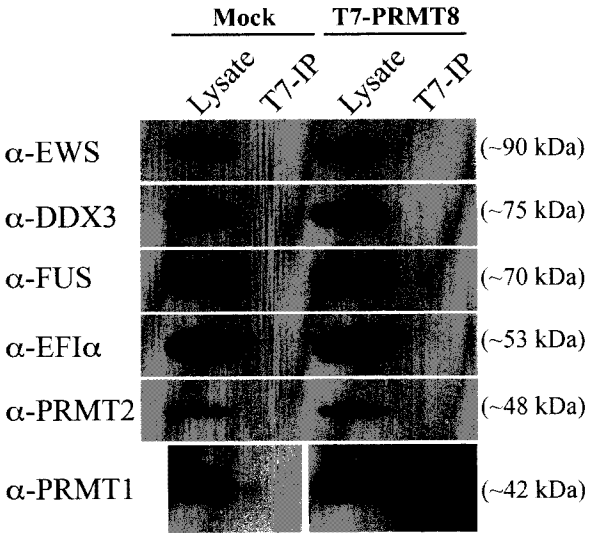
In 2008, Pahlich and colleagues identified more than 20 PRMT8-binding interactors by performing pull-down experiments using recombinant PRMT8 as bait followed by mass spectrometric identification of the bound proteins (listed in Table 3). Among the identified proteins, many contain RGG consensus methylation motifs and thus, are potential substrates of PRMT8. From the list of binding partners, we selected proteins that are known substrates of PRMTs and believed to be implicated in RNA-binding. Active transport of mRNA in ribonucleoprotein complexes along dendrites has been demonstrated as a key element of neuronal maturation (Kiebler, Bassell 2006, Rossoll, Bassell 2009). These complexes contain a number of proteins, including EWS and FUS (Pahlich, Zakaryan & Gehring 2008), thus we hypothesized that PRMT8, a neuron-specific enzyme, could participate in this process. We attempted to validate the interaction of PRMT8 with EWS, DDX3, FUS, EFIα and PRMT2 by performing coimmunoprecipitation experiments. Briefly, T7-tagged PRMT8 recombinant proteins were transiently expressed in P19 cells and immunoprecipitated using T7 antibodies coupled to sepharose beads. Immunoprecipitated proteins were assayed by western blot, but analyses did not reveal any interaction with tested proteins. Our first hypothesis was that interactions could be dependent of the methylation state of the interactors. Over expressing PRMT8 for 48 hours might have led to hypermethylation of its substrates and PRMTs are more likely to have less affinity for their substrates once they are methylated to favour enzyme turnover (Herrmann et al. 2005). To counteract this problem, T7-PRMT8 was first immunoprecipitated, and then a pull-down experiment on a mock P19 total cell lysate was performed (Figure 11). Once again, no PRMT8 interactor could be

confirmed. PRMT1, a known interactor of PRMT8 (Lee et al. 2005), was used as a positive control for coimmunoprecipitation and the signal was very strong. Transfection efficiency of T7-PRMT8 was confirmed by western blot analysis against the T7 tag.

(Primary accession number) protein name	kDa	Mascot score Exp. 1	Exp. 2	RG-rich regions
(Q99873) Protein arginine *N*-methyltransferase 1	41.5	521	378	
• (P35637) RNA-binding protein TLS/FUS	53.4	586	246	Yes
• (Q01844) RNA-binding protein EWS	68.5	498	118	Yes
(Q92804) RNA-binding protein TAF(II)68	61.8	882	217	Yes
(P51991) Heterogeneous nuclear ribonucleoprotein A3	39.6	390	24	Yes
(P31942) Heterogeneous nuclear ribonucleoprotein H3	36.9	498	307	Yes
• (P61978) Heterogeneous nuclear ribonucleoprotein K	50.9	565	1023	Yes
(Q9BQ09) Heterogeneous nuclear ribonucleoprotein U	90.5	783	200	Yes
(Q92499) ATP-dependent RNA helicase DDX1 (DEAD box protein 1)	82.4	463	140	
• (O00571) ATP-dependent RNA helicase DDX3X (DEAD box protein 3)	73.2	763	38	Yes
(Q9BV09) Caprin (cytoplasmic activation/proliferation-associated protein 1)	116	423	59	Yes
(P60709) Actin, cytoplasmic 1 (β-actin)	41.7	782	475	
(Q9NY65) Tubulin α-8 chain (α-tubulin 8)	50.1	110	352	
(P68363) Tubulin α-ubiquitous chain (tubulin K-β-1)	50.2	341	168	
(P07437) Tubulin β-2 chain	49.6	475	18	
(P38646) Stress-70 protein, mitochondrial precursor	73.7	1607	103	
(P08107) Heat shock 70 kDa protein 1	70	425	165	
(Q9HAV7) GrpE Protein homolog 1, mitochondrial precursor	24.3	264	38	
(P11586) C-1-Tetrahydrofolate synthase, cytoplasmic	101.6	519	151	
(P68104) Elongation factor 1-α 1	50.1	322	286	
(Q8WXH0) Nesprin-2 (nuclear envelope spectrin repeat protein 2)	796.4	137	29	
(P31327) Carbamoyl-phosphate synthase, mitochondrial precursor (EC 6.3.4.16)	164.9	63	58	

Table 3: Identified proteins of the GST-PRMT8 pull-down. List of proteins identified by mass spectrometry following GST-PRMT8 pull-down on hypomethylated lysate of Jurkat cells. (Adapted from Pahlich, 2008)

A


Mock
T7-PRMT8
Lysate
T7-IP
Lysate
T7-IP
α-EWS
(~90 kDa)
α-DDX3
(~75 kDa)
α-FUS
(~70 kDa)
α-EFIα
(~53 kDa)
α-PRMT2
(~48 kDa)
α-PRMT1
(~42 kDa)

