uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Lisa Hubers

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)

GRADE / DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Arginine Mehtylation of RNA-Binding Protein HuD CARM1 Regulates MN-1 Differentiation Through a Post-Transcriptional Mechanism

TITRE DE LA THÈSE / TITLE OF THESIS

Jocelyn Côté

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

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

Martin Holcik

Martin Pelchat

Gary W. Slater

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

Arginine methylation of RNA-binding protein HuD by CARM1 regulates MN-1 differentiation through a post-transcriptional mechanism

Lisa Hubers

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for the MSc degree in Cellular and Molecular Medicine
May 14, 2010

Cellular Molecular Medicine
Faculty of Medicine
University of Ottawa

Your file Votre référence
ISBN: 978-0-494-69048-2
Our file Notre référence
ISBN: 978-0-494-69048-2

Abstract

Spinal Muscular Atrophy results from loss of the survival of motor neuron (SMN) gene, leading to a deficiency in SMN protein and selective degeneration of motor neurons. We have found that coactivator-associated arginine methyltransferase 1 (CARM1) regulates the switch from proliferation to differentiation, downstream of neurotrophic signaling, in motor neuron-like cells. Here, through down-regulation of CARM1 levels, methylation of one of its substrates, HuD, is reduced, resulting in an increase in p21 mRNA. Methylation of HuD by CARM1 negatively regulates its direct interaction with p21 mRNA, the first demonstration that CARM1 can directly influence the RNA binding activity of a substrate.

We have also identified a novel interaction between HuD and SMN. In MN-1 differentiation, we propose that SMN functions as an adaptor module through arginine methylation-regulated interactions with RNA binding proteins, including HuD, and that the formation of distinct mRNP complexes on mRNA are regulated either similarly or differentially by PRMTs and/or SMN. These findings may help to elucidate the specific role of arginine methylation and SMN in regulating differentiation of motor neurons and provide crucial insights into the cell-specific pathophysiology of spinal muscular atrophy.

Table of Contents

Abstract....ii

Table of Contents....iii

List of Figures....vii

List of Abbreviations....viii

Acknowledgements....xi

Chapter 1. Introduction....1

1.1 Spinal Muscular Atrophy and the SMN protein....1

1.1.1 Epidemiology and Classification of SMA....1

1.1.2 SMN gene and protein....2

1.1.3 SMN complex: Assembly and Functions....5

1.1.4 SMN protein: The methyl-sensing Tudor domain....10

1.2 Arginine methylation....11

1.2.1 Arginine Methylation and PRMTs....11

1.2.2 Arginine Methylation and Neuronal Differentiation....14

1.2.3 CARM1: Transcriptional and Post-Transcriptional Functions....15

1.3 RNA binding proteins and mRNA stability....17

1.3.1 Role of Hu proteins and mRNA Stability in Neuronal Differentiation....17

1.3.2 CARM1 Methylation of HuD in Neuronal Differentiation....20

1.4 Rationale, Hypothesis and Objectives....23

1.4.1 Role of CARM1 in Motor Neuron Differentiation....23

1.4.1 Hypothesis....24

1.4.3 Specific Objectives....24

Chapter 2. Materials and Methods.....25

2.1 Cell culture and stable knockdown cell lines.....25

2.2 BrdU assays.....26

2.3 Isolation of Proteins, SDS-Page and Western blotting.....27

2.4 RNA Extraction, Reverse Transcription and Quantitative PCR.....28

2.5 Immunofluorescence.....29

2.6 Production of Recombinant Proteins.....30

2.7 Methylation assays.....31

2.8 Generation of a p21 construct and radioactive labeling.....32

2.9 Gel shift assays.....35

2.10 Filter binding assays.....36

2.11 UV crosslinking.....36

2.12 Immunoprecipitation and PCR.....37

2.13 Transcription and Translation (TnT) Reactions.....38

2.14 GST pulldowns and RNase treatment.....38

2.15 Site-Directed Mutagenesis.....39

2.15 Generation of an shRNA against the HuD 3'UTR.....40

Chapter 3. Results.....41

3.1 The CARM1 pathway involving HuD is active in regulating differentiation of MN-1 cells.....41

3.1.1 MN-1 cells as a model system to study the role of CARM1 in motor neuron differentiation.....41

3.1.2 CARM1 is downregulated during MN-1 differentiation.....46

3.1.3 Localization of CARM1.....48

3.1.4 Methylation of CARM1 substrates is decreased in MN-1 differentiation...48

3.1.5 Overexpression of HuD may be sufficient to promote MN-1 differentiation.....52

3.2 The pathway of HuD contribution to MN-1 differentiation downstream of CARM1.....54

3.2.1 Methylation status of HuD does not appear to affect its turnover.....54

3.2.2 CARM1 methylation of HuD reduces its direct binding to p21 mRNA 3'UTR.....55

3.3 Involvement of SMN in the pathway of MN-1 differentiation.....60

3.3.1 The role of SMN in MN-1 differentiation and neurite extension.....60

3.3.2 HuD can interact with the Tudor domain of SMN.....61

3.3.3 The interaction between HuD and the SMN tudor domain is methylation-dependent and RNA-mediated.....63

Chapter 4. Discussion.....68

4.1 Summary.....68

4.2 The CARM1 pathway involving HuD and p21 mRNA is active in regulating differentiation of MN-1 cells.....69

4.3 Regulation of CARM1 in MN-1 differentiation.....70

4.4 CARM1 regulation of HuD function in MN-1 differentiation.....73

4.5 Role of HuD in MN-1 differentiation downstream of CARM1.....76

4.6 Future Work: Determination of the pathway of HuD contribution to differentiation downstream of CARM1.....79

4.7 The potential function of SMN in the MN-1 differentiation pathway.....80

4.8 Relevance in the etiology of SMA.....86

4.9 Conclusion.....88

References.....89

Appendix 1. Supplementary Data.....103

Figure 15. Immunofluorescence of differentiation markers in MN-1 cells.....103

Figure 16. Additional attempts to determine an interaction of p21 mRNA with HuD in MN-1 cells and its regulation by CARM1.....104

Figure 17. The interaction of hnRNP K with HuD and p21 mRNA.....105

List of Figures

Figure 1. Spinal muscular atrophy.....4

Figure 2. The role of the SMN complex and arginine methylation in the snRNP biogenesis pathway.....6

Figure 3. Arginine methylation and PRMTs.....12

Figure 4. Methylation of HuD by CARM1 regulates proliferation of neuronal cells.....22

Figure 5. Optimization of MN-1 differentiation protocols.....42

Figure 6. CARM1 is required to maintain MN-1 cells in a proliferative state.....44

Figure 7. Protein and RNA analysis of CARM1 and HuD in MN-1 differentiation.....47

Figure 8. Immunofluorescence of HuD and CARM1 in MN-1 differentiation.....49

Figure 9. Methylation of HuD by CARM1 decreases in differentiation.....51

Figure 10. Overexpression of HuD in MN-1 cells may induce differentiation.....53

Figure 11. HuD has a decreased affinity to directly bind p21 mRNA when methylated *in vitro*, and HuD interacts with p21 in MN-1 cells.....57

Figure 12. SMN is involved in regulating MN-1 differentiation and neurite extension.....62

Figure 13. HuD interacts with the SMN tudor domain.....64

Figure 14. The SMN tudor domain interacts with HuD in a methylation-dependent, RNA-dependent manner.....66

Figure 15. Immunofluorescence of differentiation markers in MN-1 cells.....103

Figure 16. Additional attempts to determine an interaction of p21 mRNA with HuD in MN-1 cells and its regulation by CARM1.....104

Figure 17. The interaction of hnRNP K with HuD and p21 mRNA.....105

List of Abbreviations

^{3}H-SAM - S-[methyl-^{3}H]-adenosyl-L-methionine

AChE - Acetylcholinesterase

aDMA - asymmetrical dimethylated arginine ω-N^{G},N'^{G}

AdoMet (SAM) - S-adenosyl-L-methionine

ARE - AU-rich RNA element

ATP - Adenosine triphosphate

BSA - Bovine serum albumin

BrdU - Bromodeoxyuridine

CARM1 - Coactivator-associated arginine methyltransferase 1

CB - Cajal bodies

cDNA - complementary Deoxyribonucleic acid

ChAT - Choline acetyltransferase

DMEM - Dulbecco's modified Eagle's medium

DTT - Dithiothreitol

FBS - Fetal bovine serum

GAP-43 - Growth associated protein 43

GAPDH - Glycerol-3-phosphate dehydrogenase

GAR - glycine/arginine-rich

GDNF - Glial cell-derived neurotrophic factor

GST - Glutathione S-transferase

H3R17 - Dimethyl-histoneH3-arginine17

HB9 - Homeobox 9

hnRNP - heterogeneous nuclear Ribonucleoprotein particle

HRP - Horseradish peroxidase

JMJD6 - Jumonji domain-containing 6 protein

KSRP - KH-type splicing regulatory protein

MN-1 - motor neuron-like

mRNA - messenger Ribonucleic acid

mRNP - messenger Ribonucleoprotein particle

NGF - Nerve growth factor

NMJ - Neuromuscular junction

OD - Optical density

PBS - Phosphate buffered saline

PBS-T - Phosphate buffered saline and tween

PCR - Polymerase chain reaction

PEI - Polyethyleneimine

PGM - proline/gylcine/methionine-rich

PMSF - Phenylmethanesulphonylfluoride

PRMT - Protein arginine methyltransferase

PVDF - Polyvinylidene difluoride

qPCR - quantitative Polymerase chain reaction

RBP - RNA-binding protein

RNA - Ribonucleic acid

RNP - Ribonucleoprotein particle

RRM - RNA recognition motif

RT-PCR - Reverse transcriptase polymerase chain reaction

sDMA - symmetrical dimethylated arginine ω-N^G,N^G

SDS - Sodium dodecyl sulphate

SDS-PAGE - Sodium dodecyl sulphate - polyacrylamide gel

SMA - Spinal muscular atrophy

Smn - Survival of motor neuron gene

SMN - Survival of Motor Neuron protein

SMNΔEx7 - Survival of Motor Neuron protein without exon 7

snRNP - small nuclear Ribonucleoprotein particle

TnT - Transcription and Translation

U snRNA - Uridine-rich small nuclear RNA

UTR - Untranslated region

Acknowledgements

I would like to first thank my supervisor, Dr. Jocelyn Cote, for allowing me to have this wonderful learning experience. You have been a truly great mentor; your guidance and encouragement was both inspirational and invaluable throughout this project. I would also like to express my gratitude to my advisory committee members, Dr. Steffany Bennett and Dr. Rashmi Kothary, for their scientific contributions.

I would like to thank my lab colleagues, with whom I have had the privilege of working. Dr. G. Sanchez, Dr. H. Valderrama, H. Tadesse, I. Goulet, A. Morettin, M.E. Pelletier and B. Paquette, as well as former coworkers, S. Boisvenue and Dr. J. Deschênes-Furry, for their support, assistance and humor.

I am extremely appreciative of family and friends (Z. Tasovac, M. Favretto, M. Demers, H. Auld, and R. Lau) for their ongoing patience and support; thank you for being there for me.

Chapter 1. Introduction

1.1 Spinal Muscular Atrophy and the SMN protein

1.1.1 Epidemiology and Classification of SMA

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease and is distinguished by the selective degeneration of lower motor neurons of the spinal cord. SMA is the most common genetic cause of infant mortality (Pearn, 1980; Monani, 2005), where the more severe forms of the disease result in death before two years of age. In humans, carrier frequency is 1/35 as determined by genetic testing (Feldkotter et al., 2002; Cusin et al, 2003), and the incidence of the disease is approximately 1 in 6 000 births (Monani, 2005). Pathophysiology of SMA includes progressive weakness and wasting of the proximal voluntary muscles of the limbs, ultimately leading to paralysis, which eventually affects the entire trunk during disease progression (Melki 1997; Briese et al., 2005).

There are five clinical types of SMA as defined by the International SMA Consortium, categorized by motor capabilities and age of onset (Munsat and Davies 1992; Zerres and Rudnik-Schoneborn 1995; Briese et al., 2005, Monani, 2005).

1. Type 0 SMA, with prenatal onset, results in early neonatal death. Decreased fetal movement *in utero* and a small degree of spontaneous ventilation and movement at birth are observed.

2. Type I SMA, or Werdnig-Hoffmann disease, has an onset occurring at less then six months of age, resulting in general muscle weakness. Childen are never able to sit unaided, and have an average lifespan of eight months.

3. Type II SMA is the intermediate classification of SMA. Onset occurs between six and eighteen months; children are capable of sitting independently but not standing or walking, resulting in scoliosis of the spine. Patients usually survive past two years of age.

4. Type III SMA, or Kugelberg-Welander disease, has an onset after eighteen months. Patients are able to stand or walk short distances unassisted, and have a normal lifespan.

5. Type IV SMA, the adult-onset form of the disease, results in only mild muscle weakness. Patients develop first clinical symptoms past the age of 30.

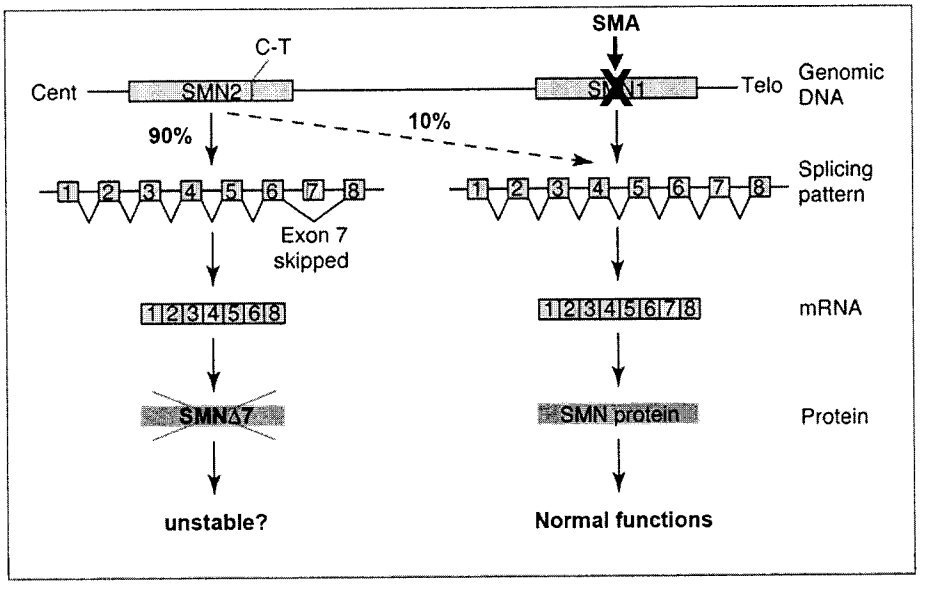
1.1.2 SMN gene and protein

The causative gene for SMA was mapped to a region of chromosome 5q13 in humans, and named the 'survival of motor neuron' (SMN) gene (Lefebvre et al., 1995). This region consists of a large inverted duplication, resulting in both telomeric (SMN1) and centromeric (SMN2) copies of the gene (Lefebvre et al., 1995). SMN1 and SMN2 genes differ by only five nucleotides, the most functionally important being a C to T transition within exon 7 splicing region of SMN2 (Lorson et al., 1999; Kashima and Manley, 2003). These mutations are found in all individuals, and cause a modification in the splicing pattern of SMN2 such that approximately 80% of the transcripts lack exon 7, and consequently produces a truncated, unstable form of the SMN2 protein (SMNΔ7)

that is rapidly degraded (Fig. 1A) (Lorson et al., 1999; Monani et al., 1999; Lorson and Androphy, 2000, Vitte et al., 2007).

The vast majority of SMA cases are attributed to homozygous deletions or loss-of-function mutations in the SMN1 gene (Lefebvre et al., 1995). Here, the SMN2 gene is able to compensate for the embryonic lethality observed in the complete absence of SMN, but somehow can not fully compensate for the function of SMN in motor neurons, and this insufficient expression of the full-length SMN protein ultimately leads to the development of SMA (see schematic in Fig. 1A). The severity of the disease is inversely correlated to the copy number of SMN2 (Coovert et al., 1997; McAndrew et al., 1997; Lefebvre et al., 1997); this gene can be considered a disease modifier due to the low levels of functional SMN protein it is able to generate (Briese et al., 2009). Interestingly, the reduced amount of ubiquitously expressed, functional, full-length SMN protein produced by the SMN2 gene is sufficient for normal activity in all cell types except motor neurons (Monani et al., 2000; Le et al., 2005). It remains unclear how a loss of function of the SMN protein results in a disorder which is selective to motor neurons. It has been proposed that motor neurons may require especially high amounts of SMN protein for proper development and/or function, and are thus particularly vulnerable to decreases in SMN levels (Battaglia et al., 1997; Bechade et al., 1999). In mammalian embryonic and early postnatal development, SMN protein levels and activity are high in the spinal cord, but declines to a steady state that is maintained during lifespan (Gabanella et al., 2005; Jablonka et al., 2000; Battaglia et al., 1997; La Bella et al., 1998). Increased levels of expression of SMN were observed during cell differentiation (Zhang et al., 2003; Burlet et al., 1998; Germain-Desprez et al., 2001; Rouget et al., 2005).

A


SMA
C-T
Cent
SMN2
SMN1
Telo
Genomic DNA
90%
10%
1 2 3 4 5 6 7 8
Exon 7 skipped
Splicing pattern
1 2 3 4 5 6 8
1 2 3 4 5 6 7 8
mRNA
SMNΔ7
SMN protein
Protein
unstable?
Normal functions

B

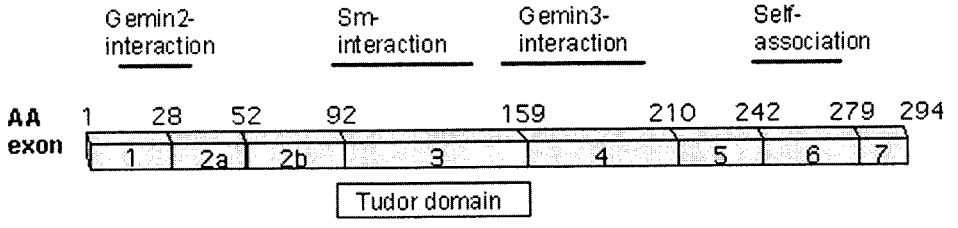

Gemin2-interaction
Sm-interaction
Gemin3-interaction
Self-association
AA
exon
1 28 52 92 159 210 242 279 294
1 2a 2b 3 4 5 6 7
Tudor domain

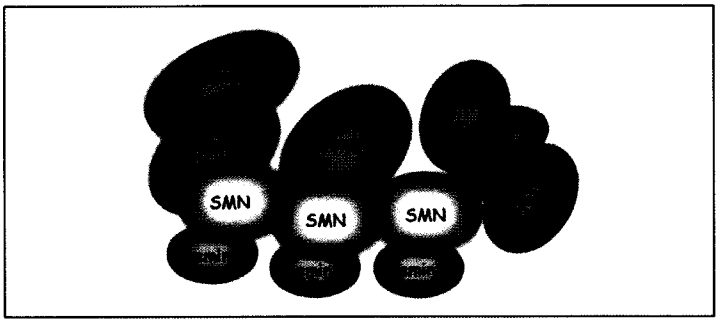
Figure 1. Spinal muscular atrophy. A, The SMN gene in healthy individuals and SMA Type I patients (modified from Khoo et al., 2003). **B,** Representation of the full length SMN protein.

1.1.3 SMN complex: Assembly and Functions

SMN has been best described to form a large macromolecular complex also known as the SMN complex. This complex contains oligomerized SMN protein as well as at least seven additional proteins, called Gemins (Liu and Dreyfuss, 1996; Charroux et al., 1999; Charroux et al., 2000; Baccon et al., 2002; Gubitz et al., 2002; Pellizzoni et al., 2002; Carissimi et al., 2006; Otter et al., 2007) and another named unrip. Together, this complex is known as the 'assemblyosome' (Paushkin et al., 2002), and is found to localize to both cytoplasm and nucleus, where it is accumulated in discrete nuclear bodies named Gems (see Fig. 2 for SMN complex schematic) (Liu and Dreyfuss, 1996).

In the cytoplasm, the SMN complex interacts with a stable, heptameric ring of Sm proteins assembled around a specific site known as the Sm site, which is a highly conserved sequence motif found in spliceosomal uridine-rich small nuclear RNAs (U snRNAs). Here, through the SMN complex, the Sm ring binds a newly exported UsnRNA at the Sm site, and formation of a small nuclear ribonucleoprotein particle (snRNP) occurs in an ATP-dependent manner (Meister et al., 2001; Meister & Fischer., 2002; Pellizzoni et al., 2002). These UsnRNAs contain specific sequence elements and structural features to which the SMN complex can interact with high affinity (Yong et al., 2004). Recently, Gemin5 was identified to be the component of the SMN complex that recognizes a specific structural feature in these snRNAs to mediate the assembly of snRNPs (Otter et al., 2007). Due to its direct interaction with both the Sm proteins and UsnRNAs, the SMN complex is responsible to ensure that Sm cores only assemble onto the proper RNA targets (Pellizzoni et al., 2002; Yong et al., 2004; Golembe et al.,

The SMN complex



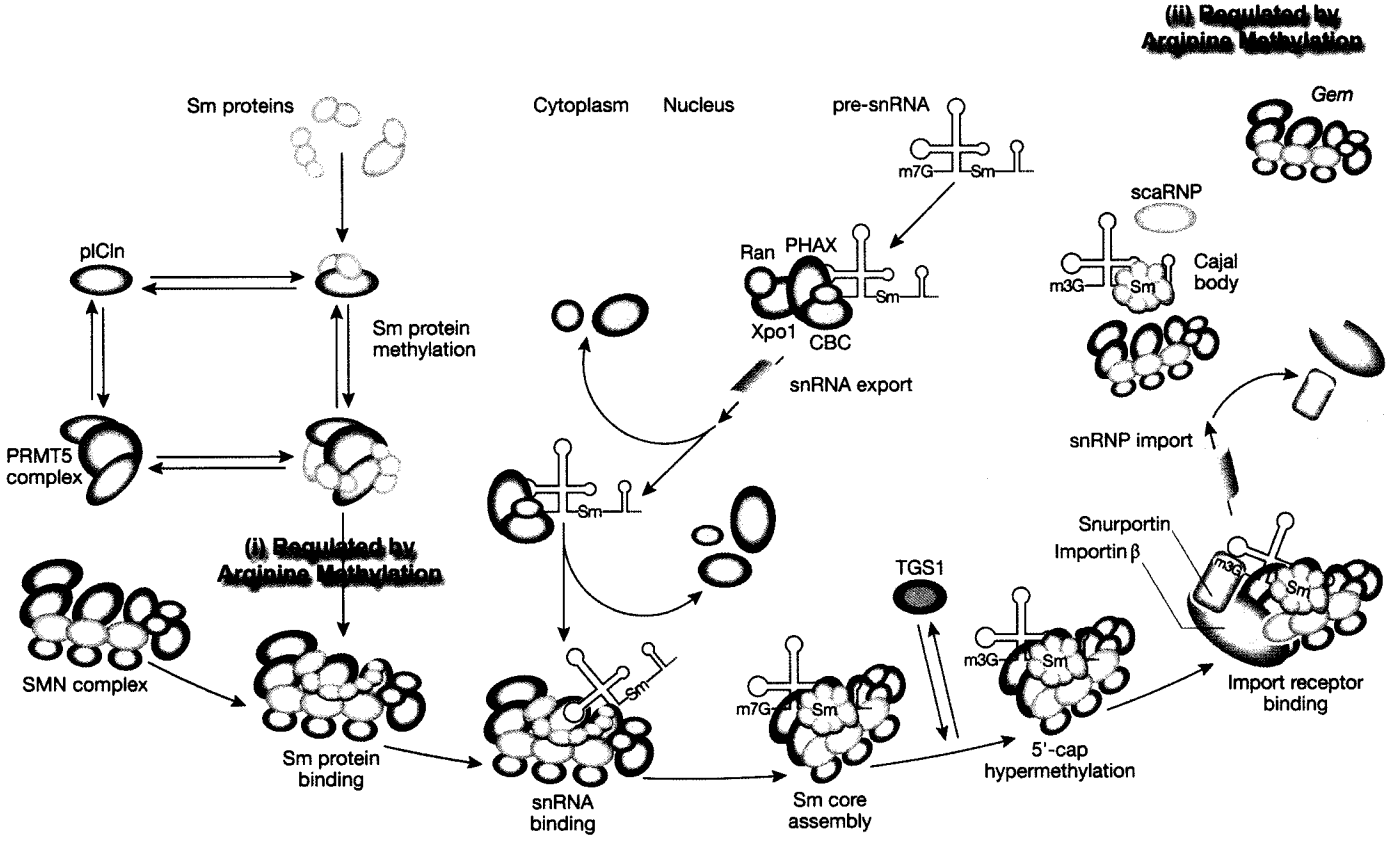


Figure 2. The role of the SMN complex and arginine methylation in the snRNP biogenesis pathway. The uridine-rich small nuclear RNAs, with the exception of U6, are transcribed by RNA polymerase II as precursors in the nucleus. Following export to the cytoplasm, U snRNAs bind to the SMN complex. SmD1, SmD3 and SmB are first methylated by the methylosome in the cytoplasm, which produces symmetrical dimethylarginines and directs them to the SMN complex. The SMN complex brings together the Sm proteins and U snRNAs and promotes proper assembly into an snRNP. Following import into the nucleus, the SMN complex and snRNPs then localize to Gems and/or Cajal bodies, where snRNPs undergo further maturation prior to functioning in pre-messenger RNA splicing (modified from Pellizzoni, *EMBO Rep.*, 2007)

2005a,b). Thus, the SMN complex acts as a chaperone of snRNPs by improving the efficiency and specificity of snRNP assembly, and inhibiting any promiscuous association of Sm proteins with other RNAs. Additionally, the affinity of the Sm proteins for SMN is enhanced by symmetrical dimethylation of some of the Sm proteins, and this modification may be important for snRNP assembly (Meister et al., 2002; Paushkin et al., 2002). This methylation is carried out by the 20S methylosome, which contains PRMT5 (Friesen et al., 2001b). Following properly assembly, the snRNP is then imported into the nucleus and concentrates in Cajal bodies (CB) (Narayanan et al., 2004). Additional snRNP-specific proteins interact with the particle to form a fully functional snRNP (Will and Luhrmann, 2001; Mattaj, 1986; Fischer and Luhrmann, 1990; Fischer et al., 1993; Hamm et al., 1990; Mattaj et al., 1993), which are then able to carry out the process of pre-mRNA splicing.