B

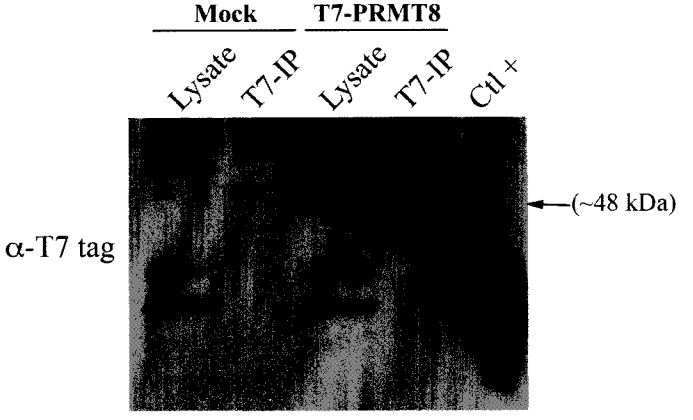
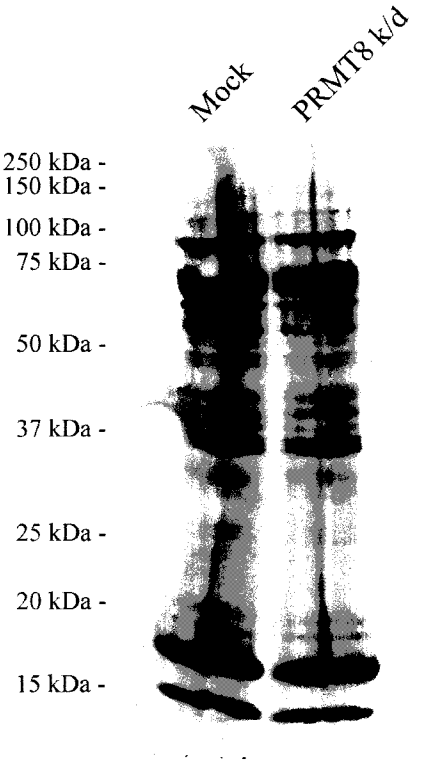

Mock
T7-PRMT8
Lysate
T7-IP
Lysate
T7-IP
Ctl+
α-T7 tag
(~48 kDa)

Figure 11: Verification of PRMT8 potential interactor(s). EWS, DDX3, FUS, EFIα, and PRMT2 have previously been identified as potential interactors of PRMT8; coimmunoprecipitation experiments were performed to verify these interactions. T7-tagged PRMT8 recombinant proteins were transiently expressed in P19 cells and immunoprecipitated using T7 sepharose. A) Western blot analyses did not confirm any interaction; PRMT1 serves as a positive control for immunoprecipitation with PRMT8. B) Transfection and immunoprecipitation efficiency of T7-PRMT8 were confirmed by western blot using T7 tag antibody. Ctl+: commercial T7 tag positive control from Novagen.

3.3.4. PRMT8 REGULATES PROTEIN INTERACTIONS WITH THE TUDOR DOMAIN OF SMN

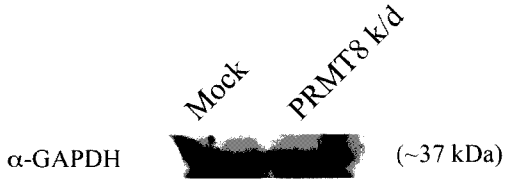
In our search for PRMT8 interactors/substrates, we decided to use the P19-pGIPZ (mock) and P19-shPRMT8 (PRMT8 knocked down) cell lines to verify if the loss of PRMT8 has an impact on the general pattern of methylated proteins. We performed an *in vivo* methylation assay to radiolabel methylated proteins using L-[methyl-^{3}H]-methionine. Total protein extracts were resolved by SDS-PAGE, transferred onto a PVDF membrane and revealed by fluorography (Figure 12A). We thought that we might be able to observe differences in the banding pattern and potentially isolate and identify by mass spectrometry proteins that are radiolabeled in mock cells but unmethylated in knocked down conditions. However, the pattern of methylated proteins is similar for both cell lines with a slight reduction around the 25 kDa region in P19-shPRMT8 cells. Western blot against GAPDH confirmed equal amount of proteins in samples (Figure 12B). Since it has been demonstrated that the same protein can be the substrate of more than one PRMT, and thus it can contain both aDMA and sDMA, we narrowed down our comparison to the pattern of aDMA in both cell lines, in order to have more specificity for the effect of PRMT8, which generates aDMA. Total cell lysates of P19-pGIPZ (mock) and P19-shPRMT8 (k/d) were immunoblotted with antibodies recognizing a subset of proteins containing aDMA (Figure 12C). Again, both lysates displayed a very similar banding pattern, with minor differences around 25 kDa. This did not allow us to isolate a protein band that could have corresponded to a protein exclusively methylated by PRMT8.

A

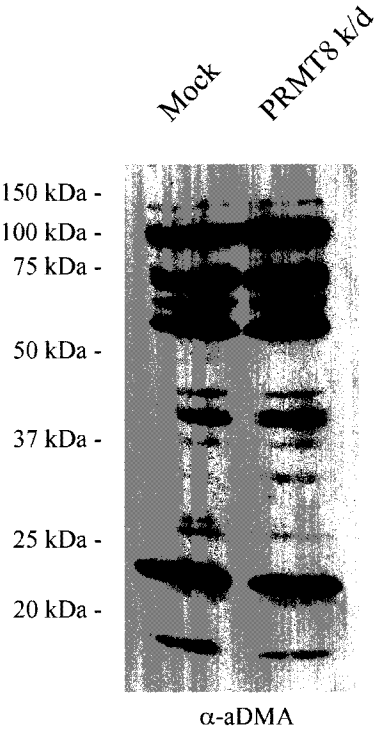


Fluorograph

B



C

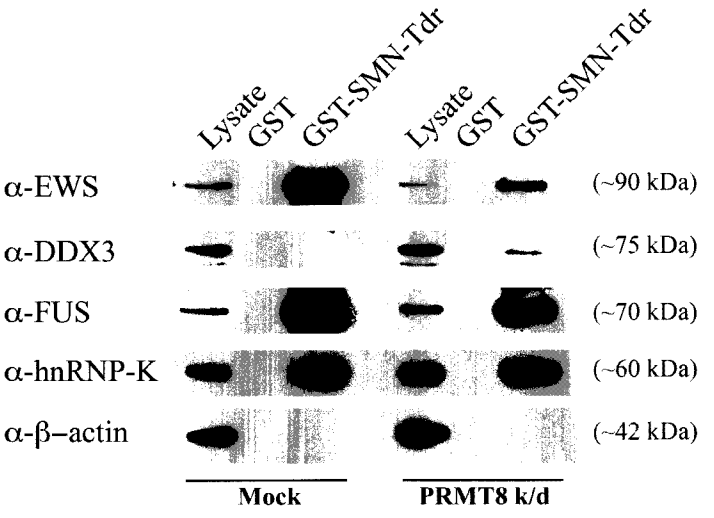


α-aDMA

Figure 12: Comparison of the pattern of methylated proteins *in vivo* when PRMT8 is knocked down. A) Methylated proteins from P19-pGIPZ (mock) and P19-shPRMT8 (k/d) cell lines were radiolabeled *in vivo* using L-[methyl-^{3}H]-methionine. Total protein extracts were resolved by SDS-PAGE, transferred onto a PVDF membrane and revealed by fluorography. The pattern of methylated proteins is similar for both cell lines. B) Western blot against GAPDH confirmed equal amount of proteins in samples. C) Total cell lysates of P19-pGIPZ (mock) and P19-shPRMT8 (k/d) were used for immunoprecipitation of aDMA-containing proteins using antibodies recognizing a subset of proteins containing asymmetric dimethylarginines. Both lysates display a very similar pattern of aDMA proteins.

Spinal Muscular Atrophy (SMA) is a genetic disease characterized by the degeneration and loss of α-motoneurons resulting in progressive muscle atrophy, paralysis and death by respiratory distress (reviewed in Rossoll, Bassell 2009). It is caused by a disruption in the survival of motor neurons (*Smn1*) gene, which leads to the expression of a truncated and unstable form of the SMN protein (Rossoll, Bassell 2009). Since PRMT8 seems to be a predominant PRMT in neurons, it was tempting to speculate that it may be an important regulator of SMN in this cell type. SMN proteins contain a Tudor domain and as described in section 1.3.1., this small conserved amino acid motif serves to mediate intermolecular protein interactions with methylarginine-containing protein (Cote, Richard 2005, Goulet et al. 2008). Thus, we decided to use the Tudor domain of SMN to compare the pattern of its binding proteins once again using the P19-pGIPZ and P19-shPRMT8 cell lines, to verify if its interactions would be altered in the absence of PRMT8. A pull-down experiment with GST-SMN-Tdr recombinant proteins bound to GST sepharose was performed in parallel with protein lysates of both cell lines. Interestingly, western blot analyses revealed that the interaction with SMN-Tdr decreases for EWS and FUS proteins when PRMT8 is knocked down, while a small enhancement of the binding is observed for DDX3 and no effect is detected with hnRNP K (Figure 13A). β-actin was used as a loading control, GST served as a negative control for interaction. Western blot against GST confirmed the use of an equivalent amount of GST proteins for pull-down (Figure 13B). It was interesting to note differences in the alteration of the affinity of tested proteins with SMN-Tdr when PRMT8 is knocked down. These results indicated a potential implication of PRMT8 as a regulator of SMN function in neuronal cells.

A


Lysate
GST
GST-SMN-Tdr
Lysate
GST
GST-SMN-Tdr
α-EWS
α-DDX3
α-FUS
α-hnRNP-K
α-β–actin
(~90 kDa)
(~75 kDa)
(~70 kDa)
(~60 kDa)
(~42 kDa)
Mock
PRMT8 k/d

B

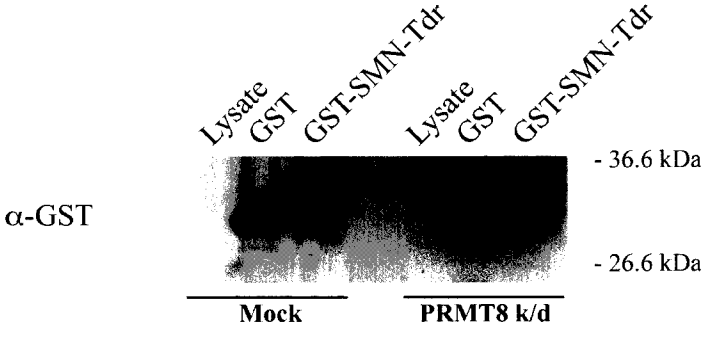

Lysate
GST
GST-SMN-Tdr
Lysate
GST
GST-SMN-Tdr
α-GST
- 36.6 kDa
- 26.6 kDa
Mock
PRMT8 k/d

Figure 13: Alteration of SMN-Tudor domain interactions in the absence of PRMT8. The tudor domain of SMN is known to interact with other protein domains containing dimethylarginines. GST and GST-SMN-Tdr recombinant proteins were expressed using a bacterial system and bound to GST sepharose. P19-pGIPZ (mock) and P19-shPRMT8 (k/d) cell lysates were incubated with GST or GST-SMN-Tdr bound to sepharose in a pull-down experiment and proteins were analyzed by western blot. A) The interaction with SMN-Tdr decreases for EWS and FUS proteins when PRMT8 is knocked down, while an induction of the binding is observed for DDX3 and no effect is detected with hnRNP K. β-actin was used as a loading control. B) Western blot against GST confirmed the use of equivalent amount of GST proteins for pull-down.

4. DISCUSSION

4.1. PRMT8 IN MOUSE TISSUES

From the outset, PRMT8 was believed to be a neuron-specific enzyme, due to the largely brain-specific expression of PRMT8 mRNA reported by Lee and colleagues in 2005, using Northern analysis. To confirm the tissue distribution of PRMT8, we performed semi-quantitative RT-PCR analysis on various adult mouse tissues and, more specifically, demonstrated that PRMT8 is a CNS transcript expressed in the brain, brain stem, cervical and lumbar segments of the spinal cord. These results were independantly confirmed by Taneda and colleagues who performed *in situ* hybridization histochemistry on central sagittal sections obtained from E16.5 mice and noted exclusive expression in the brain and spinal cord. We then had PRMT8 polyclonal antibodies generated in order to demonstrate the presence of stably translated endogenous PRMT8 proteins. In association with Cedarlane Laboratories, GST-N-terminal PRMT8 recombinant proteins were used to inoculate two rabbits (424 and 728). When comparing their antibody production by western blot, rabbit 424 showed the strongest immune response to PRMT8 and very little background to GST tag recognition. Thus, its serum was used to purify specific PRMT8 antibodies. The estimated size of full-length PRMT8 protein is 43 kDa, according to its amino acid composition. A single band at ~48 kDa was detected in mouse brain, brain stem and spinal cord, while no signal was detected in other tested tissues (heart, kidney, liver and skeletal muscle), which validated PRMT8 as a specific CNS protein. RT-PCR and western blot analyses indicated that PRMT8 expression seems to be highly regulated in mouse tissues, since there is either a strong detection or a complete absence of signal. A difference of approximately 5 kDa between the estimated