It has not yet been determined if a causal link exists between SMA pathogenesis and defects in snRNP biogenesis. It may be possible that a decrease in SMN results in defects in proper recruitment of snRNP components for generation of spliceosomal snRNPs (Pellizzoni et al., 1998), leading to improperly spliced mRNAs responsible for encoding key motor neuron-specific proteins. In vitro, Pellizzoni et al. found that wild type SMN had a stimulatory effect on splicing of pre-mRNAs, but mutant forms of SMN containing SMA-causing mutations or deletions did not (Pellizzoni et al., 1998), thus implying a function for SMN in the splicing cycle. Additional support for this possibility derives from the observation that the non-deletion SMN mutations found most commonly in SMA patients decreases the ability of SMN to oligomerize and bind Gemins and Sm proteins (Buhler et al., 1999; Pellizzoni et al., 1999). However, few splicing defects in

pre-mRNAs have been identified in SMA patients or model systems. Recently, defects in splicing have been found in SMA mice (Zhang et al., 2008; Bäumer et al., 2009), although it still remains a question whether these changes are causal or more a consequence of the disease pathology. The discovery of mRNAs whose processing may be distorted in motor neurons would be an essential next step.

A more direct function for SMN in neuronal development, specifically in neurite extension, has also been proposed. As previously mentioned, the expression of SMN was determined to upregulated during differentiation (Zhang et al., 2003; Burlet et al., 1998; Germain-Desprez et al., 2001; Rouget et al., 2005). Using mouse models of SMA, increasing evidence indicates that SMN plays a role in axon outgrowth, guidance and the formation of neuromuscular junctions (NMJs) (Rossoll et al., 2003; Kariya et al., 2008). In both cell culture and in spinal cords of mice, the SMN protein is localized in neurites and growth cones of motor neurons (Jablonka et al., 2001; Fan and Simard, 2002). Interestingly, overexpression of SMN in differentiating PC12 cells resulted in increased neurite outgrowth (Rossoll et al., 2003). Recent studies demonstrate that in neuronal processes, SMN colocalizes with the RNA-binding protein heterogeneous nuclear RNP (hnRNP) R and b-actin mRNA, but not Gemin2 (Jablonka et al., 2001), which as previously discussed is a key component in the assembly of the splicing apparatus. SMN-deficient motor neurons exhibited reduced axon growth; however, cell survival was not affected. This decrease in SMN protein resulted in a mislocalization of b-actin mRNA and protein in both axons and growth cones (Rossoll et al., 2003). Since actin is a key component in outgrowing axons, SMN may function to indirectly regulate the 'local' actin levels, and a deficiency of SMN could decrease the availability of actin monomers

for polymerization, resulting in defects in actin-dependent processes including neurite outgrowth. Thus, SMN may be required for proper assembly and targeting of specific mRNAs to the axonal transport system and their localized translation in motor neurons.

In addition to promoting neurite outgrowth, SMN may also function in regulating the proliferative state of neuronal cells. Mice containing a homozygous deletion of SMN exon 7 in neurons, causing severe motor axonal degeneration, had an increased level of p21 transcript (Olaso et al., 2005). In human SMA patients, increases in p21 mRNA was found in molecular defects that were identical to those observed in mice, though there was not complete overlap in these defects (Olaso et al., 2005). Previously, our group has demonstrated that increased levels of p21 transcript are also present in spinal cord tissues of mice with milder forms of SMA, and that this is due to a prolonged half-life of the mRNA. The p21 protein belongs to the family of cyclin-dependent kinase inhibitors (CDKIs) termed the Cip/Kip proteins. In proliferating cells, cyclin dependent kinases allow the cells to initiate the S phase in G1 as well as mitosis in G2 during the cell cycle. The Cip/Kip proteins are able to inhibit all CDK complexes and can arrest the cell cycle at either phase (Coqueret, 2003). Increases in the expression of p21 have been implicated in cell cycle arrest and differentiation of PC12 cells, as well as acceleration of neurite outgrowth (Erhardt and Pittman, 1998; Yan and Ziff, 1995; Yan and Ziff, 1997). Thus, SMN may function in both neurite elongation as well as regulation of proliferation through maintenance of p21 mRNA levels. Together, these results indicate that SMN interacts with various molecular factors in both the cytoplasm and in neurites, generating different complexes in specific subcellular regions with functions other then the

previously discussed snRNP biogenesis and pre-mRNA splicing. Thus, the range of processes that SMN is involved in may be more extensive than predicted.

1.1.4 SMN protein: The methyl-sensing Tudor domain

The SMN protein contains several motifs that are highly conserved throughout evolution as well as between species (Fig. 1B). The tudor domain of SMN is an approximately 60 amino acid motif located near the center of the SMN protein (Ponting, 1997; Talbot et al., 1998). This domain mediates numerous interactions of SMN with its binding partners, including some of the Sm proteins, namely Sm B/B', D1, and D3 (Buhler et al, 1999; Selenko et al, 2001), which as previously mentioned are components of spliceosomal snRNPs. Interestingly, these Sm proteins, as well as many other SMN interactors, contain glycine/arginine-rich (GAR) motifs, and methylation of arginine residues within these motifs increases affinity for interaction with the tudor domain (Brahms et al., 2001; Friesen et al., 2001a; Meister et al., 2001; Cote and Richard, 2005; Cheng et al., 2007).

A substitution of a glutamic acid to lysine residue at position 134 of the tudor domain, which has been found in patients with severe Type 1 SMA, strongly decreases the affinity of the SMN tudor domain for the Sm proteins (Buhler et al., 1999) and other methylated proteins (Cote and Richard, 2005). In addition, SMN was found to concentrate largely in Gems, which lack fully assembled snRNPs, when methylation is inhibited (Boisvert et al., 2002; Herbert et al., 2002). Together, these results strongly point to a key function of arginine methylation in the regulation of SMN activity, both in the formation of the spliceosomal snRNPs and potentially in other functions as well.

1.2.1 Arginine methylation and PRMTs

Arginine methylation is a post-translational modification in which nitrogen atoms within arginine residues of a polypeptide are modified to contain methyl groups (Gary and Clarke, 1998). Methylation occurs through the transfer of methyl groups from a methyl donor, S-adenosyl methionine (AdoMet), to the guanidino nitrogen in arginine. Arginine methylation increases bulkiness and hydrophobicity of the residue and inhibits hydrogen bonding without altering its charge. The two principal forms of methylated arginine found in eukaryotes are ω-N^G,N'^G-asymmetric dimethylarginines, (aDMA), and ω-N^G,N^G-symmetric dimethylarginines, (sDMA) (Fig. 3A). Arginine methylation is accomplished by a family of enzymes termed the protein arginine methyltransferases (PRMTs). Presently, nine PRMTs have been identified in mammals. All PRMTs first catalyze the formation of a monomethylated intermediate (MMA), but type I (PRMT1, 2, 3, 4, 6, and 8) and type II (PRMT 5, 7 and 9) are distinguished by their ability to produce aDMA or sDMA, respectively (Fig. 3B). All of the PRMTs are ubiquitously expressed, and it is speculated that alternative splicing may help achieve some level of tissue specificity (Scorilas et al, 2000). To date, very little is known about the regulation of the expression or the stability of PRMTs.

However, some variation exists in the specificity of substrates of each PRMT. A common feature of numerous PRMT substrates is the presence of GAR motifs (Najbauer et al, 1993). All members of the PRMT family, with the exception of PRMT4, recognize substrates containing GAR motifs. PRMT4, also referred to as coactivator-associated

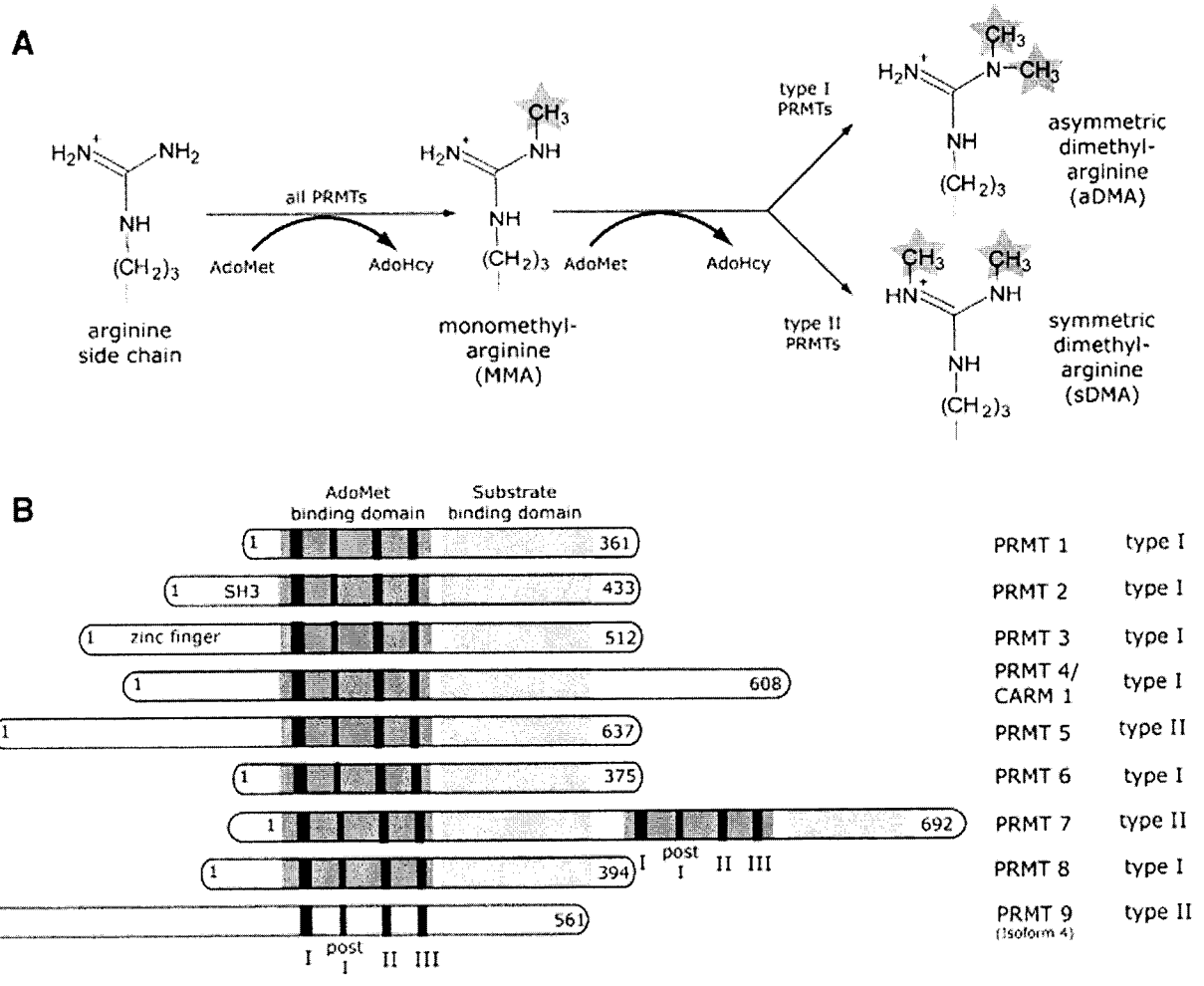

A
arginine
side chain
all PRMTs
AdoMet
AdoHcy
monomethyl-
arginine
(MMA)
AdoMet
AdoHcy
type I
PRMTs
type II
PRMTs
asymmetric
dimethyl-
arginine
(aDMA)
symmetric
dimethyl-
arginine
(sDMA)
B
AdoMet
binding domain
Substrate
binding domain
SH3
zinc finger
I
post
I
II
III
PRMT 1
type I
PRMT 2
type I
PRMT 3
type I
PRMT 4/
CARM 1
type I
PRMT 5
type II
PRMT 6
type I
PRMT 7
type II
PRMT 8
type I
PRMT 9
(Isoform 4)
type II

Figure 3. Arginine methylation and PRMTs. A, PRMT-mediated arginine methylation. Both type I and type II PRMTs first catalyze the formation of a mono-methyl-arginine intermediate. Next, the type I PRMTs transfer a second methyl group to the same nitrogen to generate asymmetric dimethylarginine. Alternatively, the type II PRMTs produce symmetric dimethylarginine. Here, the methyl donor S-adenosyl-L-methionine is converted to S-adenosyl-L-homocysteine. **B,** Members of the PRMT family. Protein length is shown by the number of amino acids. All PRMTs, with the exception of PRMT9, contain a conserved AdoMet binding domain (dark grey) and a less conserved substrate binding domain (light grey). The AdoMet binding domain of PRMT9 shares very little homology to the other members of the PRMT family (modified from Pahlich et al., *Biochimica et Biophysica Acta,* 2006).

arginine methyltransferase 1 (CARM1), exhibits a higher level of specificity, and a preferred methylation motif has yet to be determined. Recently, a few splicing and transcription elongation factors were found to be methylated by CARM1 at their proline/glycine/methionine-rich (PGM) motifs (Cheng et al., 2007). PRMT6, in addition to GAR motifs, can recognize and methylate RR motifs (Xie et al., 2007), and Type II PRMTs (PRMT5 and 7) also methylate isolated arginine residues that are not located in a known consensus region. Thus, in addition to regulation of expression of the PRMTs themselves, variations in recognition domains of each member may also modulate their functional diversity.

Arginine methylation by the PRMT family has been implicated in numerous cellular processes. Some of the best characterized roles include regulation of transcription through methylation of histones (Chen et al., 1999; Yun and Fu, 2000; Mowen et al., 2001; Wang et al., 2001), protein-protein interactions (Bedford et al., 2000, McBride and Silver, 2001), and DNA repair (Boisvert et al., 2003; Boisvert et al., 2005). Of particular interest to this project is the function of arginine methylation in RNA metabolism through methylation of RNA-binding proteins (RBPs). Numerous RBPs have been identified to be methylated by the PRMTs (Herrmann et al, 2004; Liu and Dreyfuss, 1995), which promotes processing of the interacting RNA, packaging into RNP complexes, export of the RNP into the cytoplasm and mRNA translation (Cheng et al., 2007; Xu and Henry, 2004; Yu et al., 2004). In the absence of methylation, RBPs have been found to be mislocalized (Cote et al., 2003; Lukong and Richard, 2004; Smith et al., 2004); thus, methylation by PRMTs may function as a maturation signal for these proteins (Cote et al., 2003).

1.2.2 Arginine Methylation and Neuronal Differentiation

In addition, PRMTs also function in the coordination of neuronal differentiation. Previous studies have determined that modifications occur to the pattern of methylated proteins following induction of neuronal differentiation. In PC12 cells, inhibition of methyltransferase activity impedes neurite outgrowth following addition of nerve growth factor (NGF), demonstrating that methylation is required for differentiation of these cells (Cimato, et al., 1997). Specifically, CARM1 was found to negatively regulate the differentiation of PC12 cells through maintenance of the progenitors in a proliferative state in a post-transcriptional manner (Fujiwara et al., 2006). Recently, a function for CARM1 in the regulation of cell pluripotency was shown to also involve transcriptional regulation. Methylation of histone H3 at arginine residues 17 and 26 by CARM1 was determined to play a key role in the maintenance of pluripotency in mouse embryos. Overexpression of CARM1 in embryos resulted in elevated histone H3 methylation, which induced expression of crucial pluripotency genes and directed cells to the pluripotent inner-cell-mass (Wu et al., 2009). In embryonic stem (ES) cells, a decrease in CARM1 was found to down-regulate pluripotency genes, resulting in differentiation of the cells. When CARM1 was overexpressed in the ES cells, cells became more resistant to differentiation signals (Wu et al., 2009). Together, these results indicate that CARM1 is required to maintain the pluripotency and self-renewal properties of the cells.

1.2.3 CARM1: Transcriptional and Post-Transcriptional Functions

CARM1 has been best described in the regulation of gene expression. This enzyme was first identified in a yeast-two hybrid screen as an interacting protein of glucocorticoid receptor-interacting protein-1 (GRIP1), the p160 steroid receptor coactivator (Schurter et al., 2001; Chen et al., 1999). The p160 coactivators, including GRIP1, bind directly to the hormone activated form of the nuclear receptors and are responsible for the recruitment of additional coactivators, known as secondary coactivators. These include the protein/histone acetyltransferase CBP/p300 (Chen et al., 1997; Voegel et al., 1998 Ma et al., 1999), which functions in nucleosome remodeling (Wolffe and Guschin, 2000; Cheung et al., 2000), as well as CARM1 and PRMT1 (Chen et al., 1999; Koh et al., 2001). CARM1 was first discovered to methylate histone H3 at arginine 2, 17, and 26 (Schurter et al.; 2001), but may also function in the methylation of nonhistone proteins within the transcription complex. CARM1 was recently found to methylate CBP/p300, and a mutation of the methylation sites inhibited steroid-dependent transcriptional activation (Chevillard-Briet et al., 2002; Lee et al., 2005; Xu et al., 2001). Thus, the methyltransferase activity of CARM1 as well as interaction with the p160 coactivators is required for its function with nuclear receptors (Chen et al., 2000; Lee et al., 2002), indicating that CARM1 methylation of histones and/or nonhistone proteins within the transcription complex acts synergistically with acetylation of histones and other members of the transcription complex to successfully promote chromatin remodeling, recruitment of RNA polymerase II, and finally transcription initiation.

Recently, CARM1 has also been described to function in a post-transcriptional manner. Numerous splicing factors, including CA150, SAP49, and U1C (Cheng et al., 2007), have recently been described to be methylated by CARM1. Methylation sites for all of these factors map to a common region, the PGM motif, which is responsible for many protein-protein interactions (Bedford et al., 1998). These regions surround the methylation sites, which can be regulated by CARM1 (Cheng et al., 2007). Interestingly, the PGM motif of CA150 binds the tudor domain of SMN in a CARM1 methylation-dependent manner. In addition, CARM1 methylation of CA150, a protein that functions to link transcription to splicing, promotes exon skipping in specific pre-mRNAs (Cheng et al., 2007). CARM1 has also been reported to regulate alternative splicing through an interaction with the splicing factor U1C (Ohkura et al., 2005). The discovery of splicing factors whose activity is regulated by CARM1 methylation, and impact of CARM1 methylation on protein-protein interactions, gives insights into the role that CARM1 plays in alternative splicing and suggests a potential role for this enzyme in the pathogenesis of SMA. In addition to these substrates, CARM1 also methylates some members of the Hu family of proteins, which are RNA-binding proteins known to play central roles in mRNA stability (Fujiwara et al., 2006). Thus, CARM1 may be involved in both transcriptional and post-transcriptional regulation through its methylation of chromatin remodeling factors and RNA-binding proteins, respectively.

1.3.1 Role of Hu proteins and mRNA Stability in Neuronal Differentiation

RNA-binding proteins are quickly being revealed as key components of the basic processes occurring during neurogenesis, neurite elongation, synapse formation and plasticity. Many of these proteins are found to have a direct involvement in various neurological diseases, including SMA. Hu proteins, as previously mentioned, are a family of RNA-binding proteins. In mammals, there are four members, including the neuron-specific HuD, HuC, and HuB, as well as the ubiquitously expressed HuR, all of which are encoded by several related genes (Szabo et al., 1991; Levine et al., 1993). The neuronal Hu proteins were first identified as target antigens in patients with small cell lung cancer-associated paraneoplastic neurological syndrome (Szabo et al., 1991; Sakai et al., 1994), and share a great deal of homology with the RNA-binding protein coded by the *Drosophila* gene *elav* (Embyronic Lethal, Abnormal Vision), which is required for the proper development and maintenance of the nervous system (Yao et al., 1993; Campos et al., 1985; Jimenez et al., 1987). In *Drosophila,* mutations in the *elav* gene cause neuroblasts to continuously proliferate, fail to differentiate, and migrate inappropriately (Campos et al., 1985).

Numerous studies have demonstrated that the neuronal Hu proteins are regulatory proteins required for the development, maintenance, and differentiation of neurons (reviewed by Deschênes-Furry et al., 2006). These proteins are first detectable in neurogenic precursor cells at the point of withdrawl from the mitotic cycle, and expression persists in mature neurons (Marusich et al., 1994), indicating a role for the Hu

proteins early in neurogenic differentiation. HuD in particular has been found as one of the earliest markers of neuronal cells in addition to being a crucial regulator of neuronal differentiation and survival (reviewed by Deschênes-Furry et al., 2006). Experiments in E19 rat cortical neurons, PC12 cells, and retinoic acid-induced embryonic stem cells where HuD was overexpressed resulted in accelerated neurite outgrowth (Anderson et al., 2000; Anderson et al., 2001; Kasashima et al., 1999). Inhibition of HuD in PC12 cells resulted in an inability of the cells to extend neurites upon nerve growth factor stimulation (Mobarak et al., 2000). Mice deficient in HuD have inhibited embryonic cranial nerve development and produce neurospheres that yield fewer neurons in comparison to wild type mice. However, these mice do not exhibit any morphological brain defects (Akamatsu et al., 2005).

The Hu proteins function in neuronal differentiation through post-transcriptional regulation of expression of genes involved in cell proliferation and differentiation. This is usually accomplished through recognition and high affinity interaction with AU-rich RNA elements (AREs) in the 3′-untranslated regions (UTRs) of specific target mRNAs (reviewed by Hinman and Lou, 2008). The Hu proteins all contain three RNA recognition motifs (RRMs 1-3) (Good, 1995; Szabo et al., 1991; Robinow et al., 1988) that are more than 90% identical at the amino acid level among the family members (Okano et al, 1997). These regions interact directly with the target RNA. The best characterized function of the Hu proteins occurs at the regulation of mRNA stability. Unlike many other ARE-binding proteins that predominantly destabilize transcripts, Hu proteins prevent degradation of their target mRNAs and stabilize the mRNA, significantly prolonging their half-life and allowing increased protein production (Barreau et al, 2005;

Aranda-Abreu et al., 1999; Deschênes-Furry et al., 2003; Deschênes-Furry et al., 2005; Mobarak et al., 2000). Numerous immediate early mRNAs are rapidly degraded via interactions of their AREs (Shaw et al., 1986; Chen et al., 1995), indicating that Hu proteins may play key roles in regulating mRNA turnover. Thus, through their RNA-binding abilities, Hu proteins may function by coordinately regulating their functionally-related mRNA targets. HuD interacts with numerous developmentally regulated transcripts including c-fos, c-myc, and p21 (Chagnovich and Cohn, 1996; Cuadrado et al., 2002; Joseph et al., 1998; Manohar et al., 2002). Conforming with its function in neuronal differentiation, many of the transcripts stabilized by HuD are involved in the formation of neuronal processes, including growth associated protein 43 (GAP-43) and tau (Aranda-Abreu et al., 1999; Mobarak et al., 2000; Chung et al., 1997). During neuronal differentiation, the acetylcholinesterase (AChE) transcript is also bound by HuD (Deschênes-Furry et al., 2003). AChE plays a role in promoting neurotransmission, and is also able to stimulate neurite outgrowth (Deschênes-Furry et al., 2003; Layer and Willbold, 1995; Soreq and Seidman, 2001). Thus, HuD interacts with and controls the stability of its target mRNAs, many of which have similar functions that are required for neuronal development and plasticity.