and observed size of PRMT8 could be the result of a combination of post-translational modifications such as myristoylation, phosphorylation and methylation that could slow enzyme migration in the gel. Very recently, Kousaka et al. published their results on endogenous PRMT8 in mouse central nervous system and the PRMT8 antibodies that they generated recognized a 42 kDa protein in brain extract. This discrepancy between the apparent sizes of PRMT8 on SDS-PAGE might simply be due to the use of different molecular weight markers. Protein standards contain dye to allow visualization of proteins during gel migration and these dyes can affect the migration of standard proteins. Importantly, they also verified PRMT8 protein expression in a number of different mouse tissues and, similarly to us, they only detected expression in the brain and spinal cord (Kousaka et al. 2009). However, while we observed a strong and relatively similar level of protein expression between brain and spinal cord, they observed a rather poor signal in the spinal cord compared to brain signal. Moreover, their spinal cord signal is barely more than background in liver or pancreas samples. However, the mouse strain they used to collect tissues is not specified and we cannot exclude variation of expression level from one mouse strain to another. To better characterize our PRMT8 antibodies, we tested their affinity to immunoprecipitate PRMT8. First, hexahistidine-tagged PRMT8 proteins were expressed using TNT® Coupled Rabbit Reticulocyte Lysate System (Promega), an *in vitro* eukaryotic coupled transcription / translation system, and increasing amounts of our PRMT8 antibody were used to test for its capacity to work in immunoprecipitation experiments. The antibodies seem to only have a weak affinity for native PRMT8-His since an increase of antibody concentration did not result in a significant enrichment of immunoprecipitated proteins. We then used mouse brain lysate

to test the affinity for endogenous PRMT8, but no PRMT8 protein was immunoprecipitated. Since PRMT8 is known to interact with PRMT1 and that PRMT1 forms hexamer (trimer of dimer) to be enzymatically active, endogenous PRMT8 might cluster with a PRMT1 complex in a way that the N-terminal region recognized by PRMT8 antibodies is hidden. This could also be the case to some extent in TNT lysates since PRMT1 is present in these extracts as well. We also tested our antibodies in immunofluorescence microscopy on mouse brain slices, and a speckle-like distribution rather than a membrane signal was obtained. Since our antibodies cannot immunoprecipitate endogenous PRMT8 proteins and only recognize denatured proteins on western blot, we estimate that the signal observed in IF is likely non-specific, and thus did not consider IF as a reliable method to study PRMT8 with our currently available antibodies. As previously mentioned, one of our goals with a PRMT8 antibody was to pursue our protein expression studies by performing immunofluorescence microscopy using various cellular markers for neurons, astrocytes and oligodendrocytes, to confirm the neuro-specificity of PRMT8 proteins. However, in April 2007, Taneda et al. published an extensive analysis of the regional distribution of PRMT8 mRNA in mouse brain using *in situ* hybridization (ISH) histochemistry and the PRMT8 cRNA probe was specifically hybridized within the central nervous system (CNS) and the signals were observed only in neurons (Taneda et al. 2007).

4.2. ONTOGENIC EXPRESSION OF PRMT8

Previous studies on the ontogenic expression of PRMT1 revealed the potential importance of this protein during embryonic development (Ikenaka et al. 2006).

Considering the high level of homology between PRMT1 and PRMT8, we were interested in comparing their ontogenic expression pattern. A western blot analysis on C57Bl/6 mice wt total embryos in early stages of development as well as brain and heart of mice at various stages of development uncovered a specific time-point for onset of PRMT8 expression in mouse brain. While PRMT8 signal was very weak, almost absent, in total embryos and brain sample at E15, the protein was strongly detected at E17 and increased until P21, where the high level of expression was constant in adulthood. Interestingly, the observed increase in PRMT8 expression was opposite to the constant decrease in PRMT1 over the same developmental time-points in mouse brain. Similarly to what had previously been reported, PRMT1 was strongly expressed in mouse brain during prenatal days and this level was maintained until postnatal day 7, then PRMT1 expression dramatically decreases to a low level at postnatal day 28 (Ikenaka et al. 2006). Again, Kousaka et al. performed a similar ontogenic study of PRMT8 in mouse brain. However, they only detected immunoreactivity from P3 to P28 and they did not use any development marker such as PRMT1 to support their results (Kousaka et al. 2009). Could the difference in mouse strains used for the experiments explain this discrepancy? Nevertheless, we can conclude that PRMT1 and PRMT8 both seem to carry on important functions during brain development and does so, at specific but distinct stages. In contrast, low expression of PRMT8 was observed in the early stage of heart development (E15), decreasing sharply below the detection limit if the protein is still present from E17 to P28. With these observations, we can hypothesize that a basal level of PRMT8 would be expressed in cells sustaining characteristics of pluripotent cells and when cell potentiation or differentiation is engaged there would be either up-regulation or inhibition

of PRMT8 expression depending on the cell patterning and/or lineage. However, many questions still need to be elucidated in order to understand the role of PRMT8 as well as PRMT1 at specific stages of development. Other PRMTs have also been identified as key enzymes in neuronal systems. Similarly to PRMT8, the ontogenic expression of PRMT3 in mouse brain revealed that PRMT3 becomes detectable during the perinatal stage (E15), drastically increases to a peak at P14 after which its expression is maintained even in adulthood (Ikenaka et al. 2006). CARM1 is also believed to be an important regulator of neuronal differentiation. HuD is an RNA-binding protein that has been shown to induce neuronal differentiation. It has been demonstrated that arginine methylation of HuD by CARM1 maintains PC12 cells in a proliferative state and that down-regulation of HuD methylation could be the pathway through which nerve growth factor induces neuronal differentiation of these cells (Fujiwara et al. 2006). Moreover, reduced CARM1 levels using RNA interference in MN-1 cells was shown to spontaneously induce neurite outgrowth (Tadesse et al. 2008). Additionally, methylation of histone H3 by CARM1 is required to maintain pluripotency of ES cells. Wu et al. reported that depletion of CARM1 in ES cells down-regulates pluripotency genes and leads to cell differention (Wu et al. 2009). Also, overexpressing CARM1 in ES cells delays their response to differentiation signal (Wu et al. 2009). PRMT1, PRMT3, CARM1 and now PRMT8 seem to be pivotal elements for neuronal differentiation, seemingly acting at distinct stages in this process; further studies will be required to elucidate the precise role(s) that each of these enzyme may play in this molecular mechanism.

4.3. THE P19 CELL LINE, A MODEL SYSTEM TO STUDY PRMT8

PRMT8 is a very unique member of the arginine methyltransferase family since it is the only PRMT known to be localized to the plasma membrane as well as the only one to display a tissue-specific expression pattern as being restricted to neurons of the CNS. PRMT8 was only recently discovered (2005) and very little is known about this enzyme. Moreover, no model system has yet been reported for studying endogenous PRMT8. A mammalian cell line that can be maintained in culture represents a versatile biochemical tool of major importance for understanding molecular mechanism *in vivo* that would be representative of physiological conditions. In addition, since PRMT8 is restricted to the CNS, the purification of native enzymes requires the sacrifice of laboratory animals, unlike other proteins that could be extracted from simple blood cell collections, for example. To address the role of PRMT8 in neuronal cells, we searched for a cell line expressing endogenous PRMT8. The screening of numerous cell lines under various culture conditions led us to identify the P19 mouse embryonal carcinoma cell as a good candidate model for our studies. The P19 line is particularly interesting due to its capacity to simulate the developmental decisions of stem cells to differentiate either into neuronal and glial-like cells (RA treatment) or cardiac and skeletal muscle-like elements (DMSO treatment). Therefore, we wanted to verify if the ontogenic expression profile of PRMT8 in mouse brain and heart could be recapitulated in differentiating P19 cells.

4.4. PRMT8 EXPRESSION IS REGULATED THROUGHOUT DIFFERENTIATION OF P19 CELLS

Semi-quantitative RT-PCR analyses of P19 cells throughout neuronal differentiation showed a general increase of PRMT8 transcript from Day 3 to 8, but a

very high signal on Days 5 to 7. The fold increase of PRMT8 transcript can hardly be quantified since the PCR conditions were clearly not in the linear range of amplification. In fact, the expression of PRMT8 mRNA at Day 0 is very low; 35 cycles of PCR amplification were required to visualize this band. Real-time PCR would have been a more accurate method for quantitatively comparing the relative level of PRMT8 transcript from one differentiation day to another. However, these experiments were performed while the real-time PCR technique was not easily available and mastered in our laboratory. Nonetheless, we still verified whether the high increase of PRMT8 mRNA would be correlated by an induction of protein expression. Western blot analysis revealed PRMT8 proteins at the limit of detection from Days 0 to 3. An induction of PRMT8 protein expression is observed on Day 4, followed on Day 5 by an up-regulation of the signal, which then remains elevated at a steady intensity until the end of the differentiation time course. From these results, a discrepancy between mRNA and protein levels obtained on Days 5 to 7 can be noted. This could be the result of translational regulation and/or of elevated enzyme turnover leading to rapid PRMT8 degradation at a time where its enzymatic activity would be highly required and thus compensated by the increase of its transcription rate. Mash-1 transcript was used as a neuronal marker and confirmed that PRMT8 elevation is linked with efficient differentiation of P19 cells into neurons. The increase of PRMT8 mRNA at Day 5 and the elevated PRMT8 protein level from Day 5 to 9 of neuronal differentiation coincides with the replating of cells in adherent cultures and extension of major neuronal processes. Considering that PRMT8 is localized at the plasma membrane, we can hypothesize that PRMT8 could act in concert with adhesion molecules to promote neurite outgrowth. These results also suggest that

PRMT8 would be required at a specific time-point in differentiation. Up-regulation of Mash-1 neuronal marker precedes the increase of PRMT8 transcript, these events do not occur simultaneously, meaning that PRMT8 is required and thus stimulated at a later phase of differentiation. Interestingly, this is further supported by the fact that we can observe an activation switch of PRMT8 protein during P19 neuronal differentiation in a similar manner to what we observed during mouse brain development.

We then compared the results obtained for the neuronal differentiation of the P19 cell line with the effect of the induction of the cardiac/muscular lineage of these cells. Again, similarly to the mouse heart ontogenic development, there is a loss of PRMT8 expression early after the beginning of DMSO treatment. Positive staining of DMSO-treated P19 cell with a muscular marker, myosin heavy chain, demonstrated that a fraction of the cell population did achieve differentiation. As previously reported, only a small fraction of the cell population usually achieves cardiac or skeletal muscle differentiation. Nevertheless, complete loss of the PRMT8 transcript was observed in the whole cell population upon DMSO treatment, meaning that P19 cells are able to survive with a low or absent expression of PRMT8. Moreover, loss of PRMT8 seems to be an early event in the muscular differentiation program. These observations suggested a potential implication of PRMT8 for either neuronal differentiation or maintenance of neuronal characteristics in CNS-like systems. Thus, we have demonstrated that the P19 cell line is a good mammalian model system for studying endogenous PRMT8 due to its remarkable correlation with the ontogenic expression of PRMT8 in mouse brain and heart. However, the exact function and mechanism of action of PRMT8 in the process

remain unknown. Future identification of PRMT8 interactors and substrates may give us more insight on the specific role of PRMT8 in P19 cells.

4.5. PRMT8 EXPRESSION IS REQUIRED FOR P19 CELLS TO ACHIEVE NEURONAL DIFFERENTIATION

We have demonstrated up-regulation of PRMT8 during neuronal differentiation of P19 cells and loss of expression during cardiac/muscular lineage of this same cell line. Since PRMT8 does not seem to be essential for cell survival, we investigated whether this enzyme is mandatory to achieve neuronal differentiation. Using a powerful RNAi system designed by Open Biosystem, we generated a stable cell line that has PRMT8 knocked down (P19-shPRMT8) as well as a control cell line that is resistant to puromycin, the selection marker (P19-pGIPZ). Both cell lines were treated with RA or DMSO and their phenotypes were observed. Just like parental cells, P19-pGIPZ control cells extended neuronal processes upon RA treatment and showed few positive cells to myosin heavy chain following DMSO treatment. In contrast, few P19-shPRMT8 knocked down cells survived replating and none showed neuronal extensions. P19-shPRMT8 cells proliferate in a manner similar to parental cells when grown as a monolayer. However, the induction of neuronal differentiation with RA leads to the formation of aggregates that contain a high proportion of dying cells by Day 4. The arrangement in embryoid bodies is essential for P19 to achieve differentiation and the complete disruption of these cell aggregates would interrupt the differentiation program. Thus, we could not easily evaluate the percentage of cell death by counting positive cells to trypan blue. However, a large fraction of replated P19-shPRMT8 cells did not reattach to the plate and those that