However, despite the identification of numerous Hu mRNA targets (Brennan and Steitz, 2001; Pascale et al., 2008; Lopez de Silanes et al., 2004; Barreau et al., 2005), the mechanism leading to stabilization of the interacting mRNAs has not been fully characterized. It is speculated that Hu proteins can oligomerize on their mRNA targets to inhibit further association of destabilizing proteins. Kasashima et al. have demonstrated that HuD may form dimers on its target mRNA through its third RRM. In addition,

between the second and third RRM of HuD is a less conserved basic linker domain, which has been proposed to contain a nuclear export signal that permits shuttling of the protein and bound transcripts out of the nucleus and into the cytoplasm (Keene, 1999; Kasashima et al., 1999; Fan and Steitz, 1998). When HuD shuttling is inhibited, neurite elongation is severly hindered, indicating that HuD has a key function in localization of its transcripts into the cytoplasm (Kasashima et al., 1999). Here, HuD and interacting transcripts are properly targeted, for example, to axons in the case of tau mRNA, and growth cones, in the case of GAP-43 mRNA (Aronov et al., 2002; Smith et al., 2004). In addition, HuD has also been found to associate with ribosomes and polysomes when bound to target mRNA (Smith et al., 2004; Atlas et al., 2004; Antic et al., 1999), demonstrating that HuD may also play a role in promoting translation of its target transcripts via interaction with other RBP with translational functions, either directly or indirectly. For example, HuB has been found to interact with hnRNP K, and both RBP can bind the 3'UTR of the p21 transcript (Yano et al, 2005). When only the hnRNP K protein was bound to this mRNA, translation was severely inhibited; however, recruitment of HuB abolished this effect and restored translation. HuD may act in an equivalent manner, due to the functional similarities of the Hu proteins and target transcripts.

1.3.2 CARM1 Methylation of HuD in Neuronal Differentation

Although a wealth of information now exists regarding the function of HuD in the regulation of stability of its target transcripts involved in neuronal differentiation and plasticity, relatively little information is known regarding the mechanisms of regulation

of HuD itself within neurons. As increasing evidence demonstrates that mRNA stability may be a consequence of a functional antagonism occurring between numerous stabilizing and destabilizing proteins (Barreau et al., 2005), this regulation is becoming increasingly important. The ability of HuD to bind and stabilize its mRNA targets may be regulated by posttranslational modifications of the RBP itself.

A study by Fujiwara et al. has shown that in proliferating PC12 cells, CARM1 methylates HuD at arginine residue 236, which is located in the hinge region between the second and third RRM. This correlated with reduced association of HuD with p21 mRNA in IP/RT-PCR experiments. This shifts the balance towards destabilization of the mRNA and promotes its decay, allowing the CARM1-containing cells to progress through mitosis, hence maintaining the proliferative state. The addition of NGF somehow induced a loss of HuD methylation, which led to increased levels of p21 mRNA and cellular differentiation (see Fig. 4 for schematic) (Fujiwara et al., 2006). In these cells, unmethylated HuD bound more p21 mRNA then methylated HuD, and this interaction increased the half-life of the transcript. As predicted by their model, reducing CARM1 levels through RNA interference mimicked NGF stimulation and resulted in p21 mRNA stabilization and neuronal differentiation. Thus, in PC12 cells, methylation of HuD by CARM1 is important for the regulation of neuronal differentiation. Interestingly, CARM1 was previously believed to localize predominantly in the cell nuclei, but was found to colocalize with HuD in the cytoplasm of PC12 cells (Fujiwara et al., 2006).

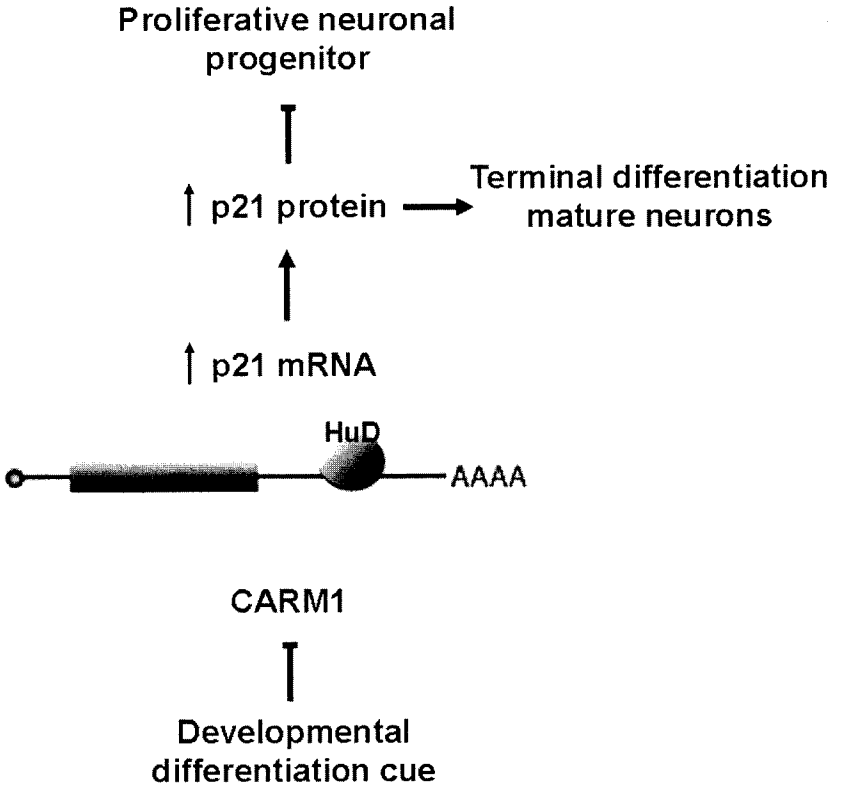

Proliferative neuronal progenitor
p21 protein
Terminal differentiation mature neurons
p21 mRNA
HuD
AAAA
CARM1
Developmental differentiation cue

Figure 4. Methylation of HuD by CARM1 regulates proliferation of neuronal cells. CARM1 methylates HuD in proliferating PC12 cells. This methylation was associated with a decreased interaction of HuD with p21 mRNA, leading to destabilization and decay of the transcript and allowing continuation through the cell cycle. A decline in HuD methylation was observed following addition of nerve growth factor, resulting in an increased interaction with p21 transcript and improved stability, as well as differentiation of the cells (generated from a study by Fujiwara et al., 2006).

1.4.1 Role of CARM1 in Motor Neuron Differentiation

Despite a large body of work elucidating SMN function, the motor neuron-specific molecular mechanisms affected by a deficiency in this protein remain unclear. Previously, our group demonstrated that KSRP, an RNA-binding protein, interacts with SMN in neuronal cells. SMN and KSRP were found to colocalize in neurites of differentiated cells. Naturally-occuring mutations in the Tudor domain of SMN seen in patients with severe forms of SMA resulted in loss of the interaction. KSRP was downregulated in SMA mice spinal cords, resulting in defects in stabilization of specific KSRP mRNA targets, including increased stability of p21 (Tadesse et al., 2008).

Additionally, the interaction between SMN and KSRP was determined to be dependent on methylation by CARM1. Previous studies by Fujiwara et al. indicate that CARM1 methylates another RNA-binding protein, HuD, and this methylation regulates the differentiation of neuronal cells. CARM1 methylation negatively affected the ability of HuD to interact with and stabilize its p21 mRNA target, either directly or indirectly, thus controlling the proliferative state of the cells. Reducing CARM1 levels in these cells caused a stabilization of p21 mRNA and induced a differentiated phenotype (Fujiwara et al, 2006).

In motor neuron-like (MN-1) cells, which retain many of the characteristics of motor neurons, knockdown of CARM1 expression was found to promote neuronal differentiation (Tadesse et al., 2008). We speculate that, in these cells, CARM1 is involved in a signaling pathway that regulates the switch from proliferation to

differentiation by regulating the properties of specific RNA binding substrates. Since reduced SMN expression resulted in defects in neuronal differentiation, and SMN can serve as an adaptor module for arginine methylated proteins involved in RNA metabolism, we propose that SMN functions in this pathway through CARM1-regulated interactions with specific RNA binding proteins. These findings may help to elucidate the specific role of arginine methylation and SMN in motor neurons, and in turn, provide crucial insights into the motor neuron-specific pathophysiology of spinal muscular atrophy.

1.4.2 Hypothesis

I hypothesize that CARM1 regulates proliferation of MN-1 cells by methylating the RNA-binding protein HuD, altering its RNA-binding ability and impacting downstream mRNA targets, including p21. I also propose that SMN will be involved in this regulatory pathway through interaction of HuD with the SMN tudor domain, and that this interaction will also be regulated by arginine methylation.

1.4.3 Specific Objectives

I) Confirm that the CARM1 pathway involving HuD and p21 mRNA is active in regulating differentiation of MN-1 cells.

II) Investigate the molecular mechanisms involved in this regulatory pathway.

III) Determine if SMN is involved in the pathway of MN-1 differentiation.

Chapter 2. Materials and Methods

2.1 Cell Culture, Differentiation and Stable Knockdown Cell Lines

Motor neuron like cells were cultured in Dulbecco's modified eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; ThermoScientific), 2mM L-glutamine and 2% penicillin streptomycin (Multicell, Wisent). Cells were maintained in 5% CO_2 at 37°C. For protein isolation and immunoprecipitation experiments, cells were passed at 50% confluency in 100mm plates and incubated overnight. Alternatively, six well plates were used for RNA extraction or immunofluorescence studies. To test various differentiation conditions, cells passed in 6-well plates were allowed to reach a confluency of 50% upon which maintenance media was removed, and replaced with media supplemented with either 10% or 2% FBS and a final concentration of 25 or 50μM trans-retinol (Sigma) and incubated for 48 hours. Following selection of the optimal condition from these trials, new plates were prepared and various growth factors were then individually added to this media, including 10ng/mL GDNF (Cedarlane), 100ng/mL BDNF (Cedarlane), or 10ng/mL NGF (Cedarlane). The cells were then incubated for approximately 48 hours prior to harvesting.

CARM1 was stably knocked down in MN-1 cells using a previously generated shRNA directed against CARM1 in pRS plasmid (OriGene). Lipofectamine 2000 reagent (Invitrogen) was used to transfect the vector into the cells according to the manufacturers protocol. Plasmid lacking the insert was transfected as a control. The cells were incubated for approximately 48 hours before selecting for cells that had successfully taken up the

vector using maintenance media containing 2μg/mL puromycin dihydrochloride (Sigma). Individual clones were isolated and maintained.

2.2 BrdU Assays and Neurite Counting

5,000 cells were plated into each well of a 96-well plate and incubated overnight in 5% CO_2 at 37°C. Wells were washed with phosphate buffered saline (1xPBS) and media was replaced with either maintenance media or differentiation media. Alternatively, pcDNA 3.1 plasmid (Invitrogen) containing myc-HuD was transfected into the MN-1 cells. 1μg of DNA was transfected using polyethyleneimine (PEI; Polysciences) transfection reagent at a DNA:PEI ratio of 1:5, according to manufacturers directions. Plasmid containing only the myc tag was transfected in an identical manner. The plates were incubated for a further 48 hours. In the transfected wells, media was removed and fresh media was added 24 hours following transfection.

BrdU assays were performed according to manufacturers protocol (Millipore). BrdU reagent was added four hours prior to addition of detection antibody. Plate reading was done at a single wavelength of 450nm. Cells treated without BrdU reagent were used as a control. Relative BrdU values were determined through comparison to the mock treated MN-1 cells.

In order to approximate the percentage of cells expressing neurites at least twice the length of the cell body, cells grown on 10cm plates were kept in maintenance media or treated with differentiation media as previously described. Alternatively, plates were transfected with 3μg of either myc or myc-HuD plasmid and again at a DNA:PEI ratio of 1:5. A minimum of five pictures of various areas of each plate was taken using a Canon

Powershot G6 camera. Total number of cells in each frame was counted, as well as the cells expressing long neurites. Percentage of cells with these neurites was taken by dividing this number by the total number of counted cells.

2.3 Isolation of Proteins, SDS-PAGE and Western blotting

Proteins were extracted from cells collected from 100mm plates. Cells were first washed in PBS, and then incubated in lysis buffer containing 10mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton, and complete protease inhibitor cocktail (Roche Applied Sciences) with continuous agitation for 30 minutes at 4°C to ensure complete lysis. The extracts were centrifuged at maximum speed for 10 minutes; resulting supernatants were stored at -20°C. The Bradford method was used to find protein concentration according to the manufacturers protocol (BioRad). Bovine serum albumin (BSA) at various concentrations was used as a standard. The optical densities (OD) were found using a spectrophotometer with a wavelength of 595nm. Concentration of protein in the samples was determined by extrapolating the OD value to the generated standard curve. Following quantification, equal amounts of each sample was combined with Laemmli reducing buffer (25% glycerol, 125mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate (SDS), 700mM β-mercaptoethanol, 0.1% bromophenol blue) and heated at 95°C for 5 minutes. Proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immunobilon-P Polyvinylidene Difluoride (PVDF; Millipore: 0.45 μm pores) membrane. 5% non-fat milk in PBS containing 0.05% Tween-20 (PBS-T) was used to block the membrane for approximately one hour at room temperature. Membranes were next incubated with primary antibody diluted in 2% non-

fat milk in PBS-T and 0.02% sodium azide at room temperature for one hour or at 4°C overnight, then washed 3 times for 5 minutes in PBS-T. Primary antibodies used include HuD (Santa Cruz), H3R17 (dimethyl-histoneH3-arginine17; Millipore), CARM1 (Bethyl Laboratories) GAPDH (glyceraldehyde 3-phosphate dehydrogenase; Covance), SMN (BD Biosciences), hnRNP K (Sigma), GST, Tuj-1 (neuronal class III B-tubulin; Millipore), Smi32 (neurofilament H, non-phosphorylated; Covance), ChAT (choline acetyltransferase; Chemicon), as well as HB9 (homeobox 9), Islet-1, and Nestin (Developmental Studies Hybridoma Bank). Secondary horseradish peroxidase (HRP) conjugated antibody (goat anti-mouse or goat anti-rabbit, Cedarlane) in 2% non-fat milk in PBS-T was then incubated with the membrane for one hour at room temperature, followed by 3 further washes in PBS-T. Bands were detected using chemiluminescent HRP substrate (Millipore) and exposure to film (Clonex).

2.4 RNA Extraction, Reverse Transcription and Quantitative PCR

Total RNA was isolated from cells using Trizol reagent (Invitrogen) according to manufacturers protocol. RNA was also extracted from cells at specific time points during differentiation and myc-HuD transfected cells. Samples were resuspended in DEPC-treated water and the OD was read on a spectrophotometer at a wavelength of 260nm. The OD of water alone was used as a control. To generate cDNA, 1μg of the respective RNA was incubated with 1μM oligo-DT for 10 minutes at 65°C, followed by reverse transcription using 0.5mM deoxyribonucleotide triphosphates (dNTPs; Invitrogen), 20U rRNasin (Promega), and 10U AMV reverse transcriptase enzyme (Promega) in AMV reverse transcriptase buffer (Promega) for one hour at 42°C.

For quantitative PCR reactions, 25ng of cDNA was combined with iQ SYBR Green Supermix (BioRad) and 400nM of the respective forward and reverse primers. Primers used were p21 (F) 5'-CAGCGATATCCAGACATTCAGA-3', p21 (R) 5'-CTCAGACACCAGAGTGCAAGAC-3', for GAPDH (F) 5'-ACCACAGTCCATGCCATCAC-3', (R) 5'-TCCACCACCCTGTTGCTGTA-3', and for CARM1 (F) 5'-AGCTGTGCAGTACTTCCAGTTCTA-3', (R) 5'-ATTGTTACTCTTCACCAGGACCTC-3'. The PCR conditions for both p21 and CARM1 consisted of heating at 94°C for 5 minutes, 32 cycles at 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 45 seconds, followed by a final incubation at 72°C for 10 minutes. The PCR program used for GAPDH was identical, except that the annealing temperature used was 55°C. Calculations of the relative amounts of p21 mRNA were done using the Livak method, using undifferentiated RNA extracts for calibration.

2.5 Immunofluorescence

Prior to plating the cells, coverslips were submerged in NaOH-saturated methanol for 30 minutes and washed in sterile water followed by ethanol. The coverslips were then dried before incubating them in coating solution containing 100μg/mL poly-D-lysine (Sigma) and 33μg/mL mouse laminin (Sigma) overnight in 5% CO_2 at 37°C. The coverslips were then placed in maintenance media for two hours before plating cells overtop. Cells to be used for differentiation experiments were allowed 24 hours to adhere to the coverslips before changing media, followed by a further 48 hours incubation. Media was removed and the coverslips were washed twice with PBS. Cells were fixed for five minutes at room temperature in a solution comprised of 48% acetone, 47% methanol,

and 5% formaldehyde, and rinsed three times in PBS. The cells were then permeabilized with 0.5% triton X-100 in PBS at room temperature for five minutes, and subsequently washed three times with PBS. Following the washes, the cells were blocked with PBS containing 0.1% BSA for 30 minutes. The selected primary antibody was then placed on the coverslips for one hour. The coverslips were washed first with 0.1% triton X-100 in PBS, then twice in PBS, before incubation with the secondary antibody in the dark for one hour at room temperature. The cells were again rinsed once with 0.1% triton X-100 in PBS and twice in PBS prior to mounting the coverslips using Vectashield mounting medium containing DAPI (Vector Laboratories Inc.) on glass slides. Imaging was done using a Zeiss Axio Imager.Z1 microscope, where the cells were viewed through a Zeiss 40X objective. The AxioCam HRm camera was used for image acquisition, and Adobe Photoshop CS2 (Adobe) was used to merge images taken using the various fluorescent filters.

2.6 Production of Recombinant Proteins

Escherichia coli BL21 cells (Stratagene) previously transformed with the appropriate constructs were cultured in lysogeny broth (LB; Fisher Scientific) containing 100μg/mL ampicillin. The cells were incubated at 37°C overnight with continuous agitation. Following addition of isopropyl-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1μM and further agitation for three hours, the cells were centrifuged, resuspended in 1xPBS and sonicated five times for ten seconds while on ice. The mixture was again centrifuged and the supernatant was tumbled with 1% Triton X-100 and glutathione agarose beads (Sigma) overnight at 4°C to allow the protein to bind. The

beads were collected and washed in 1xPBS. For GST-pulldown experiments, proteins were kept on beads in a 50% slurry in 1xPBS. Alternatively, proteins were eluted from the GST beads using 60μM glutathione in 1xPBS at a pH of 7.5. The bead solution was briefly vortexed and incubated at 37°C. The reaction was centrifuged and the supernatant was collected. Following five repetitions, the supernatants were combined and dialyzed in PBS overnight at 4°C with continuous stirring. The protein was concentrated by centrifugation of the solution through a 0.45μM filter (Millipore). Aliquots were run on 12% SDS-PAGE and stained with Commassie dye for 30 minutes to detect the produced protein. Finally, the gel was destained using methanol and acetic acid overnight with gentle agitation. The concentration of the protein was determined by Bradford assay as described in section 2.3.

2.7 Methylation Assays

For *in vitro* methylation assays, 5μg of GST-fused substrate was incubated with 25 mM Tris HCl pH7.5, 50 μM S-adenosyl-L-methionine (SAM; Perkin-Elmer), and 0.55μCi S-[methyl-^{3}H]-adenosyl-L-methionine (^{3}H-SAM; Perkin-Elmer), in the presence of 5μg GST-CARM1 or PRMT1. The reactions were incubated for two hours at 37°C with frequent mixing. Laemmli reducing buffer was added to stop the reactions, and the samples were heated at 95°C for five minutes. Reactions were then run on 12% SDS-PAGE and transferred onto PVDF membrane. The membrane was dried and sprayed with En^{3}Hance reagent (Perkin-Elmer). Bands were then visualized by fluorography, and the membrane was used for further western blot studies as described in section 2.3.

Similarly, methylation assays were completed using 25μg of total protein extract taken from MN-1 cells as a source of CARM1 enzyme. Extracts were taken from various time points during differentiation by lysing cells in 0.5 mM sodium phosphate buffer followed by five sonications for ten seconds on ice.

2.8 Generation of a p21 construct and radioactive labeling

The section of the 3'-UTR of p21 containing the three AU-rich regions found to bind to HuD was cloned into pBluescript SK vector (Stratagene). First, this region was amplified from MN-1 cells using the KOD DNA polymerase (Novatech) PCR protocol. The reaction contained 1.5mM $MgCl_2$, 0.2mM dNTPs, 12ng MN-1 cDNA, 0.4 μM of both the forward and reverse primers, and 1U KOD DNA polymerase in KOD DNA polymerase buffer. Primer sequences for the p21 3'-UTR were (F) 5'-TCACTCTGTGTGTCTTAATTA-3', and (R) 5'-AGGACTGTTCCTCCGGTATAGG-3'. The PCR program used consisted of heating at 98°C for 15 seconds, then 30 cycles at 98°C for 20 seconds and 68°C for 30 seconds. The samples were run on a 2% agarose gel, the band was visualized under UV light using the Gel Logic 200 Imaging System (Kodak) and excised, and the DNA was isolated using the DNA extraction kit (Qiagen).

To fill in the 5' protruding ends of the insert, the fragment was incubated with 62.5μM dNTPs and 2.5U Klenow enzyme (Promega) in New England Biolabs (NEB) buffer 2, followed by incubation at room temperature for ten minutes. To stop the reaction, the sample was placed at 75°C for ten minutes. Next, the insert DNA was phosphorylated to allow for later ligation with the vector. DNA from the above reaction was combined with 5μM ATP and 20U T4 polynucleotide kinase (PNK) in PNK buffer

(Promega), and incubated at 37°C for 30 minutes. Inhibition of the reaction was done using EDTA at a final concentration of 20mM. Again, the mixture was run on a 2% agarose gel, the band was excised, and the insert was purified by extraction.

To digest the pBlueScript vector, 2μg of the plasmid was incubated at 37°C for two hours with 10U EcoRV enzyme (Promega) in NEB buffer 3. Digested plasmid was isolated by gel extraction as previously described. Next, the phosphate groups were removed from the plasmid to allow for proper ligation. Here, the plasmid was treated with 1U alkaline phosphatase enzyme in dephosphorylation buffer (Roche Applied Sciences), incubated at 37°C for one hour, and again the fragment was purified.

To ligate the resulting plasmid with the p21 fragment, 50ng pBlueScript vector was incubated with 10mM ATP and 1U T4 DNA ligase enzyme in ligase buffer (Roche Applied Science). Increasing amounts of p21 insert were added to give insert:plasmid DNA ratios of 0:1, 1:1, and 3:1, in order to test for re-ligation of the vector and optimize ligation of the insert. Mixtures were incubated at 16°C overnight to allow ligation.

Ligation products were transformed into XL-1 Blue cells (Stratagene) in order to amplify the plasmid. Cells were first treated in 0.2% 2-mercaptoethanol (MP Biomedicals) on ice for ten minutes with frequent mixing. The total volume from ligation was added and cells were incubated, undisturbed, for 30 minutes on ice. Cells were then heat shocked at 42°C for 45 seconds, and placed back on ice. 200μL of LB media was added, and the mixture was incubated at 37°C for one hour with constant agitation. Cells were plated on LB agar plates containing 100μg/mL ampicillin and incubated at 37°C overnight. Individual colonies were picked and scaled up in 2mL of LB media with 100μg/mL ampicillin by shaking at 37°C overnight. Plasmid DNA was isolated from

bacteria using a PureYield plasmid miniprep kit (Promega), according to manufacturers instructions. Samples were sequenced at the StemCore Laboratories, and successful clones were further amplified using a PureLink HiPure plasmid maxiprep kit (Invitrogen).

To generate a ^{32}P-labeled p21 RNA fragment, the p21 plasmid was first linearized using 12U EcoRI restriction enzyme (Promega) and incubated at 37°C for one hour. Resulting DNA was run on a 2% agarose gel and the linearized vector was extracted as previously described. Next, 0.5μg of the plasmid was incubated for two hours at 37°C in a RNA labeling mixture containing 0.4mM ATP, 0.4mM CTP, 0.1mM GTP, 0.1mM UTP, 10mM DTT, 20U rRNasin (Promega), 20μCi ^{32}P-UTP (Perkin-Elmer), and 20U T7 polymerase (Promega) in T7 RNA polymerase buffer (Promega). 10U RQ1 DNase (Promega) was added and the reactions were incubated for a further 15 minutes at 37°C. Next, 0.3M RNase-free NaOAc with 0.2% SDS was combined with the mixture, followed by addition of a phenol, chloroform, and isoamyl alcohol mixture at a ratio of 25:24:1. The reactions were briefly shaken by hand and centrifuged at room temperature for five minutes at maximum speed. The aqueous phase containing the RNA was extracted, ethanol was added to a final concentration of approximately 75%, and the tubes were placed on dry ice for ten minutes. Reactions were centrifuged for 15 minutes at 4°C to precipitate the RNA, the supernatant was discarded and the pellet was allowed to air-dry briefly. Following resuspension in DEPC-treated water, the labeled RNA was further isolated using the RNA Cleanup Kit (Qiagen) according to the manufacturers instructions. Concentration of the radioactive RNA was found using a liquid sintillation counter.

2.9 Gel Shift Assays

In vitro methylation assays were performed as described in section 2.7. Assays in both the presence and absence of CARM1 were performed to generate a methylated and unmethylated HuD, respectively, under identical conditions. 1, 10, or 100 ng of methylated or unmethylated GST-HuD protein were incubated with 5μg/μl heparin and 2fmol ^{32}P-labeled p21 mRNA in PBS on ice for ten minutes. 1μL of RNA loading dye was added, and samples were loaded and run on a 3.5% (pre-run) bis-acrylamide gel for two hours. The gel was then dried onto Whatmann paper under heat and vacuum for one hour, exposed to film overnight, and visualized by autoradiography.