did reattach, kept a round shape and were all dead by Day 8. It would be informative in future experiments to determine if these cells die by apoptosis using e.g. annexin V or TUNEL staining. Nevertheless, these results indicate that PRMT8 expression is required to achieve neuronal differentiation in a CNS-like system. Moreover, no cell death was observed in the DMSO-treated P19-shPRMT8 cell line even though cells still formed embryoid bodies during four days before replating. This suggests that the key signal is likely to be the RA treatment and induction of that transcriptional pathway. Even though no apparent cell death was observed, cardiac or muscle differentiation could not be confirmed in DMSO-treated P19-shPRMT8 cells, since no cell was positive for the staining of MHC in three independent experiments. The yield of cardiac or skeletal myocytes differentiation is known to vary greatly from one experiment to another and usually ranges from 5% to 25% of total cells. The serum employed has a significant influence on the differentiation process of P19 cells by providing essential carrier proteins, attachment and spreading factors, low molecular weight nutrients, hormones and growth factors, but its composition can differ from one batch to another. Even with parental P19 cells, DMSO treatment constantly resulted in a low yield of myocyte formation. In contrast, neuronal differentiation always ended with a large majority of cells extending neurites. Perhaps the presence of puromycin interferes with myocytes differentiation even though it was withdrawn after replating. We have noted the inhibitory effect of puromycin on neurite outgrowth if present after replating; however, the inhibition could occur in earlier events of the myocyte formation and P19-pGIPZ cells showed a low yield of muscular cells as well. Nevertheless, we can also suppose that PRMT8 is required in P19 cells to maintain pluripotent characteristics necessary in

early stage of myocyte formation. This idea can be further supported by the mouse model. Mouse heart at E15 still expresses PRMT8 proteins while the signal disappears by E17.

4.6. Potential role of PRMT8 post-translational modifications

In this section, we want to discuss preliminary data that was collected to uncover mechanisms that could participate in the regulation of PRMT8 activity. First, we scrutinized the sequence of human PRMT8 protein using a computerized analysis tool. With the NetPhos 2.0 search algorithm (Blom, Gammeltoft & Brunak 1999, Jensen et al. 2002), a total of eight potential phosphorylation sites with a score higher than 0.95 were identified. Protein phosphorylation is recognized as the most widespread type of post-translational modification used in signal transduction and regulation of protein function (Ubersax, Ferrell 2007). Moreover, there seems to be a relation between protein phosphorylation and myristoylation, a post-translational modification identified on PRMT8. N-myristoylation promotes weak protein-membrane interaction that is reversible (Farazi, Waksman & Gordon 2001). Usually, electrostatic interactions between positively charged protein side chains and negatively charged membrane phospholipids act in concert with the hydrophobic myristate group to anchor proteins at the membrane (Farazi, Waksman & Gordon 2001, Swierczynski, Blackshear 1996). If one of these two weak biophysical interactions is disrupted, the protein's affinity for the membrane decreases and a significant proportion can be released into the cytosol (Swierczynski, Blackshear 1996). Interestingly, it has been demonstrated that phosphorylation of residues (or residues in the vicinity) implicated in the electrostatic interaction can alter

membrane association. For example, the activation of PKC in various systems leads to phosphorylation of MARCKS and its reversible translocation from the plasma membrane to the cytosol (Thelen et al. 1991). Interestingly, two potentially phosphorylated serines residues are located in the N-terminal region of PRMT8 (Ser23 and Ser28). Thus, PRMT8 may translocate as well from the plasma membrane to the cytosol, where it could participate in signaling cascades implicated in neuronal differentiation. Further investigation would be required to verify this hypothesis. For example, the analysis of subcellular localization of PRMT8 by western blot analysis on cellular protein fractionation throughout neuronal differentiation of P19 would be informative, but an immunofluorescence experiment would provide a greater perspective and this would require a PRMT8 antibody working in IF. In addition, the identification of specific PRMT8-interacting protein could help elucidating the molecular function of PRMT8.

In 2007, Sayegh and collaborators demonstrated the regulation of PRMT8 activity by its N-terminal domain. Like us, they identified two proline-rich sequences on PRMT8 that bind SH3 domain-containing proteins. They performed a similar SH3 domain pull-down experiment as us, using GST-SH3 fusion proteins with PRMT8-GFP. While we did not observe any positive interaction, they showed strong binding to the SH3 domains of Fyn and PRMT2, as well as weak binding to domains of PLCγ and p85 (Sayegh et al. 2007). We performed the experiment by using GST-SH3 proteins bound to GST sepharose and incubated them with 293T cell extracts transfected with PRMT8-GFP. On their side, they followed the same method, but transfected Hela cells with a mutated form of PRMT8 (G2A) that cannot be myristoylated. Perhaps the folding of the myristoylated protein does not render N-terminal sites available for interaction with SH3 domains

unless specific activation or modification of the protein occur and that would explain why our results were not conclusive, while removing myristoyl tail of hydrophobic carbons resulted in positive interactions. Even though they showed binding of some SH3 domains to PRMT8, interaction of the SH3 containing full-length proteins *in vivo*, still need to be demonstrated. This group also used recombinant full-length and N-terminal truncated forms of PRMT8 proteins, the latter missing the first 60 amino acids of full-length PRMT8. First, they observed that PRMT8 has a much lower methylation activity towards GST-GAR and Myelin Basic Protein (MBP) than PRMT1 (Sayegh et al. 2007). Second, they showed that both His-tagged and GST-fusion PRMT8 lacking the initial 60 amino acid residues have enhanced *in vitro* enzymatic activity versus the full-length recombinant protein (Sayegh et al. 2007). They then confirmed the regulation effect of the N-terminal domain through limited proteolysis by trypsin digestion and detected an increase of methyltransferase activity of the digested full-length PRMT8, consistent with the loss of the N-terminal domain (Sayegh et al. 2007). Finally, they demonstrated automethylation activity of PRMT8 at sites localized in and near the N-terminal domain, without determining the effect of this methylation reaction on the activity of PRMT8 (Sayegh et al. 2007). Intriguingly, it has been demonstrated that protein arginine methylation antagonized phosphorylation and vice versa (Hsu et al. 2005, Ostareck-Lederer et al. 2006). Thus, phosphorylation and arginine methylation could influence PRMT8's role in kinase-dependent signaling, as suggested by Pahlich in 2008, but it could regulate as well its anchoring at the plasma membrane.