Similarly, gel shifts were performed using GST-HuD taken from methylation assays where extracts were used as a source of CARM1 enzyme. Here, the GST-tagged protein was bound to glutathione agarose beads to allow for simplified collection. Extracts were taken at specific time points during differentiation. Following the methylation assay, the beads were collected by centrifugation for 15 seconds at maximum speed and washed three times with 1xPBS. The isolated HuD was then incubated with p21 mRNA and subjected to a gel shift assay as described above.

Gel shift assays using only differentiated extracts and p21 were also attempted. Supershifting of the complex was performed by the addition of 1μg of HuD antibody (Santa Cruz) following a ten minute incubation of the reaction described above. The mixtures were then left on ice for 30 minutes with frequent mixing before adding Laemmli reducing buffer and running on a gel.

2.10 Filter binding assays

Methylated and unmethylated HuD was prepared using methylation assays as described above. Increasing concentrations of HuD were mixed with 2fmol ^{32}P-p21 mRNA in RNA binding buffer consisting of 10mM Tris-HCl, pH 7.4, 1.5mM $MgCl_2$, 150mM KCl, 0.5mM dithiothreitol (DTT), 0.1mM phenylmethanesulphonylfluoride (PMSF), and complete protease inhibitor cocktail (Roche Applied Science). Reactions were prepared on ice and incubated for ten minutes. The 96-well apparatus containing a nitrocellulose membrane pre-soaked in RNA binding buffer was assembled. Wells were washed with binding buffer, and vacuum was applied. Following the ten minute incubation, the mixture was applied to the appropriate wells under vacuum and allowed to run through for five minutes. Wells were washed twice with RNA binding buffer to remove unbound RNA, and vacuum was applied for 30 minutes to aid in drying the membrane. The apparatus was disassembled, the membrane was removed and allowed to dry for one hour on the bench before exposure to film for two hours and visualization by fluorography.

2.11 UV crosslinking

100μg protein extracts taken from either the mock MN-1 cells or the CARM1 knockdown cells was combined with 4fmol labeled mRNA as described in section 2.9. RNA-protein mixtures were placed into wells of a 96-well plate on ice and subjected to crosslinking using a UV-Stratalinker. Following crosslinking, RNase A was added at a final concentration of 5mg/mL, and samples were incubated for 30 minutes at 37°C. Proteins were then resolved by 12% SDS-PAGE and transferred onto a PVDF membrane

as outlined in section 2.3. The dried membrane was exposed to film and stored at -80°C for up to two weeks. Subsequent western blot analysis was performed as previously described.

2.12 Immunoprecipitation and PCR

Cells were collected and lysed in lysis buffer as outlined in section 2.3. Approximately 500μg of extract was incubated with either 5μg of HuD (Santa Cruz) or 1μg IgG (Santa Cruz) antibody and tumbled overnight at 4°C. 50uL of a 50% slurry of Protein-A-Sepharose beads (Sigma) in 1xPBS were added and the mixture was allowed to tumble two hours further. The beads were collected by centrifugation, washed three times with lysis buffer and resuspended in an equal volume of Laemmli reducing buffer. The samples were run on 12% SDS-PAGE, and used for further western blotting analysis as described in section 2.3.

To study RNA that was precipitated with the protein, the RNA was extracted and reverse transcribed into cDNA as outlined in section 2.4. PCR was then performed for p21 and GAP-43 mRNA. Primers for p21 were identical to those used for qPCR as described in section 2.4. The program used consisted of heating at 94°C for five minutes, 32 cycles at 94°C for one minute, 50°C for one minute, 72°C for one minute, ending with a final heating at 72°C for ten minutes. The GAP-43 PCR program was identical, with the exception of an annealing temperature of 56°C. Primers used for this mRNA were

(F) 5'-TTTGTTTCTTGGTGTTGTTATGGC-3', and

(R) 5'-GAACGGAACATTGCACACACA-3'.

2.13 Transcription and Translation (TnT) Reactions

Reactions were prepared in the TnT Coupled Reticulocyte Lysate System (Promega) according to manufacturers instructions. Mixtures contained rabbit reticulocyte lysate, 20μM amino acids lacking methionine, 20U rRNasin, 10U T7 Polymerase, 1μg myc-HuD plasmid, and 10μCi ^{35}S-methionine (Perkin Elmer) in TnT buffer. Reactions were incubated at 30°C for two hours and run on SDS-PAGE as described in section 2.3. Gels were dried onto Whatmann paper by heating to 55°C under vacuum for one hour, exposed to film overnight at -80°C, and visualized by autoradiography.

2.14 GST pulldowns and RNase treatment

The GST tag, GST-SMN Tudor and GST-SMN Tudor E134K proteins were prepared as described in section 2.6. Proteins were not eluted from the GST beads to allow for simplified collection in pulldowns, and resuspended in 50% slurry in PBS plus complete protease inhibitor. Protein was extracted from MN-1 cells as discussed in section 2.3. For each pulldown, 400μg of the extracts diluted up to 1mL in lysis buffer was incubated with approximately 5μg of the respective GST beads. 40μg (10% of the amount used for pulldowns) of the original protein lysate was stored for later use as a total lysate sample for SDS-PAGE. Alternatively, TnT reactions described in the above section were also used for pulldowns. Again, 10% of the mixture was kept as a total reaction sample. The remainder was split evenly among each of the pulldowns, diluted in lysis buffer, and 5μg of beads were added. The mixtures were tumbled overnight at 4°C. Beads were collected by centrifugation at maximum speed for 15 seconds, and the

supernatant was discarded. The beads were washed three times with lysis buffer, tumbled for five minutes and again centrifuged. One final wash was done identically using 1xPBS. Beads were resuspended to 50% slurry in 1xPBS and run on 12% SDS-PAGE. Pulldowns done using MN-1 extracts were transferred to PVDF membrane and subjected to western blotting as described in section 2.3. As outlined in the previous section, gels containing pulldowns from the TnT reactions were dried and radioactivity was analyzed by fluorography. In pulldowns where MN-1 extracts were treated with RNase A, an identical protocol was followed, using a lysis buffer containing RNase at a final concentration of 20μg/mL added prior to cell lysis.

2.15 Site-Directed Mutagenesis

The pcDNA 3.1 myc-HuD arginine 236 to lysine or tryptophan substitutions were generated using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene), according to manufacturer's protocol. The solution used for mutagenesis consisted of 0.2mM dNTPs, 0.2μM of the respective forward and reverse primers, 50ng plasmid DNA, and 2.5U Pfu Turbo enzyme in Pfu Turbo buffer (Stratagene). The primers for the R236K mutant were (F) 5'-CCACCAGGCTCAGAAGTTCAGGCTGGACA-3',
(R) 5'-TGTCCAGCCTGAACTTCTGAGCCTGGTGG-3', and for the R236W mutant (F) 5'-CCACCAGGCTCAGTGGTTCAGGCTGGA-3',
(R) 5'-TCCAGCCTGAACCACTGAGCCTGGTGG-3'. The PCR program used began with 95°C for 30 seconds and was followed with 18 cycles at 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 22 minutes. The sequence ended with 68°C for 10 minutes. The DNA was then incubated with 20U Dpn1 restriction enzyme for one hour at 37°C

before transformation into XL-1 Blue cells and amplified as described in section 2.6. Again, sequencing of the mutants was done at StemCore Laboratories.

2.16 Generation of an shRNA against the HuD 3'UTR

First, primers were designed against a sequence contained within the 3'UTR of HuD. Primer sequences were (F) 5'-AGAGGAGACACAAGATACATTTGCT-3' and (R) 5'-AGCAAATGTATCTTGTGTCTCCTCT-3'. These complementary primers were 25bp in length, formed a hairpin structure separated by a short loop, and contain BamHI and HindIII restriction sites at either end. To anneal the oligos, 1μg of each oligo was combined and heated in saline-sodium citrate buffer (Sigma) at 95°C for ten minutes. This was followed by incubation at room temperature for one hour. The 5' end was then phosphorylated using a T4 polynucleotide kinase reaction as described in section 2.6. Next, 6μg of the pRNAT-HI.I Neo vector was linearized using 10U BamHI and HindIII enzymes in NEB buffer 2, purified on a 1% agarose gel, and de-phosphorylated using the procedures outlined in section 2.6. The insert and vector were ligated using T4 ligase. The ligation products were transformed into XL-1 cells, plated on LB plates containing 100μg/mL ampicillin, and amplified as previously described.

Chapter 3. Results

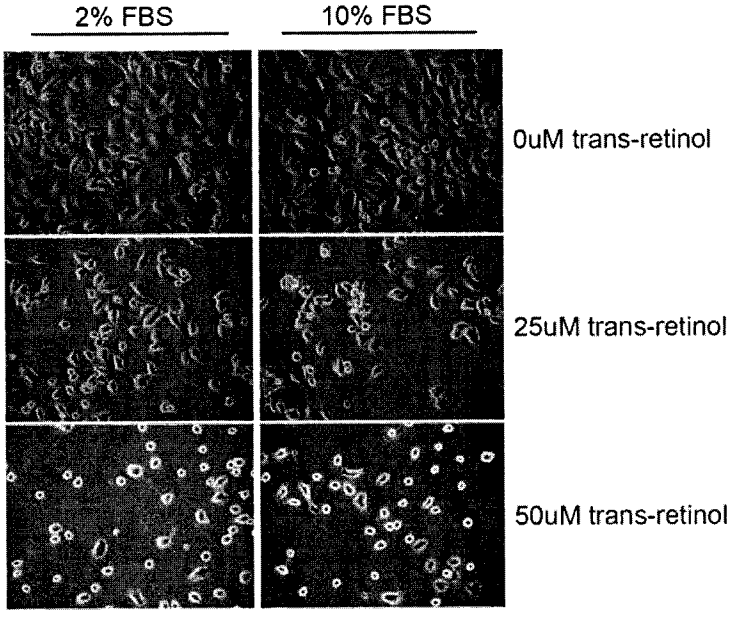
3.1 The CARM1 pathway involving HuD is active in regulating differentiation of MN-1 cells

It was previously reported that the pool of unmethylated HuD, a substrate of CARM1, increased during NGF-induced differentiation of PC12 cells, but the mechanism was not investigated (Fujiwara et al., 2006). We were interested if this phenomenon was also present in MN-1 cell differentiation. In addition, to gain insights of a potential function for arginine methylation in the motor-neuron specific etiology of SMA, we were interested in determining how protein levels and methylation activity of CARM1 are influenced in differentiation in MN-1 cells.

3.1.1 MN-1 cells as a model system to study the role of CARM1 in motor neuron differentiation

In order to determine the mechanism of how CARM1 function is regulated during differentiation of MN-1 cells, the most effective means of differentiation was first investigated. Cells were treated with media containing altered serum levels in combination with various neurotrophic factors (Figure 5). Treatments resulting in the least amount of cell injury or death and most differentiated phenotype, as indicated by cell spreading and production of multiple extensions, were determined visually. The chosen differentiation media consisted of a one-time addition of media containing 2% FBS, 25μM trans-retinol, and 10ng/mL GDNF. Following 48 hour incubation, these cells had an approximately five-fold increase in number of observed neurites that were at least twice the length of the cell body, in comparison to cells remaining in maintenance media

A


2% FBS
10% FBS
0uM trans-retinol
25uM trans-retinol
50uM trans-retinol

B

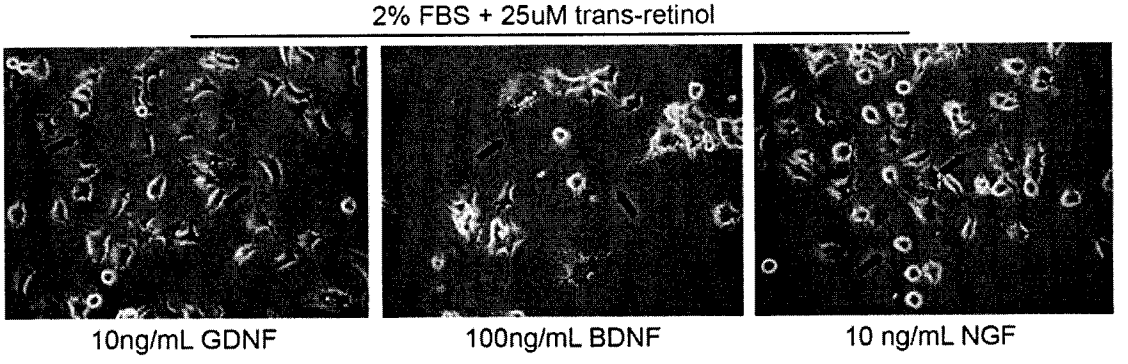

2% FBS + 25uM trans-retinol
10ng/mL GDNF
100ng/mL BDNF
10 ng/mL NGF

Figure 5. Optimization of MN-1 differentiation protocols. A, Various concentrations of trans-retinol, a well-described method for differentiating MN-1 cells, at differing serum levels. **B,** Addition of various growth factors to the optimum media chosen in A. All diagrams are taken following a one-time addition of the indicated media and 48 hour incubation. Arrows indicate neurite extensions (n=3).

(Figure 6D). BrdU assays indicated that addition of this differentiation media reduced the proliferation rate of the MN-1 cells by more then half (Figure 6C).

Next, we were interested in further characterizing this process using identified markers for various stages of motor neuron differentiation. These included markers for early differentiation (Islet 1, Nestin) and late differentiation (Tuj1, HB9, Smi32, ChAT). Antibodies recognizing the individual markers were using in immunofluorescence (Supplemental Data, Figure 15) and western blotting (data not shown); unfortunately neither technique was successful in identifying changes in protein levels between the untreated cells and cells treated with differentiation media.

As previously discussed, a transient knockdown of CARM1 in MN-1 cells also produced a differentiated phenotype (Tadesse et al., 2008). In order to study this further, a stable knockdown of CARM1 in the MN-1 cells was generated using an shRNA against CARM1 (Figures 6A and 6B). Loss of CARM1 resulted in an increase in the number of cells with long neurites and a reduced proliferation rate in comparison to cells transfected with an empty vector (Figures 6C and 6D). However, both of these effects are not as pronounced in comparison to the cells treated with differentiation media. This may be due to the selection process used when generating the stable knockdown; the cells must continue to proliferate and the most severe knockdown may have been selected against due to a greatly slowed or inhibited proliferation rate. When the stable knockdown cells were treated with differentiation media, the proliferation rate was further decreased compared to the mock knockdown cells. Thus, loss of CARM1 alone was sufficient to slow growth of MN-1 cells and mimics a partially differentiated phenotype, indicating a function for CARM1 in the regulation of MN-1 cell differentiation.

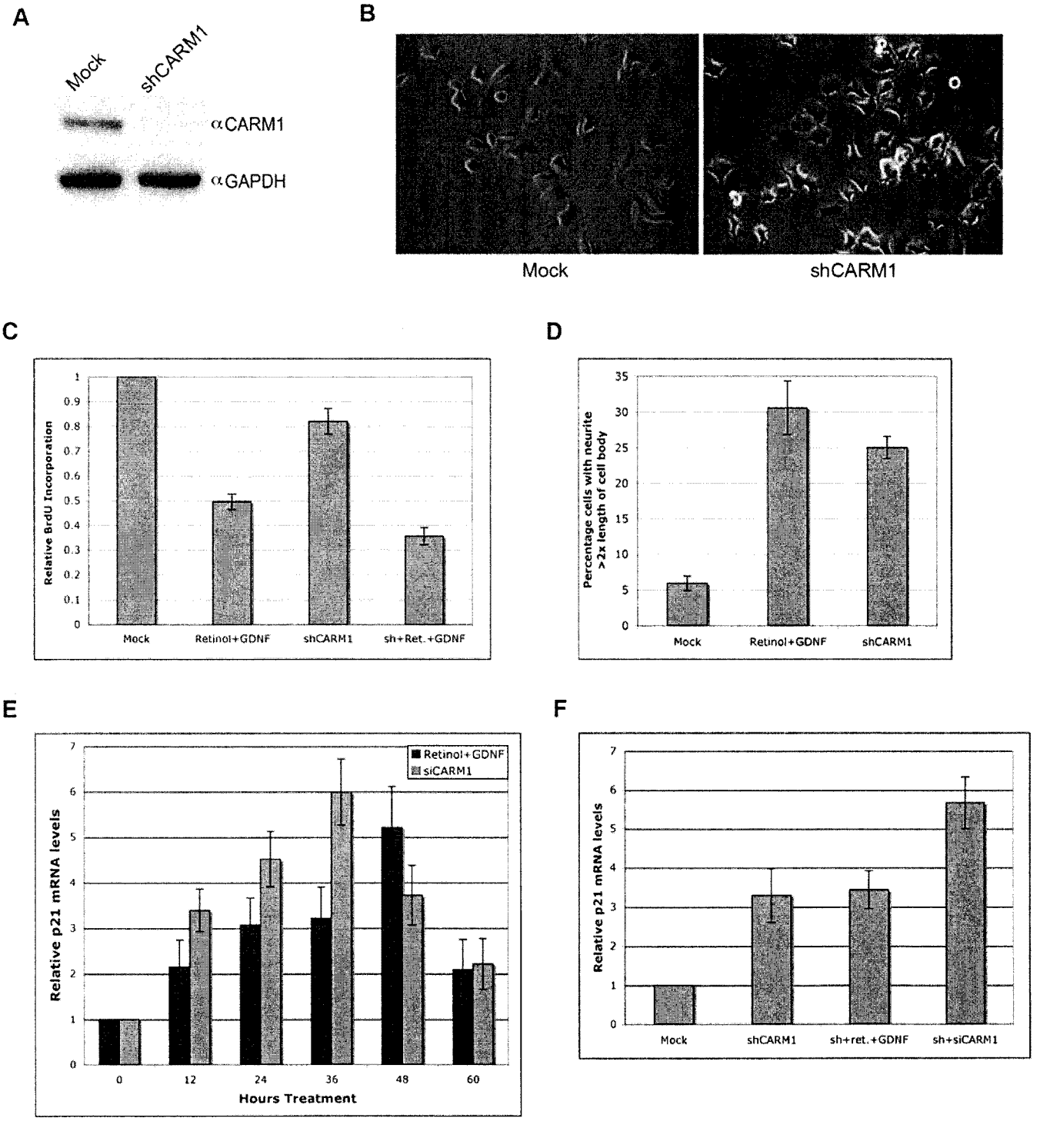

A
Mock
shCARM1
αCARM1
αGAPDH
B
Mock
shCARM1
C
Relative BrdU Incorporation
Mock
Retinol+GDNF
shCARM1
sh+Ret.+GDNF
D
Percentage cells with neurite >2x length of cell body
Mock
Retinol+GDNF
shCARM1
E
Retinol+GDNF
siCARM1
Relative p21 mRNA levels
Hours Treatment
F
Relative p21 mRNA levels
Mock
shCARM1
sh+ret.+GDNF
sh+siCARM1

Figure 6. CARM1 is required to maintain MN-1 cells in a proliferative state. A, Stable knockdown of CARM1 in MN-1 cells. Total protein extracts from cells transfected with empty vector and CARM1 knockdown cells were immunoblotted with CARM1 and GAPDH antibodies. **B,** CARM1 knockdown cells exhibit a differentiated phenotype in comparison to cells containing empty vector. Arrows indicate neurite extensions. **C,** BrdU assays demonstrate that cells treated with differentiation media, as well as the CARM1 knockdown cells, have a reduced proliferation rate. Knockdown cells in the presence of differentiation media produced a further decrease in proliferation. **D,** Addition of differentiation media, or alternatively reducing CARM1 levels, is sufficient to promote neurite extension. **E** and **F,** p21 mRNA levels are greatly increased in MN-1 differentiation and in transient CARM1 knockdown, as well as the stable and double knockdown, as determined by pPCR (n=5).

Next, the impact on downstream mRNA targets predicted to play a role in motor neuron differentiation was investigated. Changes to p21 mRNA levels were of primary interest due to its involvement in PC12 differentiation via CARM1-methylated HuD (Fujiwara et al., 2006). Quantitative PCR (RT-qPCR) demonstrates that the amount of p21 mRNA increases by more than five-fold 48 hours following addition of differentiation media compared to mock treated cells. In a transient knockdown of CARM1, the increase in p21 levels was even more pronounced, and occurred within a shorter amount of time (Figure 6E). Following a peak at these time points, the levels of mRNA decreased rapidly. In the stable CARM1 knockdown cells, p21 levels increased by more than three-fold (Figure 6F). It was predicted that the p21 levels would not be as elevated in these cells in contrast to the other tested conditions, since the CARM1 knockdown cells are still cycling at a higher rate in comparison to the transient CARM1 knockdown as well as the cells treated with differentiation media. Addition of differentiation media to the stable knockdown did not further increase the p21 mRNA level, despite the decrease in proliferation rate observed in BrdU assays. Transient knockdown of CARM1 remaining in the stable knockdown resulted in a further increase in the p21 level to the same extent observed in transient knockdown in wild type cells. Unfortunately, attempts at western blotting using numerous antibodies against p21 were unsuccessful. These results demonstrate that p21, known to inhibit neuronal proliferation and promote neurite outgrowth, is an important cell cycle regulator in MN-1 cells. Loss of CARM1 can elevate the expression of p21 mRNA independently of neurotrophic factors, imply a role for CARM1 in maintaining proliferation of MN-1 cells through downstream effects on p21 mRNA.

3.1.2 CARM1 is down-regulated during MN-1 differentiation

Since reducing CARM1 levels through RNA interference induced a differentiated phenotype, we were curious to assess if CARM1 levels and/or activity were decreased during MN-1 differentiation induced by trans-retinol/GDNF treatment. Western blotting for CARM1 demonstrates that protein levels are decreased as early as 12 hours following addition of differentiation media (Figure 7A); however, this regulation may occur at the level of transcription, translation, and/or protein stability. To determine if CARM1 expression is regulated at the transcriptional level, quantitative PCR was used to monitor the mRNA levels during differentiation. The mRNA levels of CARM1 were found to remain constant (Figure 7B). The discrepancy between RNA and protein suggests that regulation may take place at the level of translation and/or stability of the protein. Interestingly, addition of a protease inhibitor, MG132, to the MN-1 cells stabilized the levels of CARM1 protein (Sanchez, G., unpublished data). Thus, addition of neurotrophic factors to MN-1 cells somehow causes a down-regulation of CARM1 protein levels, further indicating a key role for this methyltransferase in the maintenance of proliferation of MN-1 cells. In addition, regulation of CARM1 may occur at the level of protein stability.

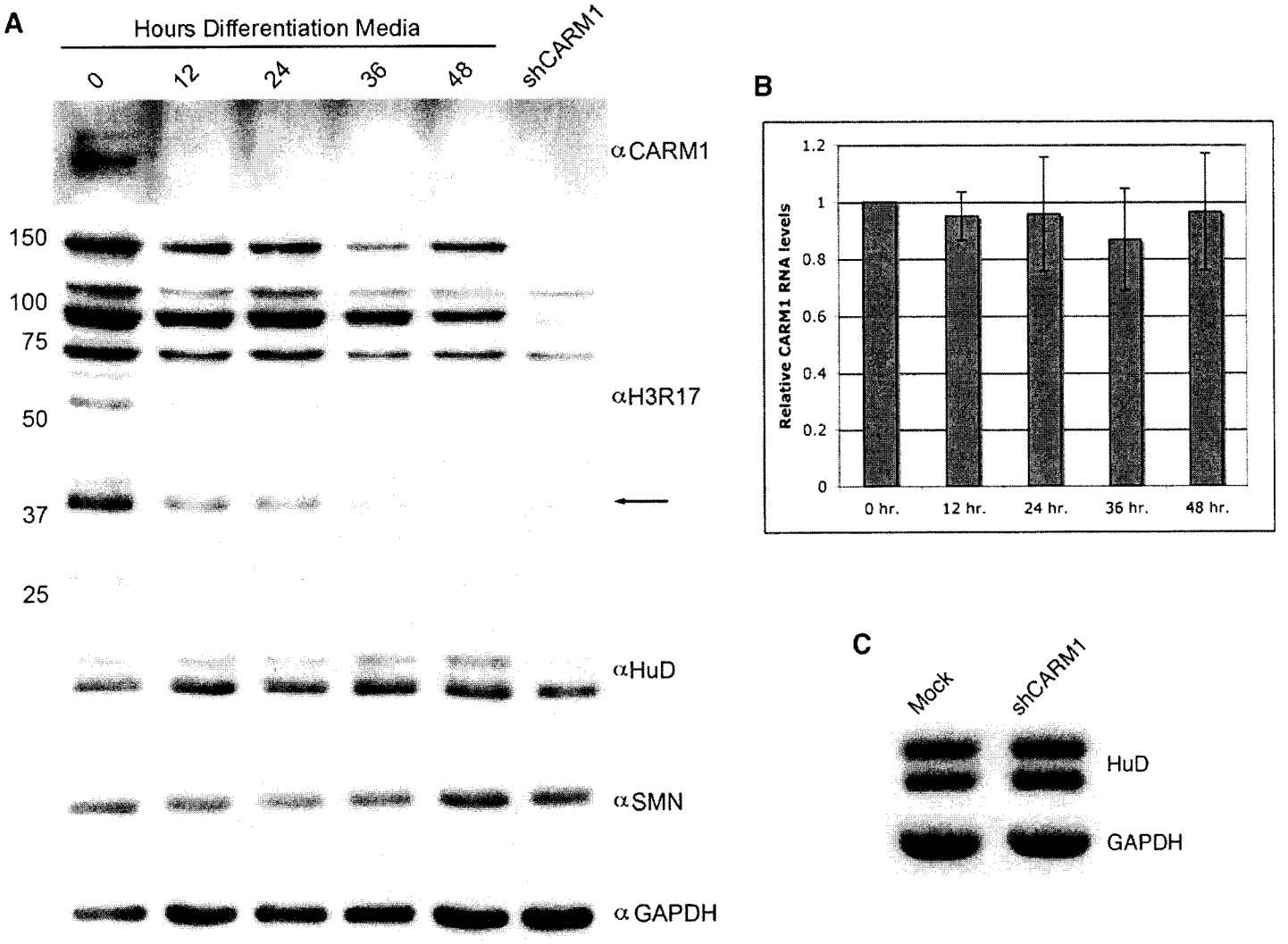

A
Hours Differentiation Media
0
12
24
36
48
shCARM1
αCARM1
150
100
75
αH3R17
50
37
25
αHuD
αSMN
αGAPDH
B
Relative CARM1 RNA levels
1.2
1
0.8
0.6
0.4
0.2
0
0 hr.
12 hr.
24 hr.
36 hr.
48 hr.
C
Mock
shCARM1
HuD
GAPDH

Figure 7. Protein and RNA analysis of CARM1 and HuD in MN-1 differentiation. **A,** Western analysis of MN-1 cells treated with differentiation media and CARM1 knockdown cells. CARM1 protein level decreases drastically within 12 hours treatment. H3R17, an antibody that specifically recognizes CARM1 methylated proteins, shows that the methylation status of numerous substrates is decreased in this process, as well as the CARM1 knockdown. One of the bands appears at a molecular weight that is identical to HuD, as indicated. Immunoblotting for SMN and HuD indicate that the levels of these proteins do not change during differentiation. Two bands were detected using the HuD antibody in all cells except the CARM1 knockdown. **B,** CARM1 mRNA levels remain constant in MN-1 differentiation, and **C,** HuD is alternatively spliced to produce two distinct isoforms in an approximately equal ratio, which is not altered in the absence of CARM1. These isoforms differ in size by about 75bp (n=4).

3.1.3 Localization of CARM1

The localization of CARM1 may also be important in determining its activity. Previously, CARM1 was believed to reside in the nucleus of many cell types (Frankel et al., 2002; Yadav et al., 2003), but it was recently reported to colocalize with at least one substrate, HuD, in the cytoplasm of proliferating PC12 cells (Fujiwara et al., 2006). Using immunofluorescence, CARM1 was found in the cytoplasm and is mostly concentrated around the periphery of the nucleus in cycling MN-1 cells. Following induction of differentiation, CARM1 appears to further concentrate around the nucleus, then disperses throughout the cytoplasm in late differentiation (Figure 8). However, these results do not correlate with the decrease in CARM1 protein that was observed using western blotting (Figure 7A); this will be addressed further in the discussion. Interestingly, before addition of differentiation media, HuD was found in both the nucleus and cytoplasm. In late differentiation, HuD is found exclusively in the cytoplasm, implying that this substrate may have specific subcellular functions.

3.1.4 Methylation of CARM1 substrates is decreased in MN-1 differentiation

Since CARM1 protein levels were found to decrease during differentiation, we also assessed if this correlated with a reduction in the methylation status of CARM1 substrates. The H3R17 antibody, which specifically recognizes CARM1 methylated motifs, was used to monitor the overall methylation profile of CARM1 substrates during differentiation treatment of MN-1 cells. A decrease in the methylation status of almost all of the substrates was observed (Figure 7A); however, it is important to note that this may be due to reduced levels of the CARM1 substrates themselves, and not necessarily a

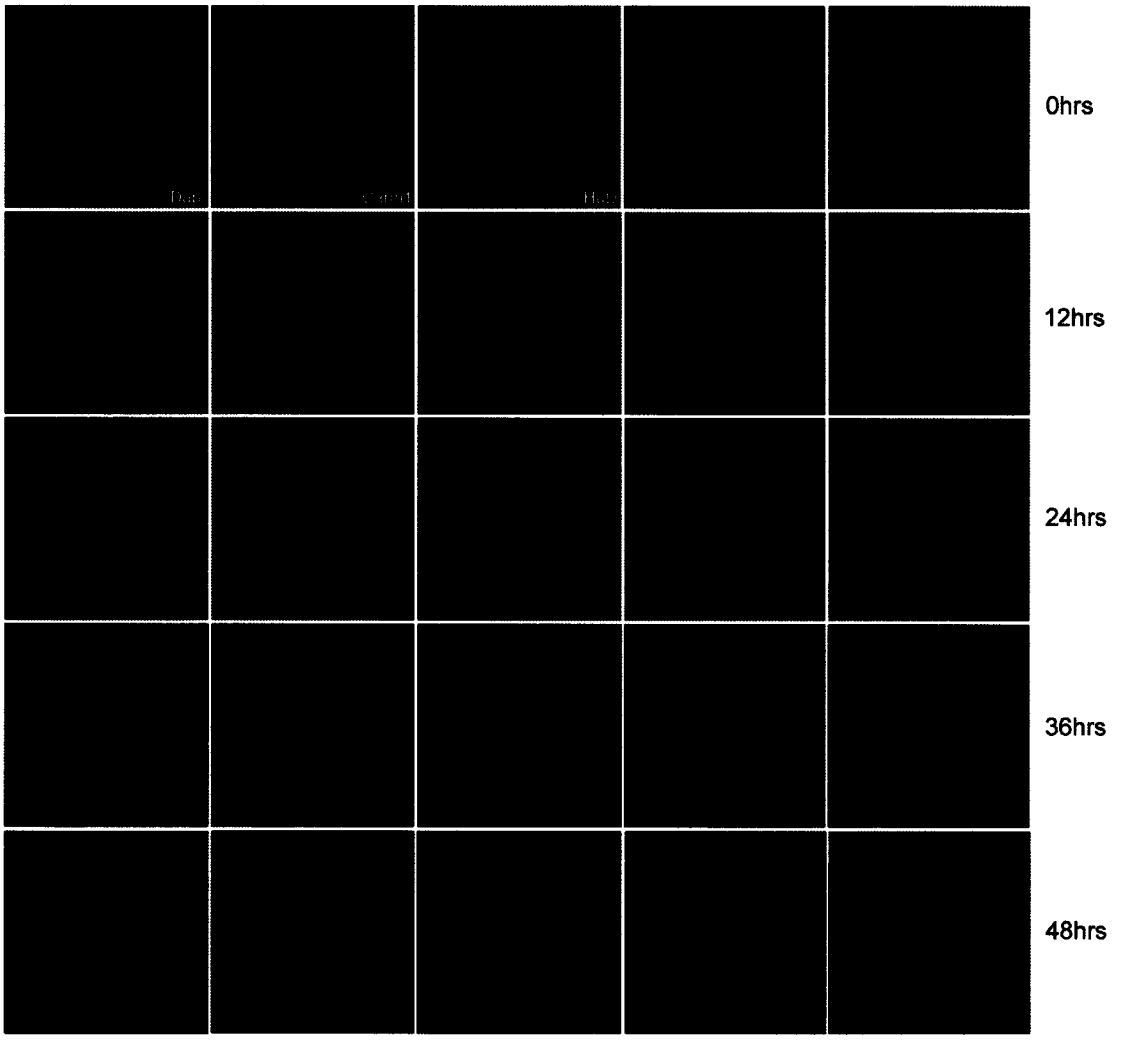

0hrs
12hrs
24hrs
36hrs
48hrs

Figure 8. Immunofluorescence of HuD and CARM1 in MN-1 differentiation. Both proteins are largely cytoplasmic in cycling cells. Upon differentiation treatment, CARM1 concentrates along the nuclear periphery, and both proteins are found in the generated extensions (n=3).

reduction in methylation status. The methylation profile of the CARM1 knockdown cells was also studied. As predicted, methylation of very few proteins was detected with the H3R17 antibody.

As previously discussed, in PC12 cells, HuD has been identified as a CARM1 substrate involved in promoting differentiation (Fujiwara et al., 2006). To monitor the methyltransferase activity of CARM1 on this particular substrate using the MN-1 cells, a methylation assay was completed using GST-tagged HuD, the radioactive methyl donor ^{3}H-SAM, as well as MN-1 protein extracts as a source of enzyme (Figure 9). Here, extracts taken from both the mock and CARM1 knockdown cells were incubated with the methyl donor and the GST-tagged substrate. In the absence of CARM1 within the MN-1 cells, exogenous HuD was significantly less methylated compared to the cells transfected with an empty vector. Another assay was performed using cellular protein extracts prepared at various time points following addition of differentiation media. Interestingly, methylation of GST-HuD appears to decrease rapidly following induction of MN-1 differentiation.

However, as previously discussed, this may be due to a decrease in the amount of HuD protein following induction of differentiation, and not in its methylation status. In the MN-1 cells, western blotting for HuD during differentiation demonstrates that the levels of the protein are not altered in this process (Figure 7A). Interestingly, using the H3R17 antibody, the methylation of a protein with approximately the same molecular weight as HuD was found to be greatly reduced, both in the CARM1 knockdown as well as in differentiation. Unfortunately, immunoprecipitation to confirm the identity of the protein using either the HuD or H3R17 antibody was unsuccessful. Together, these

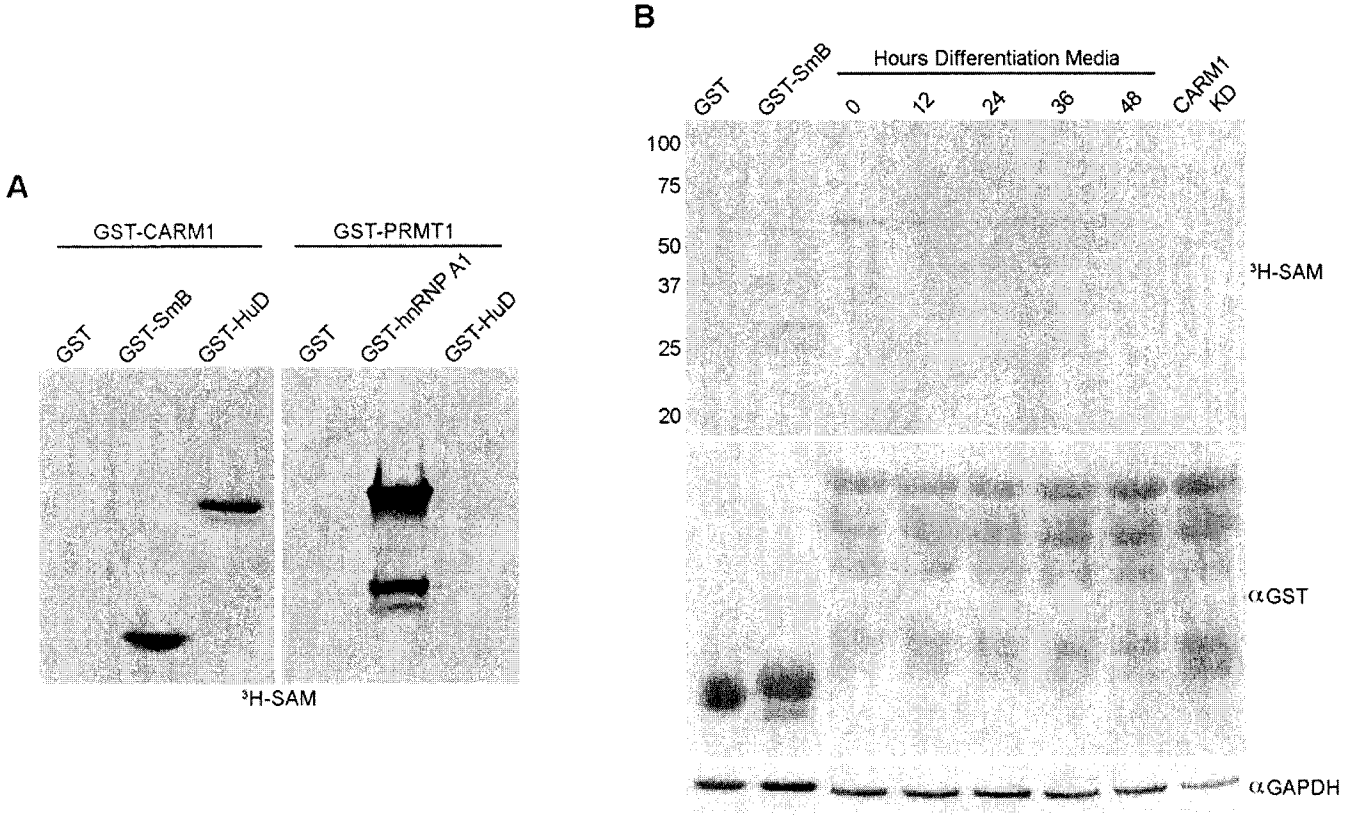

A
GST-CARM1
GST-PRMT1
GST
GST-SmB
GST-HuD
GST
GST-hnRNP A1
GST-HuD
³H-SAM
B
GST
GST-SmB
Hours Differentiation Media
0
12
24
36
48
CARM1 KD
100
75
50
37
25
20
³H-SAM
αGST
αGAPDH

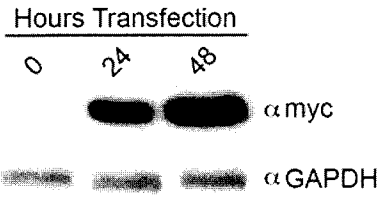
Figure 9. Methylation of HuD by CARM1 decreases in differentiation. Methylation assays using GST-HuD fusion protein and **A,** GST-CARM1 or PRMT1, and **B,** extracts from cells treated with differentiation media, as well as the CARM1 knockdown, as a source of enzyme. Proteins were incubated in the presence of the radioactive methyl donor AdoMet (n=4).

results indicate that HuD is a substrate of CARM1 in MN-1 cells, and addition of neurotrophic factors reduces its methylation status during differentiation. Due to its known function in regulating neuronal differentiation, the inhibited methylation of HuD may function to decrease growth rate and promote neurite outgrowth.

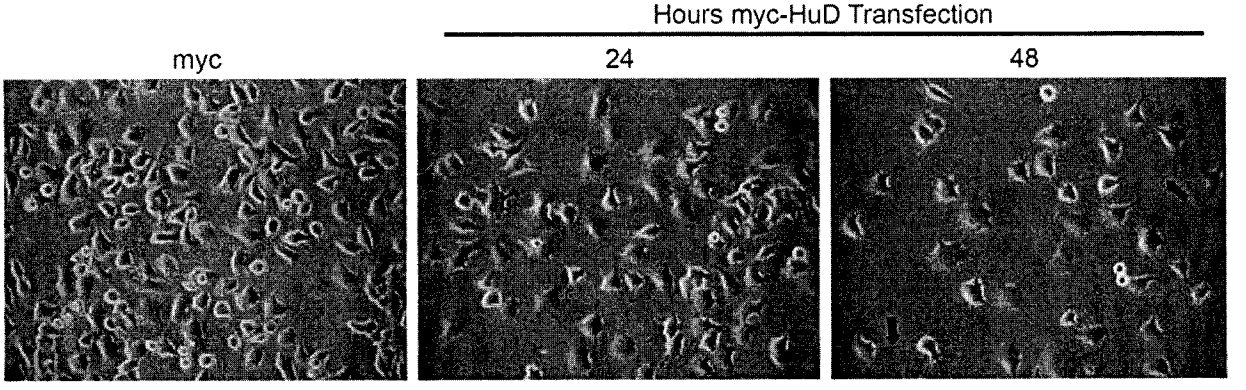
3.1.5 Overexpression of HuD is sufficient to promote MN-1 differentiation

In order to further study the potential function for HuD in the differentiation of MN-1 cells, a vector containing myc-HuD was transfected into the cells. Overexpression of HuD resulted in a differentiated phenotype (Figures 10A and 10B). In addition, the number of neurites at least twice the length of the cell body was increased two and a half fold in comparison to transfection of the myc tag alone (Figure 10C). However, the proliferation rate of both types of transfected cells was almost identical (data not shown). RT-qPCR shows p21 mRNA levels are increased approximately five fold in the cells expressing myc-HuD in contrast to cells transfected with empty vector (Figure 10D), but the amount of CARM1 mRNA and protein (data not shown) remain constant. The increased ratio of HuD in comparison to CARM1 may result in a lower proportion of HuD being methylated, hence mimicking the situation observed during differentiation. Together, these results imply that an increased proportion of unmethylated HuD up-regulates p21 and promotes neurite outgrowth. Thus, downstream of CARM1, it may be the relative amount of unmethylated to methylated HuD that regulates its function in MN-1 differentiation. To further investigate this, the H3R17 antibody could be used in order to monitor CARM1 activity.

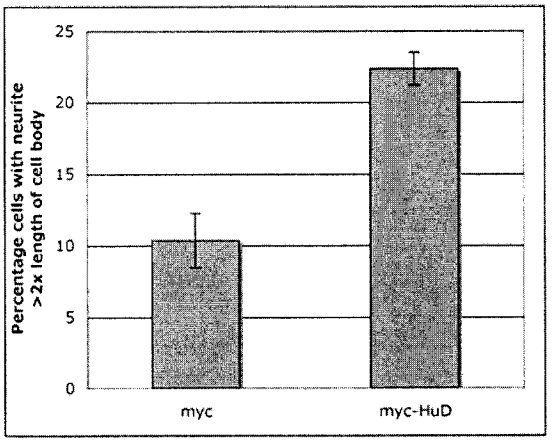
A


Hours Transfection
0
24
48
αmyc
αGAPDH

B


Hours myc-HuD Transfection
myc
24
48

C


Percentage cells with neurite >2x length of cell body
25
20
15
10
5
0
myc
myc-HuD

D

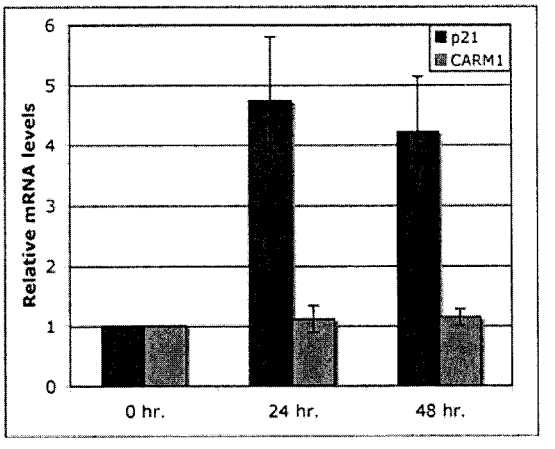

Relative mRNA levels
6
5
4
3
2
1
0
p21
CARM1
0 hr.
24 hr.
48 hr.

Figure 10. Overexpression of HuD in MN-1 cells may induce differentiation. A, Protein extracts from myc-HuD transfected cells were immunoblotted with myc and GAPDH antibodies. **B,** Cells overexpressing HuD exhibit a differentiated phenotype in comparison to cells transfected with empty vector, and **C,** produce an increased number of long neurites. Arrows indicate neurite extensions. **D,** Transfection of HuD increases p21 mRNA levels, but not CARM1 (n=4).

3.2 The pathway of HuD contribution to MN-1 differentiation downstream of CARM1

Next, we hoped to study the molecular events that occur directly downstream of CARM1 in MN-1 cells. This includes investigation of the effect of methylation on the function and expression of HuD during differentiation.

3.2.1 Methylation status of HuD does not appear to affect its turnover

Next, we hoped to determine how the expression of HuD is regulated during MN-1 differentiation. Due to the observation that overexpression of HuD promoted differentiation, we were interested in verifying if HuD protein levels are increased during differentiation. However, HuD protein levels remained constant. Interestingly, two bands were detected for HuD at approximately 39 and 42 kDa in cells that were induced to differentiate (Figure 7A). This could be a result of alternative splicing of HuD, resulting in different isoforms, or post-translational modifications of the protein, such as phosphorylation. Multiple isoforms for HuD have been described, and PCR using primers for flanking exons demonstrate that two isoforms are present in the MN-1 cells (Figure 7C). Interestingly, the larger protein observed on the Western blot was greatly diminished in the CARM1 knockdown, but the mRNA splicing pattern was identical in both the wild type and CARM1 knockdown cells. This could be due to modification of a signaling pathway that affects HuD translation or protein/mRNA stability, or another post-translational modification that first requires CARM1 methylation. This could occur either through GDNF-induced differentiation, or alternatively caused by the knockdown of CARM1, and will be discussed in later sections.

It may also be possible that CARM1 activity may regulate the half-life of HuD. It was determined using HeLa cells that methylation of p/CIP by CARM1 was associated with an increased rate of degradation (Naeem et al., 2007). Upon MN-1 differentiation, CARM1-methylated HuD may similarly be targeted for degradation at a more rapid rate. New substrate can be resynthesized in the differentiating cells, but will not be methylated due to inactivation of CARM1. Thus, the overall cellular pool of the substrate will remain the same, but the amount of methylated HuD will be relatively decreased compared to proliferating cells. We assessed the half-life of HuD briefly by incubating GST-HuD with protein extracts taken from differentiated MN-1 cells as a source of CARM1, as well as the methyl donor AdoMet at 37°C for up to nine hours. As previously shown in methylation assays and using the H3R17 antibody, less methylation is observed using these differentiated extracts. The amount of exogenous HuD protein is not altered, as determined by Western blotting for GST (data not shown), indicating that methylation of HuD does not affect its rate of degradation. Thus, the amount of HuD protein and its turnover rate do not appear to change following induction of differentiation. However, these results are preliminary and need to be corroborated. Potential experiments to confirm this are explored in the discussion.

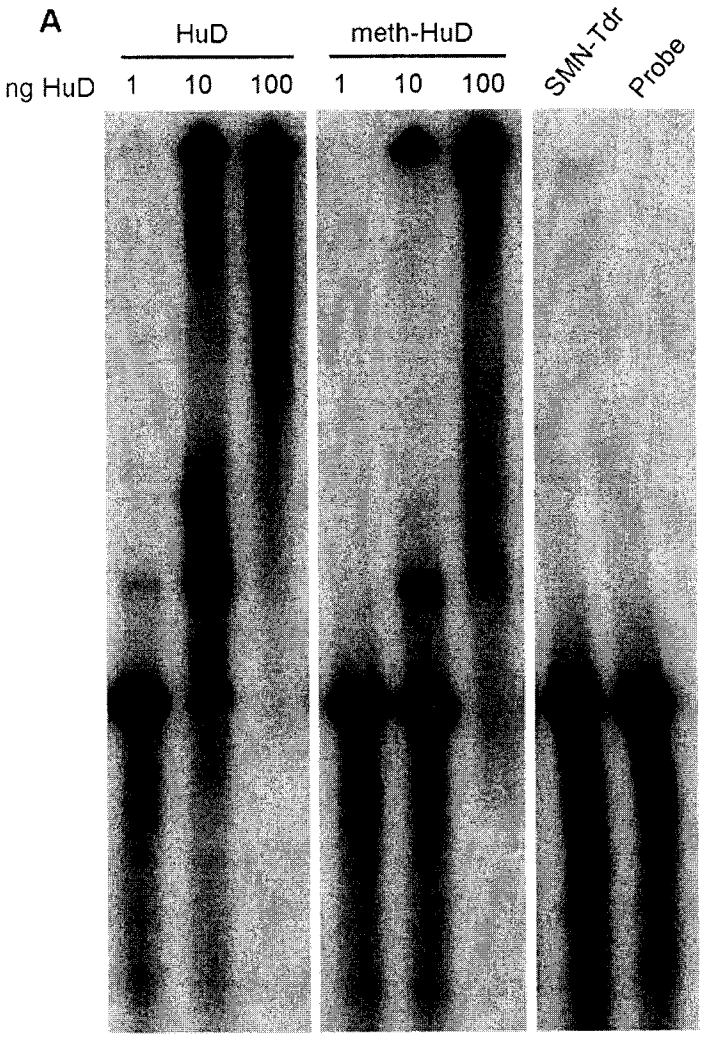
3.2.2 CARM1 methylation of HuD reduces its direct binding to p21 mRNA 3'UTR

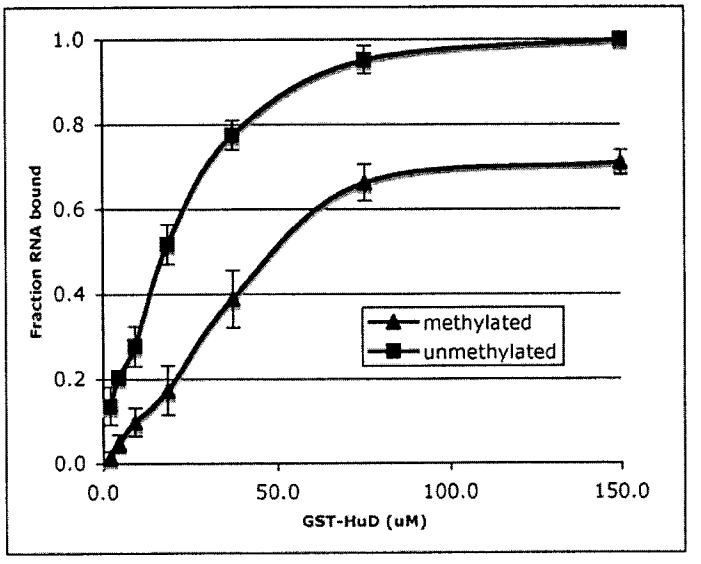
As mentioned previously, studies in PC12 cells demonstrated that unmethylated HuD immunoprecipitated more p21 mRNA than methylated HuD (Fujiwara et al., 2006). However, these results were obtained using IP/RT-PCR experiments, and do not confirm a direct influence of methylation of HuD on its RNA binding abilites. Therefore, we

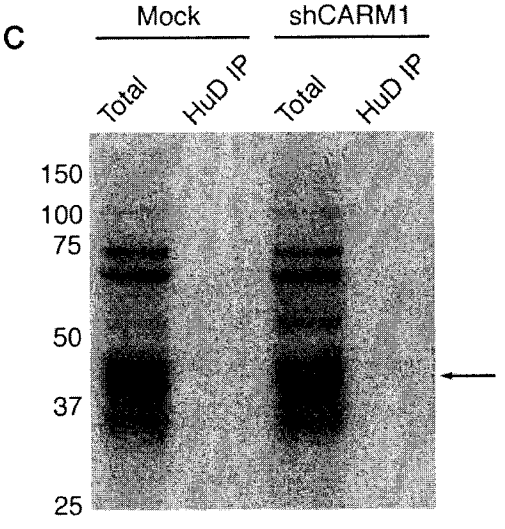
hoped to determine the direct effect of HuD methylation on its binding to p21 mRNA, as well as to study this interaction within the MN-1 cells.