4.7. IDENTIFICATION OF PROTEINS INTERACTING WITH PRMT8

In our search for PRMT8 interactors/substrates, we performed coimmunoprecipitation experiments in order to confirm the interaction of PRMT8 with specific proteins that were first identified by Pahlich et al. in a GST-PRMT8 pull-down experiment. However, we did not coimmunoprecipitate EWS, DDX3, FUS, EFIα or PRMT2 with recombinant T7-PRMT8 transiently transfected in P19 cells. Most tested proteins are RNA-binding proteins, but it had been demonstrated that, in neurons, mRNA is transported to dendrites in granules containing, among others, EWS and FUS proteins (Pahlich, Zakaryan & Gehring 2008). Thus, we thought that there could be a correlation between mRNA transport in neuron dendrites and PRMT8, a neuron-specific enzyme. Only PRMT1, a known PRMT8-binding protein, was immunoprecipitated. We could not confirm any PRMT8-interacting protein, but the efficiency of our immunoprecipitation method was confirmed by the presence of PRMT1. Also, a recent study combining quantitative SILAC-based mass spectrometry and bead proteomes, identified a number of proteins binding unspecifically to the most commonly used affinity matrices, such as GST sepharose (Trinkle-Mulcahy et al. 2008). Interestingly, the majority of PRMT8-binding interactors identified by Pahlich et al., using mass spectrometry, are among the contaminant proteins listed by Trinkle-Mulcahy et al. These results could also explain why we were not able to confirm any PRMT8 interactor.

In contrast with our results, two different groups reported the interaction of PRMT8 with the Ewing sarcoma (EWS) RNA-binding protein (Pahlich, Zakaryan & Gehring 2008, Kim et al. 2008). The reaction conditions described in both papers differ significantly from ours and they might not have been stringent enough, which could have

resulted in artefactual interactions. Specifically, the pull-down experiment followed by mass spectrometry done by Pahlich et al. was performed using a very high concentration of purified GST-PRMT8 proteins (10μg) as well as a lysis buffer with low detergent (0.1% Triton X-100) and a minimum amount of salt (100 mM NaCl) (Pahlich, Zakaryan & Gehring 2008). On the contrary, we consider that immunoprecipitation of T7-PRMT8 from transfected cells reflects more physiological conditions and that 1% Triton X-100 and 150 mM of NaCl in the lysis buffer are more appropriate to prevent unspecific interactions. On their side, Kim et al. did pull-down and immunoprecipitation experiments using both PRMT8 and EWS tagged proteins. For example, coimmunoprecipitation was performed on whole cell extracts from HEK293T cells that had been co-transfected with HA-PRMT8 and Flag-tagged EWS. Overexpression of both proteins may have resulted in various artefactual behavior such as important alteration of the expression levels and protein modifications as well as mislocalization due to the saturation of some post-translational machinery (Marks et al. 1996). Moreover, EWS can be phosphorylated in cells and this modification regulates transcriptional activity (Olsen, Hinrichs 2001). Both phosphorylation and methylation states of a protein can influence protein-protein interactions; thus, simultaneous overexpression of tagged PRMT8 and EWS could have led to a degree of affinity that is unrepresentative of physiological conditions due to incomplete protein processing through the post-translational machinery. Moreover, these two groups reported interaction of EWS with PRMT8 without exactly agreeing on its capacity to act as a substrate for this methyltransferase. Using *in vitro* methylation assay, Pahlich et al. observed a rather poor arginine methyltransferase activity of PRMT8 towards EWS in comparison to PRMT1. In contrast, Kim and

colleagues suggested, using a similar *in vitro* assay, that EWS is a substrate for PRMT8, as efficient as for PRMT1. Furthermore, it has been demonstrated that EWS binds to PRMT8 independently of its methylation state; the interaction is maintained even when EWS is completely methylated (Pahlich, Zakaryan & Gehring 2008). This can be surprising since PRMTs are more likely to have less affinity for their substrates once these are methylated, in order to favour enzyme turnover (Herrmann et al. 2005). Both manuscripts report the use of GST-PRMT1 and GST-PRMT8 to perform *in vitro* methylation assay towards GST-EWS full-length or truncated proteins, but their results do not correlate. At this point, there is no strong evidence to invalidate our coimmunoprecipitation results which did not confirm other PRMT8-interactor than endogenous PRMT1. One hypothesis that has been proposed by the group of Pahlich and Gehring is that PRMT8 could act as an adaptor to direct specific proteins to the plasma membrane when they lack membrane crossing helices or other post-translational modifications usually required for membrane targeting (Pahlich, Zakaryan & Gehring 2008). However, if there is a shuttling of PRMT8 between the membrane and cytosol or even the nucleus, the role of adaptor protein to the plasma membrane could be limited to particular cellular states. Since then, we have identified the P19 cells as a mammalian system where PRMT8 is strongly induced during neuronal differentiation. Moreover, PRMT8 seems to have an essential function in this process. Thus, a future step in the search for PRMT8-binding partners could be to perform coimmunoprecipitation experiments using whole cell extracts from differentiated neuronal P19 cells and perform mass spectrometric analysis.

4.8. CONTRIBUTION OF PRMT8 ACTIVITY TO OVERALL ASYMMETRIC DIMETHYLATION IN NEURONAL CELLS

Most of the known methylated proteins are substrates of PRMT1, which is the predominant type I PRMT in mammalian cells, accounting for ~85% of cellular PRMT activity, but we should consider the fact that other arginine methyltransferases are less well characterized and that this estimate was made using crude biochemical assays at a time when few PRMTs were identified (Boisvert, Chenard & Richard 2005, Pahlich, Zakaryan & Gehring 2006). Here we have reported the antagonistic ontogenic expression of PRMT1 versus PRMT8 and have established a parallel between the increases of PRMT8 proteins in developing mouse brain and in P19 neuronal differentiation. Hence, we wanted to verify whether in a CNS-like cellular model expressing PRMT8, less overall type I methyltransferase activity would be attributed to PRMT1 and a larger proportion would be of PRMT8 contribution. We have generated a P19 cell line derivative that stably expresses a shRNA targeting PRMT8 mRNA which reduces its expression below detection level (Figure 9A). This cell line was used to study the pattern of *in vivo* methylated proteins. As previously described, we performed *in vivo* methylation assays to radiolabel methylated proteins using L-[methyl-^{3}H]-methionine in the presence of translation inhibitors (method adapted from Desrosiers, Tanguay 1988) on P19-pGIPZ (mock) and P19-shPRMT8 (PRMT8 knocked down) cell lines, and then resolved proteins by SDS-PAGE before visualizing bands by fluorography. Our goal was to detect either significant reduction or even disappearance of methylation signal on specific protein bands in P19-shPRMT8 cells versus mock (P19-pGIPZ). This would have revealed the importance PRMT8 methylation activity in P19 cells and indicated the