To first determine if HuD interacts with p21 mRNA in the MN-1 cells, myc or myc-HuD was overexpressed in the cells as previously described. The protein was immunoprecipitated using the myc antibody, followed by extraction of RNA from the isolated complex. Reverse transcription and PCR using primers against p21 show that this mRNA does interact with HuD. GAP-43 mRNA, which has been identified to bind HuD in neurons (Mobarak et al., 2000; Chung et al., 1997), was also immunoprecipitated (Figure 11D). Both interactions were not detected following transfection with a plasmid containing only the myc tag. However, as mentioned, these interactions may not be direct, and could occur through an unknown intermediate. Due to the differentiated phenotype observed following overexpression of HuD, comparison with the CARM1 knockdown or cells treated with differentiation media could not be performed. Thus, this potentially increased proportion of unmethylated HuD, through interaction with p21, may act to increase levels of the transcript and produce a differentiated phenotype.

To study if binding between the HuD protein and p21 mRNA is direct, a p21 construct was generated containing the three AU-rich regions within the 3'UTR found to bind to HuD (Joseph et al., 1998). The construct was then transcribed in the presence of a ^{32}P-labeled UTP to generate a radioactive form of the p21 mRNA. Similar to previous experiments, a methylation assay was carried out using GST-tagged HuD and CARM1 proteins in the presence or absence of a methyl donor. A gel shift assay was then performed by incubating increasing amounts of HuD with ^{32}P-p21, and the mixtures were run on a native gel. CARM1 alone was also used to ensure that the enzyme itself does not


A
HuD
meth-HuD
ng HuD
1
10
100
1
10
100
SMN-Tdr
Probe


B
1.0
0.8
0.6
0.4
0.2
0.0
Fraction RNA bound
methylated
unmethylated
0.0
50.0
100.0
150.0
GST-HuD (uM)


C
Mock
shCARM1
Total
HuD IP
Total
HuD IP
150
100
75
50
37
25

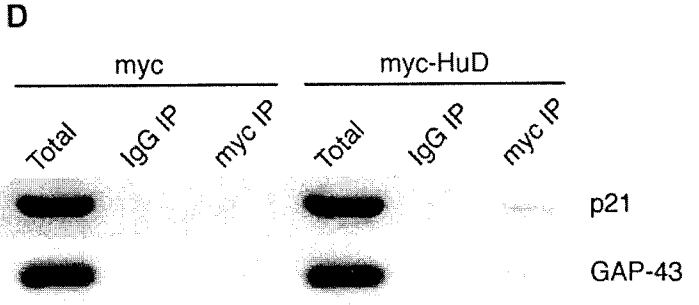

D
myc
myc-HuD
Total
IgG IP
myc IP
Total
IgG IP
myc IP
p21
GAP-43

Figure 11. HuD has a decreased affinity to directly bind p21 mRNA when methylated *in vitro*, and HuD interacts with p21 in MN-1 cells. A, Gel shift assays monitoring the ability of methylated and unmethylated HuD to bind ^{32}P-p21 mRNA. *In vitro* methylation assays were performed in the presence and absence of CARM1. **B,** Binding curves of both methylated and unmethylated HuD for p21 using nitrocellulose filter-binding assays. **C,** Crosslinking of the ^{32}P-p21 mRNA with protein extracts from mock and CARM1 knockdown cells. Immunoblotting for HuD, as shown, indicates that this protein corresponds to the molecular weight of a protein found to bind p21 mRNA. Attempts at immunoprecipitating endogenous HuD were unsuccessful. **D,** Immunoprecipitation of transfected myc or myc-HuD followed by RNA extraction and RT-PCR indicate that HuD interacts with p21, as well as GAP-43, in MN-1 cells (n=3).

bind to the p21 mRNA. In both cases, increased HuD-p21 interactions were observed by increasing the amount of protein present (Figure 11A). However, a shift was observed in the unmethylated HuD mixture when a much smaller amount of protein was present in comparison to methylated HuD, indicating that HuD protein has a higher affinity for p21 mRNA when it is not methylated by CARM1. In order to quantify this interaction, filter binding assays were performed. Again, increasing concentrations of HuD obtained from methylation assays were incubated with ^{32}P-p21, and the mixtures were passed through a nitrocellulose membrane under vacuum. Conforming to results observed in the gel shift assays, at almost all concentrations of HuD, more protein was bound to p21 mRNA in the absence of methylation (Figure 11B). Again, this demonstrates that when HuD is not methylated by CARM1, it has an increased ability to directly bind p21. Though these are in vitro studies, they further imply an important role for arginine methylation in the regulation of this RNA-binding protein, and a potential downstream effect in differentiation.

To determine if this was also the case in MN-1 cells, equal amounts of protein extracts taken from both mock and CARM1 knockdown cells were crosslinked with the ^{32}P-p21 mRNA and run on SDS-PAGE. A number of proteins within the extracts were found to bind to p21 mRNA, and one of the proteins corresponded to the molecular weight of HuD (Figure 11C). However, immunoprecipitation of HuD from the reaction mixture to confirm this interaction was unsuccessful. To further investigate the effect of methylation from MN-1 cells on this interaction, methylation assays were performed as previously described using GST-HuD and differentiated extracts as enzyme. HuD was then isolated through pulldown of the GST protein, incubated with ^{32}P-p21 mRNA and

subjected to gel shift (Supplementary Data, Figure 16A). During differentiation, where it was previously determined that HuD was less methylated by CARM1, the protein was determined to interact with p21 with higher affinity. Though this does not confirm an identical impact of methylation of HuD on interaction with p21 within MN-1 cells, we speculate that the decreased level of methylated HuD observed in differentiation promotes p21 expression through a direct and stabilizing interaction, resulting in the observed increase in p21 transcripts. To try to further characterize this interaction using only MN-1 cells, total protein extracts alone were used in a gel shift assay (Supplementary Data, Figure 16B). Complexes were observed to form, but unfortunately a number of bands caused by folding of the ^{32}P-p21 probe may have interfered with detection. Attempts to supershift HuD to confirm an interaction of p21 with the protein within the extracts were unsuccessful.

3.3 Involvement of SMN in the pathway of MN-1 differentiation

Next, we wished to determine a potential function for SMN in the MN-1 differentiation pathway. As previously discussed, numerous RNA binding proteins associate with the SMN Tudor domain, and these interactions can be enhanced by arginine methylation (Cheng et al., 2007; Boisvert et al., 2002). Because the methylation status of HuD by CARM1 was shown to decrease during differentiation, we were also interested in monitoring its interaction with the SMN Tudor domain.

3.3.1 The role of SMN in neuronal differentiation

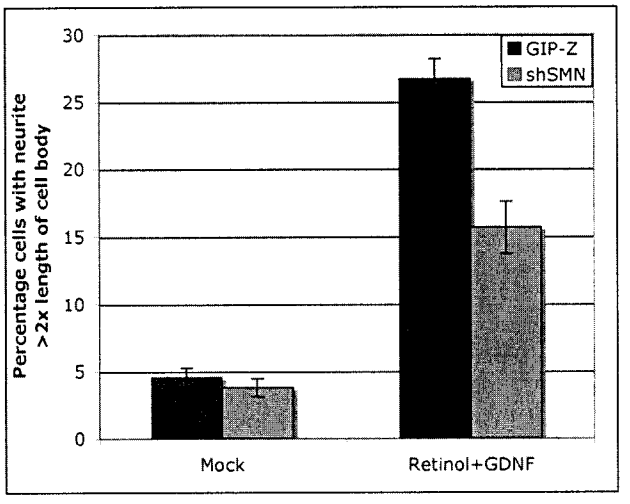
It was previously reported that neurospheres from mice lacking SMN generated neural stem cells that had an increased proliferation rate when induced to differentiate, and that these cells had fewer and shorter neurites (Shafey et al., 2008). In the absence of SMN from PC12 as well as N2a cells, these cells exhibited defects in neuritogenesis, as demonstrated by RNA interference (Bowerman et al., 2007; Tadesse et al., 2008). In addition, motor neurons from SMA mice display decreased axon growth, though their survival is not affected. (Rossoll et al., 2003). Given that our pathway of interest appears to function in regulating the switch from proliferation to differentiation, we were next interested in determining at which point SMN may be regulating differentiation. Consistent with the abovementioned findings, SMN may act only in regulating neuritogenesis, or may function more broadly in proliferation of MN-1 cells. A stable knockdown of SMN was generated in MN-1 cells with a 60% reduction in SMN protein. We first monitored the percentage of cells producing neurites at least twice as long as the cell body. When induced to differentiate, the percentage of SMN knockdown cells that

generated neurites was approximately half that of the empty vector (Figure 12A), indicating that SMN functions in promoting neurite extension, similar to that observed with other neuronal cells. In maintenance media, the SMN knockdown cells had a proliferation rate identical to cells transfected with empty vector, as determined by BrdU assay. When treated with differentiation media, the reduction in proliferation rate was less pronounced in cells with decreased SMN protein (Figure 12B). Additionally, qPCR demonstrates that reducing SMN protein in cells grown in maintenance media does not impact the amount of p21 mRNA in cells. Upon differentiation, SMN knockdown cells had reduced p21 mRNA levels in comparison to cells transfected with empty vector (Figure 12C). Thus, these results demonstrate that upon addition of neurotrophic factors, SMN is required for the transition of MN-1 cells into differentiation, in addition to extension of neurites. Interestingly, Western blotting for CARM1 demonstrates that the levels of this protein are increased in the absence of SMN (Sanchez, G., unpublished data). This indicates a potential function for SMN in MN-1 differentiation that is opposite to CARM1, and will be discussed in later sections.

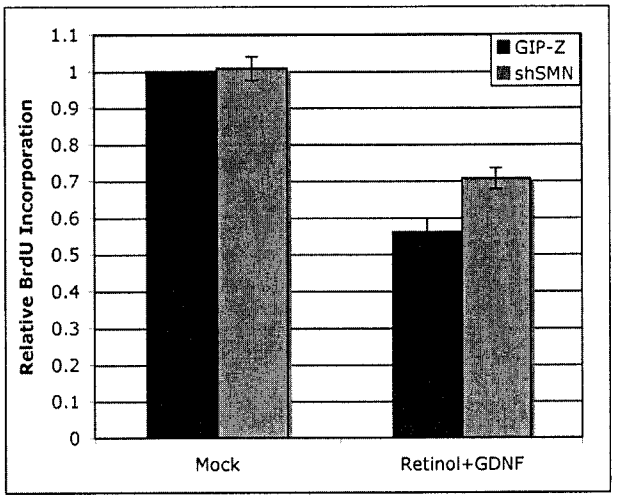
3.3.2 HuD can interact with the Tudor domain of SMN

SMN has been described to function as a chaperoning protein for numerous RNA-binding proteins (Tadesse et al., 2008; Cheng et al., 2007; Buhler et al, 1999; Selenko et al, 2001). Thus, we were next interested in determining if SMN may act in regulating MN-1 differentiation through an interaction with HuD. We first investigated if an interaction occurs between HuD and the SMN tudor domain, due to the affinity of this domain to bind other proteins (Tadesse et al., 2008; Cheng et al., 2007). In order to

A


Percentage cells with neurite >2x length of cell body
30
25
20
15
10
5
0
GIP-Z
shSMN
Mock
Retinol+GDNF

B


Relative BrdU Incorporation
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
GIP-Z
shSMN
Mock
Retinol+GDNF

C

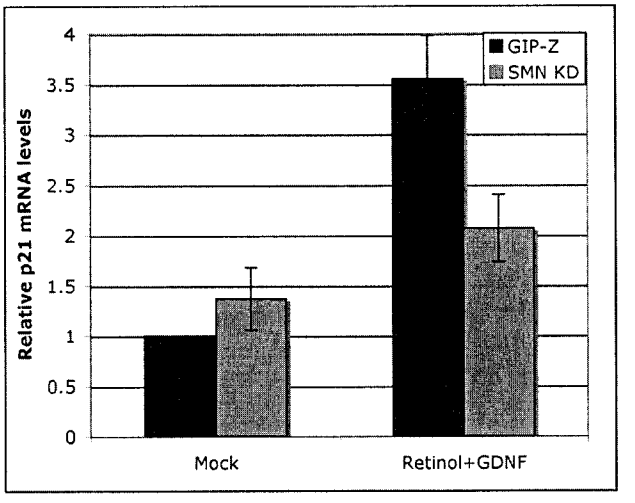

Relative p21 mRNA levels
4
3.5
3
2.5
2
1.5
1
0.5
0
GIP-Z
SMN KD
Mock
Retinol+GDNF

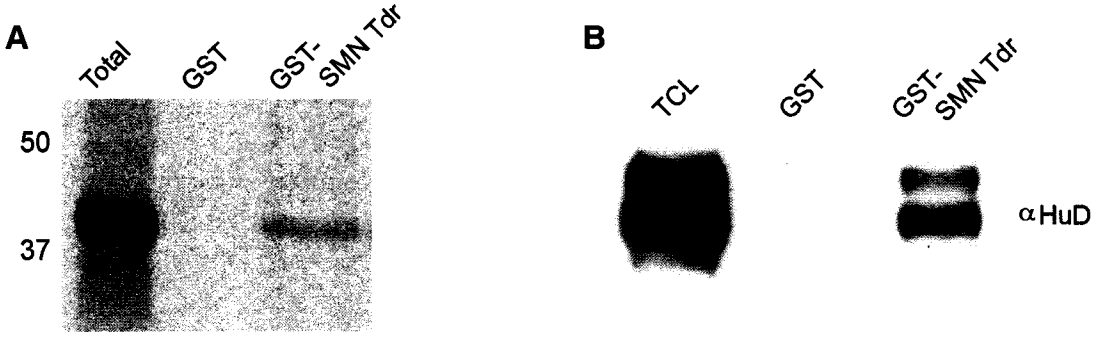
Figure 12. SMN is involved in regulating MN-1 differentiation and neurite extension. A, MN-1 cells lacking SMN produced fewer long neurites following treatment with differentiation media in comparison to cells transfected with GIPZ vector. **B,** In maintenance media, the proliferation of SMN knockdown cells is not altered. Addition of differentiation media to the SMN knockdown cells does not reduce proliferation as much in comparison to cells containing only the GIPZ plasmid. **C,** Differentiation treatment of the SMN knockdown cells does not increase p21 mRNA levels to the same extent observed in cells transfected with GIPZ vector, as determined by qPCR (n=4).

determine if these proteins interact *in vitro*, a myc-tagged HuD protein was generated using rabbit reticulocyte lysates in a transcription and translation (TnT) reaction. Incubation with GST-SMN Tudor protein and pulldown of the complex indicates that under these reaction conditions, the two proteins do interact as determined by western blotting (Figure 13A). An interaction was not detected using the GST protein tag alone.

To investigate this interaction using MN-1 proteins, GST-Tudor pulldowns were performed using MN-1 wild type extracts as a source of SMN binding proteins. Again, using western blotting, HuD was found to precipitate with the Tudor domain (Figure 13B). However, this does not confirm an interaction within the MN-1 cells. In order to verify that this interaction occurs in the cells, co-immunoprecipitation of SMN and HuD from MN-1 cells was attempted. Unfortunately, these experiments have thus far been unsuccessful, and the interaction remains to be determined. To assess localization of the two proteins, immunofluorescence was used. Both SMN and HuD were found to localize in an even distribution throughout the cytoplasm of MN-1 cells (Figure 13C); however, a colocalization remains to be clarified. Together, these results identify a novel *in vitro* interaction between these two proteins, and a potential role for SMN in the pathway of MN-1 differentiation through interaction with HuD.

3.3.3 The interaction between HuD and the SMN tudor domain is methylation-dependent and RNA-mediated

Next, we wished to determine if CARM1 methylation regulates this interaction. Methylation of SMN-binding proteins has been reported to enhance interaction between the methylated substrate the SMN Tudor domain; for example, SMN functions in


A
Total
GST
GST-SMN Tdr
50
37
B
TCL
GST
GST-SMN Tdr
αHuD

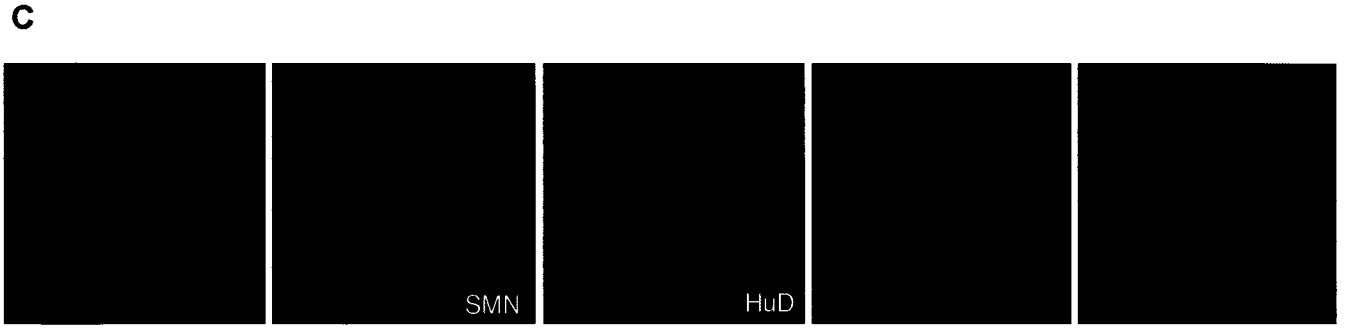

C
SMN
HuD

Figure 13. HuD interacts with the SMN tudor domain. **A,** Transcription and translation reactions of myc-HuD using ^{35}S-methionine labeling, followed by GST-SMN tudor pulldowns demonstrate that the proteins interact in this system. **B,** Interaction of the GST-tagged tudor domain with HuD in MN-1 protein extracts, using GST pulldowns and immunoblotting for HuD (n=4). **C,** Both SMN and HuD were found to localize to the cytoplasm of MN-1 cells. SMN was also found to be concentrated in granules within the nucleus (n=3).

chaperoning KSRP, a RNA-binding protein, in a CARM1-methylation dependent manner (Tadesse et al., 2008). In the TnT reactions discussed earlier, where HuD was found to precipitate with the tudor domain, HuD is methylated by CARM1 present in the lysate. To first investigate the requirement for CARM1 methylation in the interaction of the tudor domain with HuD, a tudor protein containing a mutation from glutamine to lysine at position 134 on this protein (E134K) was used in a GST-pulldown experiment in combination with the MN-1 wild type protein extract. This substitution is found in human patients with severe SMA, and reduces interactions of the Tudor domain with numerous arginine-methylated proteins (Cote and Richard, 2005). Using this mutated protein, interaction with HuD appears to be completely lost (Figure 14A). This indicates that this residue, due to its methyl-binding abilities, is required for the interaction of the SMN Tudor domain and HuD. Loss of interaction using SMA-causing mutations suggests that this binding may be functionally relevant to the etiology of SMA.

To further investigate the dependence of this interaction on CARM1 methylation, extracts from the CARM1 knockdown cells were used as a source of binding proteins and subjected to GST-Tudor pulldown as previously described. The interaction with HuD was severely diminished in comparison to the amount of protein that precipitated from the wild type extract (Figure 14B), further demonstrating that methylation of HuD enhances its interaction with the SMN Tudor domain. Thus, these results demonstrate an additional role for CARM1 methylation in regulating the interaction of these two proteins, and a potential downstream function in differentiation. It is important to note that these observations may represent a bias by overexpression of the SMN tudor domain, and must be corroborated through co-immunoprecipitation in MN-1 cells.

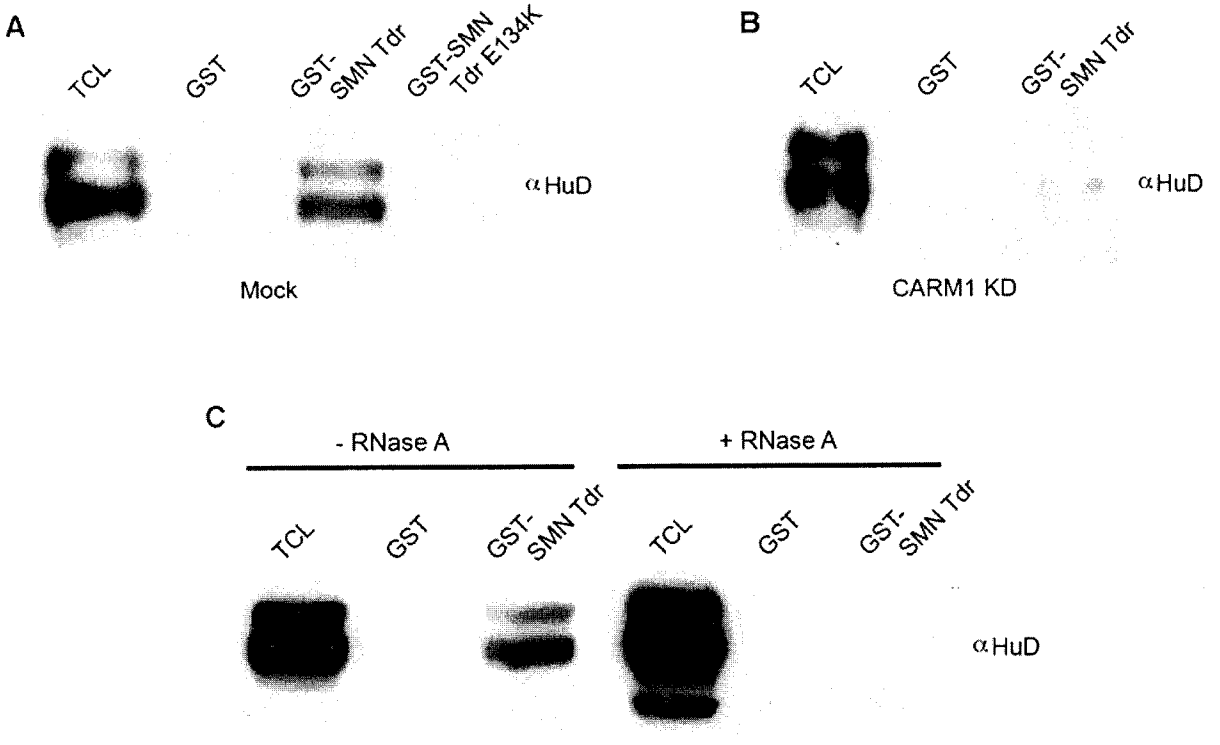

A
TCL
GST
GST-SMN Tdr
GST-SMN Tdr E134K
αHuD
Mock
B
TCL
GST
GST-SMN Tdr
αHuD
CARM1 KD
C
- RNase A
+ RNase A
TCL
GST
GST-SMN Tdr
TCL
GST
GST-SMN Tdr
αHuD

Figure 14. The SMN tudor domain interacts with HuD in a methylation-dependent, RNA-dependent manner. A, HuD from MN-1 cell extracts interacts with GST-tagged wild type SMN tudor domain, but not an E134K mutant, determined using GST pulldowns and immunoblotting for HuD. **B,** A decreased interaction is detected in pulldowns using CARM1 knockdown extracts. **C,** Wild type extracts treated with or without RNase A indicate that this interaction is RNA-dependent in this environment (n=4).

Since both SMN and HuD are capable of binding to RNA, we next wanted to examine if the observed interaction was RNA-dependent. To determine if an RNA intermediate was required, RNase was added to the wild type MN-1 lysate to degrade RNA. Interestingly, very little HuD protein was precipitated with the Tudor domain in a GST-pulldown (Figure 14C), demonstrating that this interaction is largely dependent on RNA. However, as previously mentioned, these results need to be confirmed using MN-1 co-immunoprecipitation. The identity and specific role of this RNA in facilitating an interaction between SMN and HuD remains to be determined.

Chapter 4. Discussion

4.1 Summary

We have determined that the CARM1 pathway involving HuD and p21 mRNA is active in regulating differentiation of MN-1 cells, downstream of trans-retinol and neurotrophic signaling. Here, through down-regulation of CARM1 levels, methylation of one of its substrates, HuD, is reduced, which results in an increase in levels of p21 mRNA. We have also demonstrated that CARM1 methylation of HuD negatively regulates its direct interaction with the RNA. Additionally, we also identified a novel interaction between HuD and SMN. We propose that SMN functions in MN-1 differentiation as an adaptor protein through arginine methylation-regulated interactions with specific RNA binding proteins, including HuD, and that distinct mRNP complexes on mRNA may be regulated either similarly or differentially by PRMTs and/or SMN. Together, these results suggest a key role for arginine methylation and SMN in regulating differentiation of motor neurons and may present fundamental insights into the cell-specific etiology of SMA.

4.2 The CARM1 pathway involving HuD and p21 mRNA is active in regulating differentiation of MN-1 cells

Previously, a study by Fujiwara et al. demonstrated a post-transcriptional function for CARM1 in maintaining the proliferative state of PC12 cells. Addition of NGF reduced the methylation status of a CARM1-specific substrate, HuD, which led to increased levels of p21 mRNA and cellular differentiation. Overexpression of a methylation-resistant form of HuD bound more p21 mRNA then methylated HuD, and this interaction stabilized the transcript. Reducing CARM1 levels through RNA interference mimicked NGF stimulation and resulted in p21 mRNA stabilization and neuronal differentiation (Fujiwara et al., 2006). We first determined that this pathway was active in MN-1 cells using trans-retinol and GDNF to induce differentiation, where we observed a decrease in proliferation rate and increased production of neurite extensions. Methylation assays monitoring the ability of CARM1 within MN-1 total protein extracts to methylate HuD demonstrated that the methylation status of this protein decreases rapidly during differentiation, which correlated with an increase in p21 mRNA.

We were next interested to determine if knockdown of CARM1 from MN-1 cells would produce a phenotype similar to that observed following addition of neurotrophic factors. Depletion of CARM1 in a stable knockdown was sufficient to reduce the growth rate of MN-1 cells and promote neurite outgrowth, independent of neurotrophic factors. The ability of the extracts to methylate HuD was abolished, and both a CARM1 transient knockdown and the stable knockdown greatly increased the amount of p21 transcript in the cells. Thus, the loss of CARM1-dependent methylation of HuD may a key process in which these neurotrophic factors induce the differentiation of MN-1 cells.

4.3 Regulation of CARM1 in MN-1 differentiation

As mentioned, the methylation status of the CARM1-specific substrate HuD was somehow reduced following addition of neurotrophic factors to the MN-1 cells; however, the mechanism remains unclear. To determine how neurotrophic factors alter the methylation abilities of CARM1, we monitored CARM1 levels following induction of differentiation. Here, the amount of CARM1 protein was rapidly down-regulated, demonstrating that a pathway exists upstream of CARM1 that is activated by addition of these neurotrophic factors and is responsible for the decline in amount of enzyme. Interestingly, CARM1 mRNA levels were found to remain constant through this process, and addition of a proteasome inhibitor stabilizes the amount of the protein. Together, these results strongly suggest that upon differentiation, regulation of CARM1 takes place at the level of protein stability. CARM1 protein may be targeted for degradation by a signaling cascade activated by addition of neurotrophic factors. Higashimoto et al. have demonstrated that phosphorylation of CARM1 can lead to its inactivation *in vitro*. Our lab has found that CARM1 is increasingly phosphorylated during differentiation of C2C12 myoblasts (Paquette, B., unpublished data). We have so far failed to detect a slower-migrating band that could represent a phosphorylated form of CARM1 in MN-1 cells, suggesting that distinct mechanisms may exist in different cell types to regulate CARM1 levels or activity.

We were next interested in monitoring the localization of CARM1. CARM1 was previously believed to localize predominantly in the cell nuclei, but demonstrated cytoplasmic localization, as well as intense nuclear staining, in PC12 cells (Fujiwara et al., 2006). In MN-1 cells, CARM1 was found to localize to the cytoplasm and is largely

concentrated at the nuclear periphery. When the cells were treated with differentiation media, CARM1 further concentrates in the perinuclear space, than appears to diffuse into the cytoplasm in late differentiation. However, it is important to note that there does not appear to be a decrease in CARM1 protein following addition of differentiation media, as was observed using western blotting. Other experiments have indicated that the antibody used to detect CARM1 may not recognize the native form of the protein. Thus, the proteins detected here may be the result of non-specific interactions with the antibody, impeding speculation of the localization of CARM1 in the MN-1 cells. Immuno-fluorescence studies using the CARM1 knockdown cells could be used in order to confirm the specificity of the antibody. Alternatively, western blotting may not detect all CARM1 protein in the cells, as only the soluble fraction is used for SDS-PAGE following lysis. Following induction of differentiation of MN-1 cells, if CARM1 is only located in the insoluble fraction, it would not be detected by western blotting as previously observed. However, the protein may still be present in the insoluble fraction through interaction with chromatin, for example. Thus, it may be CARM1 present in the insoluble regions of the cell that is detected by immunofluoresence. In order to test this possibility, both the soluble and insoluble fraction from cell lysis could be run on SDS-PAGE. If CARM1 is present in the insoluble fraction during MN-1 differentiation, it would be detected by western blotting.

As previously mentioned, Fujiwara et al. demonstrated that the methylation status of HuD was decreased following NGF treatment, but they did not investigate methylation of other CARM1-specific substrates. Due to the decrease in CARM1 protein observed by western blot following addition of neurotrophic factors, we monitored the methylation

status of the overall pool of CARM1 substrates in this process. Upon induction of differentiation, we observed a decline in the CARM1-specific methylation of numerous substrates using the H3R17 antibody, indicating that the ability of this methyltransferase to maintain the methylation status of is substrates is reduced during differentiation. One of these proteins had a molecular weight that corresponded to HuD; however, the identity of the protein must be confirmed using immunoprecipitation and western blotting with H3R17.

However, it is important to consider that this increased pool of unmethylated CARM1 substrates may be the result of a number of different processes. The substrates may have altered turnover rate in the absence of methylation, and the reduction in methylated proteins may be due to reduced levels of CARM1 substrates themselves, not necessarily a decrease in methylation. It has been previously shown that in HeLa cells, CARM1 methylation of p/CIP resulted in an increased degradation rate of the protein (Naeem et al., 2007). We investigated if HuD may also be degraded at a more rapid rate in the CARM1-containing MN-1 cells. In preliminary experiments, we found that methylation of the GST-tagged HuD protein did not impact its rate of degradation. Thus, it appears at least in the case of this substrate that CARM1 methylation does not affect substrate turnover. HuD protein containing mutations at its methylation site, either to a seemingly constitutively methylated form or a methylation-resistant form of the protein, have been generated and could be used in similar experiments to confirm the effect of methylation on HuD turnover (outlined in section 4.4). Additionally, western blotting for HuD following inhibition of translation with cycloheximide in the proliferating, differentiated and CARM1 knockdown cells would help to verify this observation in

cells. Another prospective mechanism of how methylation status of CARM1 substrates could be decreased during differentiation, without altering the total amount of the protein, is that the substrates could be actively demethylated upon addition of neurotrophic factors. The first arginine demethylase, JMJD6, was recently described (Chang et al., 2007). However, the conditions used for methylation assays performed in order to study methylation status of HuD are not compatible with JMJD6 demethylase activity. Hence, we can rule out the contribution of demethylase activity in the overall methylation of HuD, at least in these assays. Whether a demethylase contributes to the regulation of HuD methylation status *in vivo* remains to be determined.

Thus, these results support the emerging concept that CARM1 functions as a key regulator of the transition from a stem cell or proliferative state into differentiation in numerous lineages. While many studies have focused on a transcriptional role for CARM1 through methylation of histones, there now seems to be a post-transcriptional aspect to CARM1 regulatory function in this process, part of which is characterized here.

4.4 CARM1 regulation of HuD function in MN-1 differentiation

HuD has been described to bind AREs in the 3'UTR of its mRNA targets to stabilize the transcript (reviewed by Hinman and Lou, 2008). In PC12 cells, Fujiwara et al. determined by immunoprecipitation of HuD and RT-PCR of interacting mRNA that CARM1 methylation of HuD correlated with reduced association of the protein with p21 mRNA in neuronal cells. However, these experiments do not provide a very quantitative analysis of the interaction. Using gel shift and filter binding assays, we have proven that methylation of HuD by CARM1 negatively regulates its interaction with the p21 ARE-

containing fragment. In addition, gel shift assays show that a number of complexes with increasing molecular weights form between HuD and the 3'UTR of p21 mRNA, indicating that the protein oligomerizes on this target. This is consistent with a previous report that HuD is capable of forming dimers on its target mRNA through its third RRM (Kasashima et al., 2002).

These results are the first to demonstrate that CARM1 can directly influence the RNA binding activity of a substrate. To date, very little is known about the role of arginine methylation in regulating RNA binding. Similar to our results using HuD, the methylation of SAM68 by PRMT1 decreased its poly(U) binding abilities *in vitro* (Rho et al., 2007), and methylation of hnRNP A1 also functioned to reduce its RNA binding ability (Rajpurohit et al., 1994). Thus, it appears that at least in these cases, arginine methylation may be potential control mechanism to negatively regulate RNA binding. However, other studies have found that protein-RNA interaction is not affected by arginine methylation (Valentini, et al., 1999; Raman et al., 2001). Aside from methylating chromatin-associated factors, such as histones and p300, CARM1 also methylates a number of RNA binding proteins, including KSRP, U1C, and CA150 (Tadesse et al., 2008; Cheng et al., 2007), and our results could indicate an additional regulatory function for arginine methylation on function of these proteins.

We next investigated if HuD interacts with p21 in MN-1 cells. Attempts at IP/RT-PCR experiments were unsuccessful; however, overexpression of HuD determined that this RNA-binding protein bound the p21 transcript. To determine the methylation-dependent regulation of the interaction between endogenous HuD protein and p21 in these cells, UV crosslinking was performed using MN-1 protein extracts and the p21

3'UTR. This revealed numerous interacting proteins, one of which had a molecular weight that corresponds to that of HuD. In the absence of CARM1 from cells, it appears that a slightly increased amount of this protein binds the mRNA. Unfortunately, attempts to confirm the identity of this protein using immunoprecipitation were unsuccessful. In addition, methylation of GST-HuD using CARM1 in MN-1 extracts also demonstrates that HuD has a higher affinity for p21 following induction of differentiation, where we have shown that methylation of HuD is decreased (Supplementary Figure 16). Together, these results are consistent with our proposed model that in MN-1 cells, CARM1 methylation decreases the affinity of HuD for p21 mRNA.

p21 is a cell cycle inhibitor that blocks proliferation and promotes neurite outgrowth (Erhardt and Pittmann, 1998; Yan et al., 1995; Yan et al., 1997). Fujiwara et al. demonstrated that the interaction of HuD with p21 stabilizes the transcript and increased its half-life in PC12 cells, leading to up-regulated levels of the mRNA and protein, thus promoting a differentiated phenotype. In MN-1 cells, we determined that p21 mRNA levels are also greatly increased in differentiation, and speculate that similar to PC12 cells, this is due to increased stability of this mRNA by HuD.

We were also interested to determine if other RNA-binding proteins functioned in regulating p21 mRNA levels. As previously mentioned, results of UV crosslinking demonstrated that multiple proteins within MN-1 cells bound the 3'UTR of p21. One protein, at approximately 55kDa, had a higher binding capacity for the mRNA target in the CARM1-deficient cells. We speculated that this protein may be a member of the hnRNP family of RNA-binding proteins. Like the Hu proteins, hnRNPs are involved in mRNA stability, as well as transport and translation (Schenkel, 1988), and can be

methylated by the PRMTs (reviewed in Pahlich et al., 2006). One member of this family, hnRNP K, was discovered to bind the 3'UTR of p21 to repress its translation in proliferating neuronal cells. Upon inducing differentiation, HuB interacts with the p21 mRNA as well as hnRNP K. Here, the Hu protein antagonizes hnRNP K to allow p21 protein production, demonstrating an activity balance between the two RNA-binding proteins in the regulation of p21 translation during neurogenesis (Yano et al., 2005). Though this model does not appear to correlate to the results of the crosslinking, the potential role of hnRNP K in this pathway was of interest. We found that hnRNP K interacts with the p21 3'UTR *in vitro*. In addition, co-immunoprecipitation experiments using MN-1 cells demonstrated that hnRNP K also interacts with HuD (Supplementary Figure 17). Thus, hnRNP K may also be a member of a distinct mRNP complex containing HuD and p21, and CARM1 methylation of the proteins may function in regulating these interactions. Though this remains to be confirmed in MN-1 cells, these results further indicate the potential for an activity balance occurring between numerous components involved in the regulation of MN-1 differentiation.

4.5 Role of HuD in MN-1 differentiation downstream of CARM1

HuD has been best characterized to function in promoting differentiation in a number of neuronal cell lines, as well as in vivo (reviewed by Deschênes-Furry et al., 2006). Previous studies have found that in NGF induced PC12 cells, HuD protein levels are increased slightly as cells differentiate and extend neurites (Dobashi et al., 1998). We next investigated the levels of this protein in MN-1 cells; however, the amount of HuD does not change following induction of differentiation. Interestingly, two bands are

detected using Western blotting at approximately 39 and 42 kDa. Both bands are expressed at consistent levels throughout differentiation. In the CARM1 knockdown cells, only the lower protein is present. HuD is known to be alternatively spliced to produce two major transcript variants (Okano and Darnell, 1997; Inman et al., 1998; Steller et al., 1996; Tora et al., 2000), which we determined to be present in both the wild type and CARM1-deficient MN-1 cells. The resulting protein products would differ by approximately 2.5kDa; thus, the observed proteins may each represent an isoform. However, the relative ratio of these transcripts did not change in CARM1-deficient cells in comparison to the wild type cells, indicating that a signaling pathway activated upon transfection of the shRNA could alter HuD translation or protein stability, resulting in loss of expression of the larger protein. Alternatively, these pair of bands could be caused by post-translational modifications of HuD, including phosphorylation. HuD contains numerous sites that can be phosphorylated by serine/threonine kinases (Pascale et al. 2005; Mobarak et al., 2000). This pathway could be somehow downregulated due to CARM1 knockdown. The phosphorylation of HuD could be investigated using a phosphatase assay.

To gain insights into potential specific subcellular functions for HuD, we next investigated if any changes occurred in its localization during MN-1 differentiation. HuD has been reported to localize with its mRNA targets in the cytoplasm of the neuronal cells to stimulate neurite outgrowth (Kasashima et al., 1999; Kasashima et al., 2002). In undifferentiated MN-1 cells, HuD localized to both the cytoplasm and the nucleus. Following induction of differentiation, HuD is found largely in the cytoplasm as well as in extensions. Since we predict that HuD has an increased affinity for interaction with

p21 in differentiation, these observations are consistent with previous reports that HuD interacts with target transcripts in the cytoplasm during this process.

In PC12 cells, overexpression of HuD is sufficient to promote neurite outgrowth (Anderson et al., 2000; Kasashima et al., 1999). We wanted to assess if this was the case in MN-1 cells, which would indicate that HuD is downstream of CARM1 in the differentiation pathway. A greater percentage of HuD-transfected cells were found to express neurites and amount of p21 mRNA was increased in comparison to MN-1 cells expressing an empty vector. Based on these results, we speculate that this effect of HuD overexpression may be explained by an increase the pool of unmethylated HuD, thus mimicking the situation observed when CARM1 levels are reduced. In addition, we demonstrated that the transfected HuD also interacts with GAP-43 mRNA, which has been implicated in the formation of neuronal processes (Mobarak et al., 2000). Hence, it is possible that CARM1 methylation may regulate the interaction of HuD with other mRNAs important for neuronal differentiation, although this will require further experimentation. It is important to consider that there may be other CARM1 substrates that may also function in this differentiation pathway. However, our observations demonstrate that overexpression of HuD alone is sufficient to reproduce the differentiated phenotype, indicating that, downstream of CARM1, HuD has a key function and is likely dominant over other potential RNA-binding proteins in this pathway.

4.6 Future Work: Determination of the pathway of HuD contribution to differentiation downstream of CARM1

To further investigate the function of HuD in MN-1 differentiation downstream of CARM1, an shRNA against the 3' end of HuD mRNA has been generated. Stable transfection of this vector into the MN-1 cells should remove endogenous HuD, but will also allow for transfection of mutated forms of the protein. Due to the observed functions of HuD in promoting neuronal differentiation and neurite extension, we speculate that loss of HuD from the MN-1 cells will reduce or inhibit the ability of the cells to properly differentiate following addition of neurotrophic factors. These predictions will be verified by monitoring proliferation rate and ability to generate neurites, as well as qPCR to study p21 transcript levels.

To investigate the affect of methylation on HuD function, two mutants have been generated, both containing mutations at the arginine 236 residue which is methylated by CARM1. An R236K mutant, a methylation resistant form of the protein, as well as a R236W mutant, whose side chain mimicks the methylated form of the arginine residue, will be used. These mutants, as well as the wild-type HuD, will be used to rescue the HuD-deficient MN-1 cells, upon which the abovementioned parameters will be monitored. We predict that introduction of the methylation-resistant form of HuD will interact with highest affinity with p21 mRNA. This would therefore stabilize the transcript and promote the differentiated phenotype at a more increased level in comparison to the wild type HuD, which can still be methylated by CARM1 in the cells. The R236W mutant, which appears as a constitutively methylated form of the protein, is predicted to behave in a manner similar to the HuD-deficient cells. This protein is

expected to have decreased affinity for p21, resulting in degradation of the transcript and allowing the cells to continue through the cell cycle. Thus, this experiment will confirm that arginine methylation of HuD is involved in its function in the regulation of MN-1 differentiation.

4.7 The potential function of SMN in the MN-1 differentiation pathway

Distinct RNA-binding proteins recognizing 3'-UTR targets within mRNAs can assemble together with translation factors and ribosomal subunits to form mRNPs, also referred to as mRNA granules (Kiebler and Bassell, 2006; Rossoll and Bassell, 2009). These mRNPs are targeted to dendrites and axons of neurons to allow local translation of the bound mRNA (Rossoll et al., 2009), and this localization is an important component of axonal guidance and synaptic plasticity. Recently, impairment of mRNPs has been linked to SMA (Lin and Holt, 2008; Rossoll and Bassell, 2009). SMN has been described to function as a chaperone for assembly of numerous RNA-binding proteins into various mRNPs (Rossoll and Bassell, 2009). Thus, we were also interested to study a potential role of SMN in regulating HuD. As previously discussed, the tudor domain is responsible for numerous protein-protein interactions. We first determined that the tudor domain alone is capable of directly binding HuD *in vitro*, using GST-tudor pulldown assays with rabbit reticulocyte lysates, as well as using extracts from wild type MN-1 cells as a source of HuD. However, we have yet to characterize this interaction and its regulation in MN-1 cells through co-immunoprecipitation. Using immunofluorescence, both SMN and HuD were found to localize in an even distribution throughout the cytoplasm with a great deal of overlap in cycling MN-1 cells; however, the interaction remains to be confirmed.

To study this further and establish a potential function for HuD within mRNPs, we could investigate if a direct interaction occurs between the two proteins in RNA granules using BiFC or FRET techniques. In addition, we have determined that the interaction between HuD and SMN appears to be RNA-dependent, although a weak direct interaction was detected. This suggests that these proteins likely interact in a functional context, perhaps in the formation of an mRNP. The weak direct interaction between the proteins could serve to stabilize the mRNP once it is formed on the RNA. Co-immunoprecipitation of SMN with HuD in the presence of RNase could be used to further investigate the RNA-dependence of this interaction.

SMN has been implicated in promoting neuronal development, particularly in outgrowth of neurites, where it interacts with RNA-binding proteins (Rossoll et al., 2003, Bowerman et al., 2007; Tadesse et al., 2008). In motor neuron cells, SMN is found to localize to neurites and growth cones, and motor neurons from SMA mice have deceased ability to generate axons (Jablonka et al., 2001; Fan and Simard, 2002; Rossoll et al., 2003). SMN-deficient PC12 and N2a cells exhibited defects in neuritogenesis, as determined through RNA interference (Bowerman et al., 2007; Tadesse et al., 2008). Since the pathway we have investigated thus far seems to act mainly at the level of the switch from proliferation to differentiation, we wanted to assess in our cell system at which level SMN was regulating differentiation, an important question that still remains unclear. We were interested in investigating the potential role for SMN in regulating proliferation, or to determine if it simply functioned in neurite extension. Similar to other neuronal cell lines, MN-1 cells stably lacking SMN were found to display shorter neurite extension upon differentiation. In maintenance media, SMN knockdown cells had the

same proliferation rate and expressed the same level of p21 mRNA as did the control vector. Following induction of differentiation, p21 levels did not increase as much as seen in the control cells. In addition, the proliferation rate of the SMN-deficient cells was not decreased to the same extent as the control cells in differentiation. Thus, it appears that in MN-1 cells, SMN is required for neurite extension, as has been shown in PC12 and N2a cells (Bowerman et al., 2007; Tadesse et al., 2008). However, in these models, an alteration in the proliferation rate of the cells was not detected. Thus, in MN-1 cells, SMN may also have additional functions that contribute to the proper transition from proliferation into differentiation through regulation of p21.

SMN has been reported to interact with RNA-binding proteins in neurites of PC12 and N2a cells (Rossoll et al., 2003; Tadesse et al., 2008). SMN colocalizes with hnRNP R in neuronal processes, where this RNA-binding protein is believed to promote b-actin mRNA localization and translation in neuronal growth cones (Rossoll et al., 2002; Rossoll et al., 2003; Jablonka et al., 2001). Motor neurons deficient in SMN mislocalized b-actin mRNA and protein in both axons and growth cones, resulting in reduced axon growth (Rossoll et al., 2003). Due to the observed decrease in neurites produced in SMN-deficient MN-1 cells, we speculate that b-actin mRNPs may also be important in neurite extension in these cells. Since overexpression of HuD promotes the generation of neurites, we predict that HuD may somehow function within these mRNPs as well, possibly through chaperoning by SMN. To gain further insight into this relationship, HuD could be overexpressed in the SMN-deficient MN-1 cells. We predict that if SMN is necessary for transition of MN-1 cells into differentiation, the absence of SMN may inhibit the normal differentiated phenotype observed following HuD transfection.

Alternatively, overexpression of HuD may rescue the differentiated phenotype despite the lack of SMN. These experiments will help to determine if SMN functions in regulating differentiation upstream or downstream of HuD, thus further elucidating the role of SMN in motor neuron differentiation.

Interestingly, in the SMN-deficient cells, an increase in CARM1 protein levels was observed (Sanchez, G., unpublished data). Together with the observation that CARM1 maintains proliferation of MN-1 cells and the speculated function for SMN in promoting differentiation, this indicates a role for SMN that is opposite to CARM1. We speculate that CARM1 may function to delay differentiation in the SMN-deficient cells. Future experiments to further investigate this could include knocking down CARM1 in the SMN-deficient cells. Similar to experiments overexpressing HuD, we speculate that if SMN is required for differentiation of MN-1 cells, the SMN deficiency will inhibit the differentiated phenotype observed following CARM1 knockdown. Following addition of neurotrophic factors, this additional knockdown may rescue the differentiated phenotype, allowing production of extensions and a reduced proliferation rate similar to that found in wild type MN-1 cells. Alternatively, even in the presence of neurotrophic factors, SMN may still be required for neurite extension. Again, the phenotypes observed in these experiments would help to place SMN upstream or downstream of CARM1 in the regulatory pathway of MN-1 differentiation.

Due to the speculated function for SMN opposite to CARM1 in regulating MN-1 differentiation, we were also interested in determining if the interaction of SMN with HuD was mediated by CARM1. The tudor domain of SMN interacts with numerous arginine-methylated proteins, including various RNA-binding proteins, with a higher

affinity when the substrate is methylated. One such example is the CARM1-dependent interaction between KSRP and SMN in RNA granules (Tadesse et al., 2008). KSRP, in addition to regulating the localization of the b-actin mRNA to growth cones, is known to bind and destabilize the p21 transcript, resulting in degradation of the mRNA. Loss of interaction between SMN and KSRP resulted in an increase in p21 mRNA levels. These results suggest that SMN, as well as CARM1, are required for proper assembly of a distinct RNP complex containing KSRP, and that these interacting proteins may function similarly in motor neuron differentiation. Preliminary results indicate that this was also the case for HuD, where pulldown of GST-SMN tudor with MN-1 cells deficient in CARM1 did not precipitate as much HuD as did the mock knockdown cells. Thus, in this context, interaction of HuD with the tudor domain of SMN appears to be CARM1-methylation dependent, although this effect may be indirect. However, we have yet to confirm this interaction and its regulation in MN-1 cells using co-immunoprecipitation. Identification of this novel interaction and possible methylation-dependent regulation provides insights into the potential function for SMN in mediating the differentiation of MN-1 cells through interaction with HuD.