existence of unique PRMT8 substrates. When comparing the pattern of methylated proteins of both cell lysates, we noticed only a slight reduction of the signal intensity between 20 kDa and 30 kDa in the absence of PRMT8, but no band was completely decreased. Since we were comparing total cell lysates, which means that each band visualized probably contained a number of different proteins and that a single protein can be the substrate of more than one PRMT, we could not evaluate the importance of PRMT8 activity. Thus, we sought more specificity of the analysis by immunoprecipitating a subset of proteins containing aDMA and comparing the protein pattern in both P19-pGIPZ and P19-shPRMT8 cell lines. Again no conclusive result was obtained since both cell lines displayed a very similar banding pattern, with a small reduction in the signal for P19-shPRMT8 cells. However, we should also consider the fact that the aDMA antibodies used recognize only a subset of protein that harbors a conserved motif or structure around the methylated sites. Also, the fact that the absence of PRMT8 could have been compensated by other important type I PRMTs such as PRMT3 and PRMT6 has not been excluded. It would have been really interesting to perform these same experiments with differentiated P19 cells when PRMT8 is substantially increased, but knocked down PRMT8 cells do not survive the differentiation process.

4.9. PRMT8 MODULATES PROTEIN BINDING TO THE TUDOR DOMAIN OF SMN

The survival of motor neurons protein (SMN) is the product of the *Smn1* gene and a disruption in both alleles of this gene results in proximal spinal muscular atrophy (SMA), a disease characterized by the selective degeneration of α-motoneurons (Tadesse

et al. 2008). Depending on the severity of the disease, clinical characteristics include muscular weakness, muscle atrophy, paralysis and death from respiratory distress (Tadesse et al. 2008). The SMN protein is expressed ubiquitously but, since only α-motoneurons are affected in the disease, one view is that SMN may exert neuro-specific activities.

Specific mRNA binding proteins and accessory factors can recognize *cis*-acting sequences within the 3'-unstranslated region of mRNA and assemble together with translation factors and ribosomal subunits into large messenger ribonucleoprotein (mRNP) complexes, also termed mRNA granules (Kiebler, Bassell 2006, Rossoll, Bassell 2009). These granules can be actively transported along microtubules and several mRNAs are targeted to dendrites and/or axons of neurons where local protein translation can occur (Rossoll, Bassell 2009). This RNA localization mechanism is a key element for neuronal polarity, axon guidance and synaptic plasticity and some impairment of RNA granules have been linked to SMA (Lin, Holt 2008, Rossoll, Bassell 2009). Many years of research on mRNP led to the elaboration of a proposed model in which SMN may facilitate the assembly of RNA cargo molecules with different RNA-binding proteins, adaptor proteins, molecular motor proteins, translational components and auxiliary factors required for efficient transport and/or local translation (Rossoll, Bassell 2009). In addition, SMN contains a Tudor domain, which recognizes methylarginine-containing protein, and it is thought that arginine methylation can regulate SMN activities by modulating its protein-protein interactions (Cote, Richard 2005). In 2006, Elvira and collaborators isolated and analyzed the composition of RNA granules expressed in rat developing brain using a proteomic approach, an automated system by LC Q-TOF

MS/MS (Liquid Chromatography, Accurate-Mass Quadrupole Time-of-Flight, Tandem Mass Spectrometry). Some dimethylarginine-containing proteins were identified in that study and, among them, known SMN interactors were listed (Elvira et al. 2006). Since our results have shown that PRMT8 is an important PRMT for neuronal differentiation, we wanted to assess whether it could contribute to the regulation of SMN in neuronal cells. According to antibody availability, we compared the interaction of SMN-Tdr with various proteins believed to be present in RNA granules, and that had also been identified by others as potential PRMT8 interactors/substrates. We did a pull-down experiment with GST-SMN-Tdr recombinant proteins using again the P19-pGIPZ and P19-shPRMT8 cell lines and compared its affinity for various mRNP proteins in presence of absence of PRMT8. Interestingly, western blot analyses revealed that EWS and FUS have a decreased affinity for SMN-Tdr in PRMT8 knocked down cells. Thus EWS and FUS binding region with SMN-Tdr are most likely hypomethylated in the absence of PRMT8. In contrast, DDX3 shows an induction of SMN-Tdr association potentially accompanied with a higher level of arginine methylation, while interaction with hnRNP K is not altered. These results suggest that PRMT8 may be responsible for methylation of specific RNA-binding proteins in neurons which, as we suspected, could not be detected using the approaches described in the previous section. In addition, PRMT8 might be implicated as a regulatory element of enzymatic activity of other PRMTs since its decrease does not unilaterally result in a reduction of arginine methylation of specific proteins but can also lead to an increase of SMN-Tdr recognition of binding partners. For example, PRMT8 has been demonstrated to form heterodimers with PRMT1 (Lee et al. 2005), however, homodimerization of PRMT1 is known to be essential for AdoMet methyl donor binding

(Zhang, Cheng 2003). We do not know if heterodimerization with PRMT8 still allows for the recruitment of AdoMet by PRMT1, but preventing the binding of the methyl donor cofactor in arginine methylation could be a way for PRMT8 to negatively regulate PRMT1 activity and explain the results observed in the case of DDX3.

5. CONCLUSION

Our research on PRMT8 suggests an important role for PRMT8 in neuronal differentiation and/or function in the CNS. An ontogenic analysis of PRMT8 in mouse brain revealed that its expression is induced during the perinatal stage and is maintained in adulthood. Interestingly, PRMT1 is expressed during the early embryonic stage then is drastically reduced in adult mouse brain. These results suggest an important role of PRMT1 as well as PRMT8 at specific stages of the development. We first hypothesized an implication for PRMT8 in neuronal differentiation due to its unique tissue-specific expression pattern in the CNS. In this manuscript we demonstrated the requirement of PRMT8 in P19 cells to achieve neuronal processes. Furthermore, we have established the P19 mouse embryonal carcinoma cell line as a good model system to study endogenous PRMT8. This represents a versatile biochemical tool of major importance that will hopefully allow understanding of molecular mechanisms *in vivo* that would be representative of physiological conditions in the CNS, since the exact role, function and regulation mechanism of PRMT8 still remain to be elucidated.

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