Alternatively, it may be possible that in addition to CARM1, another PRMT can methylate HuD, and that interaction with SMN is regulated through methylation by this other PRMT and not by CARM1. Methylation of HuD by this PRMT would increase in differentiation, opposite to the decline in methylation by CARM1 as observed. Here, the balance between activity of PRMTs would dictate which SMN-containing complex is stabilized/chaperoned. Other potential SMN interactors, including KSRP, are dependent on CARM1; when levels of this PRMT are reduced, SMN is released from these

complexes. KSRP is known to act as a destabilizer for many transcripts, including p21 (Barreau et al., 2005; Gherzi et al., 2004). The SMN-KSRP complex could act to maintain low levels of this mRNA. When CARM1 levels are decreased, SMN could then be transferred to HuD, an interaction independently promoted by methylation of HuD by a distinct PRMT, where it would form a stabilizing complex on p21.

Recently, a few CARM1 substrates have also been found to be methylated by PRMT5. CA150, a splicing factor mentioned previously, is methylated by both PRMTs, but its interaction with SMN is regulated exclusively by CARM1. SmB, a component of the core complex involved in snRNP biogenesis, interacts with SMN in a PRMT5-dependent manner, despite methylation by both abovementioned PRMTs (Cheng et al., 2007). We speculate that a similar differential regulation may be true for other SMN interactors, including HuD, and/or additional combinations of PRMTs.

We were next interested to determine which other PRMTs may be responsible for this alternate methylation of HuD. As previously discussed, methylation assays using GST-HuD and MN-1 extracts as a source of PRMTs indicated that methylation of this protein decreased rapidly upon induction of differentiation. Western blotting for various PRMTs demonstrated that both PRMT5 and PRMT1 were present at constant levels throughout differentiation, indicating that these PRMTs are not likely responsible for additional methylation of HuD. Interestingly, our lab previously determined that PRMT8 is upregulated during P19 cell differentiation. Previously, our group demonstrated that PRMT8-deficient cells are unable to differentiate upon stimulation, further indicating that this enzyme is required for proper transition into differentiation (Pelletier, M., unpublished data). Recently, PRMT8 has been reported to be highly expressed in the

developing mouse brain, and is maintained throughout adulthood (Kousaka et al, 2009). In MN-1 differentiation, PRMT8 is not detected; however, this was determined prior to optimization of the differentiation protocol, and may be due to insufficient differentiation of the cells. In addition, PRMT3 was shown to have a similar expression profile to PRMT8 in mouse brain (Ikenaka et al., 2006). Thus, it may be possible that either of these PRMTs, or another PRMT that is expressed in a similar manner, is responsible for the methylation of HuD and regulation of its activity in vivo.

4.8 Relevance in the etiology of SMA

Recently, a dual dysfunction hypothesis for SMA has been proposed. This theory explains the wide spectrum in clinical severity of the disease by suggesting that two distinct mechanistic and temporal defects may cause motor neuron degeneration in SMA (Pellizoni, 2007). Here, greater reductions in functional SMN, resulting in early developmental defects and more severe forms of SMA, would largely be caused by a decrease in snRNP biogenesis. Milder forms of the disease, in contrast, may develop due to impaired axonal mRNP metabolism, not snRNP assembly. At all decreased levels of SMN, the unifying premise of both mechanisms is that the pathological consequence is due to an imbalance of the homeostasis of mRNAs that are uniquely expressed or vital in motor neurons. Alterations in processing, localization or translation of these mRNAs would temporally and spatially modify the expression of either a single or numerous proteins necessary for development and function of the neuromuscular system.

It has been suggested that heterogeneity in protein and RNA components of the various mRNP transport granules may play a role in regulating differential translation and

neuronal plasticity (Vanderklish and Edelman, 2005). We propose that arginine methylation, in addition to SMN, functions in the formation of specific mRNP complexes involved in regulating motor neuron differentiation, and speculate that this could also be the case in mouse SMA models and human tissues. We have identified a potential component of one such distinct mRNP to be the RNA-binding protein HuD, which functions in regulating differentiation of MN-1 cells through increasing p21 mRNA. This up-regulation in p21 levels, likely caused by stabilization of the transcript, is negatively regulated by CARM1-methylation of HuD. p21 functions in inhibiting the cell cycle and promoting neurite outgrowth (Erhardt and Pittman, 1998); thus, this methylation maintains the proliferative state of the MN-1 cells. p21 mRNA has been reported to be up-regulated in human SMA patients and spinal cord tissues of mice with mild forms of SMA (Olaso et al., 2005; Tadesse et al., 2008). We have found that the inhibited proliferation of SMN knockdown cells may be explained by higher CARM1 levels, although further experimentation will be required to clarify the mechanism(s) involved in this up-regulation. It would also be crucial to monitor for potential misregulation of HuD in SMA tissues. To date, the importance of alterations in p21 stability in the pathophysiology of SMA has yet to be determined. Several mRNAs, in addition to p21, are increased in human SMA patients as well as mouse models (Balabanian, 2007; Olaso, 2005; Anderson, 2004). Thus, in addition to the misregulation of alternative splicing in SMA, and it is possible that numerous transcripts, including p21, could also be abnormally regulated at the level of mRNA stability or transport, and play a role in the etiology of SMA. Clarifying which of these defect(s) are directly causal in SMA will be the next big challenge in understanding and hopefully finding a cure for this disease.

4.9 Conclusion

We have identified an RNA-binding protein that interacts with SMN and whose RNA-binding abilities are altered in a methylation-dependent manner during differentiation of motor neuron-like cells. We have implicated a potential role for discrete mRNP complexes in this process that may be regulated either similarly or differentially by arginine methylation and/or SMN, and thus may be misregulated in the pathophysiology of SMA. The findings presented in this thesis support our initial hypothesis and fulfill the specific objectives stated for this project. Using this data, we hope to help further understand the underlying mechanisms resulting in the motor neuron-specific degradation observed in SMA patients in order to find a prospective future treatment of this deadly disease.

References

Anderson, K.D., Morin, M.A., et al. (2000). "Overexpression of HuD, but not of its truncated form HuD I+II, promotes GAP-43 gene expression and neurite outgrowth in PC12 cells in the absence of nerve growth factor." J Neurochem 75: 1103-1114.

Anderson, K.D., Sengupta, J., et al. (2001). "Overexpression of HuD accelerates neurite outgrowth and increases GAP-43 mRNA expression in cortical neurons and retinoic acid-induced embryonic stem cells in vitro." Exp Neurol 168: 250-258.

Anderson, K.N., Baban, D., et al. (2004) "Expression profiling in spinal muscular atrophy reveals an RNA binding protein deficit." Neuromuscul Disord 14: 711-722.

Akamatsu, W., Fujihara, H., et al. (2005). "The RNA-binding protein HuD regulates neuronal cell identity and maturation." Proc Natl Acad Sci USA 102: 4625-4630.

Antic, D., Lu, N. and Keene, J.D. (1999). "ELAV tumor antigen, Hel-N1, increases translation of neurofilament M mRNA and induces formation of neurites in human teratocarcinoma cells." Genes Dev 13: 449-461.

Aranda-Abreu, G.E., Behar, L., et al. (1999). "Embryonic lethal abnormal vision-like RNA-binding proteins regulate neurite outgrowth and tau expression in PC12 cells." J Neurosci 19: 6907-6917.

Aronov, S., Aranda, G., et al. (2002). "Visualization of translated tau protein in the axons of neuronal P19 cells and characterization of the tau RNP granules." J Cell Sci 115: 3817-3827.

Atlas, R., Behar, L., et al. (2004). "The insulin-like growth factor mRNA binding-protein IMP-1 and the Ras-regulatory protein G3BP associate with tau mRNA and HuD protein in differentiated P19 neuronal cells." J Neurochem 89: 613-626.

Baccon, J., Pellizzoni, L., et al. (2002). "Identification and characterization of Gemin7, a novel component of the survival of motor neuron complex." J Biol Chem 277: 31957-31962.

Balabanian, S., Gendron, N.H. and Mackenzie, A.E. (2007). "Histologic and transcriptional assessment of a mild SMA model." Neurol Res 29: 413-424.

Barreau, C., Paillard, L., and Osborne, H.B. (2005). "AU-rich elements and associated factors: are there unifying principles?" Nucleic Acids Res 33: 7138-7150.

Battaglia, G., Princivalle, A., et al. (1997). "Expression of the SMN gene, the spinal muscular atrophy determining gene, in the mammalian central nervous system." Hum Mol Genet 6: 1961-1971.

Bäumer, D., Lee, S., et al. (2009). "Alternative Splicing Events Are a Late Feature of Pathology in a Mouse Model of Spinal Muscular Atrophy." PLoS Genet 5(12):e1000773.

Bechade, C., Rostaing, P., et al. (1999). "Subcellular distribution of survival motor neuron (SMN) protein: possible involvement in nucleocytoplasmic and dendritic transport." Eur J Neurosci 11: 293-304.

Bedford, M.T., Reed, R., and Leder, P. (1998). "WW domain-mediated interactions reveal a spliceosome-associated protein that binds a third class of proline-rich motif: the proline glycine and methionine rich motif." Proc Natl Acad Sci USA 95: 10602-10607.

Bedford, M.T., Frankel, A., et al. (2000). "Arginine methylation inhibits the binding of proline-rich ligands to Src homology 3, but not WW, domains." J Biol Chem 275(21): 16030-6.

Bedford, M.T., and Richard, S. (2005). "Arginine methylation: an emerging regulator of protein function." Mol Cell Rev 18: 263-272.

Boisvert, F.M., Cote, J., et al. (2002). "Symmetrical dimethylarginine methylation is required for the localization of SMN in Cajal bodies and pre-mRNA splicing." J Cell Biol 159(6): 957-969.

Boisvert, F.M., Cote, J., et al. (2003). "A proteomic analysis of arginine-methylated protein complexes." Mol Cell Proteomics 2: 1319-1330.

Boisvert, F.M., Chenard, C., et al. (2005). "Protein interfaces in signaling regulated by arginine methylation." Sci STKE 271: p. re2.

Bowerman, M., Shafey, D. and Kothari, R.M. (2007) "SMN depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity." J Mol Neurosci 32(2): 120-131.

Brahms, H., Meheus, L., et al. (2001). "Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B' and the Sm-like protein LSm4, and their interaction with the SMN protein." RNA 7(11): 1531-1542.

Brennan, C.M. and Steitz, J.A. (2001). "HuR and mRNA stability." Cell Mol Life Sci 58: 266-277.

Briese, M., Esmaeili, B., et al. (2005). "Is spinal muscular atrophy the result of defects in motor neuron processes?" Bioessays 27(9): 946-57.

Briese, M., Esmaeili, B., et al., (2009). "Deletion of smn-1, the Caenorhabditis elegans ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan." Hum Mol Genet 18(1): 97-104

Buhler, D., Raker, V., et al. (1999). "Essential role for the tudor domain of SMN in spliceosomal U snRNP assembly: implications for spinal muscular atrophy." Hum Mol Genet 8: 2351-2357.

Burlet, P., Huber, C., et al. (1998). "The distribution of SMN protein complex in human fetal tissues and its alteration in spinal muscular atrophy." Hum Mol Genet 7: 1927-1933.

Campos, A.R., Grossman, D. and White, K. (1985) "Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects: A developmental-genetic analysis." J Neurogenet 2: 197-218.

Carissimi, C., Baccon, J., et al. (2005). "Unrip is a component of SMN complexes active in snRNP assembly." FEBS Lett 579(11): 2348-2354.

Carissimi, C., Saieva, L., et al. (2006). "Gemin8 is a novel component of the survival motor neuron complex and functions in small nuclear ribonucleoprotein assembly." J Biol Chem 281(12): 8126-8134.

Chagnovich, D. and Cohn, S. (1996). "Binding of a 40-kDa protein to the N-myc 3'untranslated region correlates with enhanced N-myc expression in human neuroblastoma." J Biol Chem 271: 33580-33586.

Chagnovich, D., Fayos, B. and Cohn, S. (1996). "Differential activity of ELAV-like RNA-binding proteins in human neuroblastoma." J Biol Chem 271: 33587-33591.

Chang, B., Chen, Y., et al. (2007). "JMJD6 is a histone arginine demethylase." Science 318 (5849): 444-447.

Charroux, B., Pellizzoni, L., et al. (1999). "Gemin 3: A novel DEAD box protein that interacts with SMN, the spinal muscular atrophy gene product, and is a component of gems." J Cell Biol 147(6): 1181-94.

Charroux, B., Pellizzoni, L., et al. (2000). "Gemin4: A novel component of the SMN complex that is found in both gems and nucleoli." J Cell Biol 148(6): 1177-1186.

Chen, C.Y., Xu, N. and Shyu, A.B. (1995). "mRNA decay mediated by two distinct AU-rich elements from c-fos and granulocyte-macrophage colony-stimulating factor transcripts: different deadenylation kinetics and uncoupling from translation" Mol Cell Biol 15: 5777-5788.

Chen, D., Ma, H., et al. (1999). "Regulation of transcription by a protein methyltransferase." Science 284(5423): 2174-2177.

Chen, D., Huang, S.M. and Stallcup, M.R. (2000). "Synergistic, p160 coactivator-dependent enhancement of estrogen receptor function by CARM1 and p300." J Biol Chem 275: 40810-40816.

Chen, H., Lin, R.J., et al. (1997). "Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300." Cell 90: 569-580.

Cheng, D., Cote, J., et al. (2007). "The arginine methyltransferase CARM1 regulates the coupling of transcription and mRNA processing." Mol Cell 25(1): 71-83.

Cheung, W.L., Briggs, S.D. and Allis, C.D. (2000). "Acetylation and chromosomal functions." Curr Opin Cell Biol 12(3): 326-333.

Chevillard-Briet, M., Trouche, D. and Vandel, L. (2002). "Control of CBP co-activating activity by arginine methylation." EMBO 21: 5457-5466.

Chung, S., Eckrich, M., et al. (1997). "The Elav-like proteins bind to a conserved regulatory element in the 3'-untranslated region of GAP-43 mRNA." J Biol Chem 272: 6593-6598.

Cimato, T.R., Ettinger, M.J., et al. (1997). "Nerve growth factor-specific regulation of protein methylation during neuronal differentiation of PC12 cells." J Cell Biol 138: 1089-1103.

Coovert, D., Le, T., et al. (1997) "The survival of motor neuron protein in spinal muscular atrophy." Hum Mol Genet 6(8): 1205-1214.

Coqueret, O. (2003). "New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment?" Trend Cell Biol 13(2): 65-70.

Cote, J., Boisvert, F.M., et al. (2003). "Sam68 RNA binding protein is an in vivo substrate for protein arginine N-methyltransferase 1." Mol Biol Cell 14: 274-287.

Cote, J. and Richard, S. (2005). "Tudor domains bind symmetrical dimethylated arginines." J Biol Chem 280(31): 28476-28483.

Cuadrado, A., Navarro-Yubero, C., et al. (2002). "HuD binds to three AU-rich sequences in the 3'-UTR of neuroserpin mRNA and promotes the accumulation of neuroserpin mRNA and protein." Nucleic Acids Res 30: 2202-2211.

Cusin, V., Clermont, O., et al. (2003). "Prevalence of SMN1 depletion and duplication in carrier and normal populations: implication for genetic conselling." J Med Genet 40(4): e39.

Deschênes-Furry, J., Bélanger, G., et al. (2003). "Post-transcriptional Regulation of Acetylcholinesterase mRNAs in Nerve Growth Factor-treated PC12 Cells by the RNA-binding Protein HuD." J Biol Chem 278: 5710-5717.

Deschênes-Furry, J., Bélanger, G., et al. (2005). "The RNA-binding Protein HuR Binds to Acetylcholinesterase Transcripts and Regulates Their Expression in Differentiating Skeletal Muscle Cells." J Biol Chem 280: 25361-25368.

Deschênes-Furry, J., Perrone-Bizzozero, N. and Jasmin, B. (2006). "The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity." BioEssays 28(8): 822-833.

Dobashi, Y., Shoji, M., et al. (1998). "Expression of HuD protein is essential for initial phase of neuronal differentiation in rat pheochromocytoma PC12 cells." Biochem Biophys Res Comm 244: 226-229.

Erhardt, J.A. and Pittman, R.N. (1998). "$p21^{WAF1}$ induces permanent growth arrest and enhances differentiation, but does not alter apoptosis in PC12 cells." Oncogene 16: 443-451.

Fan, L. and Simard, L.R. (2002). "Survival motor neuron (SMN) protein: role in neurite outgrowth and neuromuscular maturation during neuronal differentiation and development." Hum Mol Genet 11: 1605-1614.

Fan, X.C. and Steitz, J.A. (1998). "HNS, a nuclear-cytoplasmic shuttling sequence in HuR." Proc Natl Acad Sci USA 95: 15293-15298.

Feldkotter, M., Schwarzer, V., et al. (2002). "Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy." Am J Hum Genet 70(2): 358-368.

Fischer, U. and Luhrmann, R. (1990). "An essential signaling role for the m3G cap in the transport of U1 snRNP to the nucleus." Science 249: 786-790.

Fischer, U., Sumpter, V., et al. (1993). "Nucleo-cytoplasmic transport of U snRNPs: definition of a nuclear location signal in the Sm core domain that binds a transport receptor independently of the m3G cap." EMBO 12: 573-583.

Frankel, A., Yadav, N., et al. (2002). "The novel human protein arginine *N*-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity." J Biol Chem 277: 3537-3543.

Friesen, W., Massenet, S., et al. (2001a). "SMN, the product of the spinal muscular atrophy gene, binds preferentially to dimethylarginine-containing protein targets." Mol Cell 7(5): 1111-1117.

Friesen, W., Paushkin, S., et al. (2001b). "The methylosome, a 20S complex containing JBP1 and pICln, produces dimethylarginine-modified Sm proteins." Mol Cel Biol 21(24): 8289-8300.

Fujiwara, T., Mori, Y., et al. (2006). "CARM1 Regulates Proliferation of PC12 Cells by Methylating HuD." Mol Cell Biol 26(6): 2273-2285.

Gabanella, F., Carissimi, C., et al. (2005). "The activity of the spinal muscular atrophy protein is regulated during development and cellular differentiation." Hum Mol Genet 14(23): 3629-42.

Gary, J. and Clarke, S. (1998). "RNA and protein interactions modulated by protein arginine methylation." Prog Nucleic Acid Res Mol Biol 61: 65-131.

Germain-Desprez, D., Brun, T., et al. (2001). "The SMN genes are subject to transcriptional regulation during cellular differentiation." Gene 279: 109-117.

Golembe, T., Yong, J., et al. (2005a). "Lymphotropic herpesvirus saimiri uses the SMN complex to assemble Sm cores on its small RNAs." Mol Cell Biol 25: 602-611.

Golembe, T., Yong, J., et al. (2005b). "Specific sequence features, recognized by the SMN complex, identify snRNAs and determine their fate as snRNPs." Mol Cell Biol 25: 10989-11004.

Good, P.J. (1995). "A conserved family of elav-like genes in vertebrates." Proc Natl Acad Sci USA 92: 4557-4561.

Gubitz, A.K., Mourelatos, Z., et al. (2002). "Gemin 5, a novel WD repeat protein component of the SMN complex that binds Sm proteins." J Biol Chem 277(7): 5631-5636.

Hamm, J., Darzynkiewicz, E., et al. (1990). "The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal." Cell 62: 569-577.

Herbert, M., Shpargel, K., et al. (2002). "Coilin methylation regulates nuclear body formation." Dev Cell 3(3): 329-337.

Herrmann, F., Bossert, M., et al. (2004). "Arginine methylation of scaffold attachment factor A by heterogeneous nuclear ribonucleoprotein particle-associated PRMT1." J Biol Chem 279: 48774-48779.

Higashimoto, K., Kuhn, P., et al. (2007). "Phosphorylation-mediated inactivation of coactivator-associated arginine methyltransferase 1." Proc Natl Acad Sci USA 104(30): 12318-12323.

Hinman, M.N. and Lou, H. (2008). "Diverse molecular functions of Hu proteins." Cell Mol Life Sci 65(20): 3168-3181.

Ikenaka, K., Miyata, S., et al. (2006). "Immunohistochemical and western analysis of protein arginine N-methyltransferase 3 in the mouse brain." Neurosci 141(4): 1971-1982.

Inman, M.V., Levy, S., et al. (1998). "Gene organization and chromosome location of the neural-specific RNA-binding protein Elavl4." Gene 208: 139-145.

Jablonka, S., Schrank, B., et al. (2000). "Reduced survival motor neuron (Smn) gene dose in mice leads to motor neuron degeneration: an animal model for spinal muscular atrophy type III." Hum Mol Genet 9: 341-346.

Jablonka, S., Bandilla, M., et al. (2001). "Coregulation of survival of motor neuron (SMN) protein and its interactor SIP1 during development and in spinal muscular atrophy." Hum Mol Genet 10: 497-505.

Jimenez, F. and Campos-Ortega, J.A. (1987). "Genes in subdivision 1B of the Drosophila melanogaster X-chromosome and their influence on neural development." J Neurogenet 4: 179-200.

Joseph, B., Orlian, M. and Furneaux, H. (1998). "p21(waf1) mRNA contains a conserved element in its 3'-untranslated region that is bound by the Elav-like mRNA-stabilizing proteins." J Biol Chem 273(32): 20511-20516.

Kariya, S., Park, G.H., et al. (2008). "Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy." Hum Mol Genet 17(16): 2552-2569.

Kasashima, K., Sakashita, E., et al. (2002). "Complex formation of the neuron-specific ELAV-like Hu RNA-binding proteins." Nucleic Acids Res 30: 4519-4526.

Kasashima, K., Terashima, K., et al. (1999). "Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation." Genes Cells 4: 667-683.

Kashima, T. and Manley, J. (2003). "A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy." Nat Genet 34: 460-463.

Keene, J.D. (1999). "Why is Hu here? Shuttling of early-response-gene messenger RNA subsets." Proc Natl Acad Sci USA 96: 5-7.

Koh, S.S., Chen, D., et al. (2001). "Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities." J Biol Chem 276: 1089-1098.

Kousaka, A., Mori, Y., et al. (2009). "The distribution and characterization of endogenous PRMT8 in mouse central nervous system." Neurosci 163(4): 1146-1157.

La Bella, V., Cisterni, C., et al. (1998). "Survival motor neuron (SMN) protein in rat is expressed as different molecular forms and is developmentally regulated." Eur J Neurosci 10: 2913-2923.

Layer, P.G. and Willbold, E. (1995). "Novel functions of cholinesterases in development, physiology, and disease." Prog Histochem Cytochem 29: 1-94.

Le, T., Pham, L., et al. (2005). "SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN." Hum Mol Genet 14(6): 845-857.

Lee, D.Y., Teyssier, C., et al. (2005). "Role of protein methylation in regulation of transcription." Endocrine Rev 26(2): 147-170.

Lee, Y.H., Koh, S.S., et al. (2002). "Synergy among nuclear receptor coactivators: selective requirement for protein methyltransferase and acetyltransferase activities." Mol Cell Biol 22: 3621-3632.

Lee, Y.H., Coonrod, S.A., et al. (2005). "Regulation of coactivator complex assembly and function by protein arginine methylation and demethylimination." Proc Natl Acad Sci USA 102: 3611-3616.

Lefebvre, S., Burglen, L., et al. (1995). "Identification and characterization of a spinal muscular atrophy determining gene." Cell 80: 155-165.

Lefebvre, S., Burlet, P., et al. (1997). "Correlation between severity and SMN protein level in spinal muscular atrophy." Nat Genet 16(3): 265-269.

Levine, T.D., Gao, F., et al. (1993). "Hel-N1: an autoimmune RNA-binding protein with specificity for 3′ uridylate-rich untranslated regions of growth factor mRNAs." Mol Cell Biol 13: 3494-3504.

Liu, Q. and Dreyfuss, G. (1995). "In vivo and in vitro arginine methylation of RNA-binding proteins." Mol Cell Biol 15: 2800-2808.

Liu, Q. and Dreyfuss, G. (1996). "A novel nuclear structure containing the survival of motor neurons protein." EMBO 15: 3555-3565.

Lopez de Silanes, I., Zhan, M., et al. (2004). "Identification of a target RNA motif for RNA-binding protein HuR." Proc Natl Acad Sci USA 101: 2987-2992.

Lorson, C.L. and Androphy, E.J. (2000). "An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN." Hum Mol Genet 9: 259-265.

Lorson, C., Hahnen, E., et al. (1999). "A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy." Proc Natl Acad Sci USA 96: 6307-6311.

Lukong, K.E. and Richard, S. (2004). "Arginine methylation signals mRNA export." Nat Struct Mol Biol 11: 914-915.

Ma, H., Hong, H., et al. (1999). "Multiple signal input and output domains of the 160-kDa nuclear receptor coactivator proteins." Mol Cell Biol 19: 6164-6173.

Manohar, C., Short, M., et al. (2002). "HuD, a neuronal-specific RNA-binding protein, increases the in vivo stability of MYCN RNA." J Biol Chem 277: 1967-1973.

Marusich, M.F., Furneaux, H.M., et al. (1994). "Hu neuronal proteins are expressed in proliferating neurogenic cells." J Neurobiol 25: 143-155.

Mattaj, I. (1986). "Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding." Cell 46: 905-911.

Mattaj, I., Boelens, W., et al. (1993). "Nucleocytoplasmic transport and snRNP assembly." Mol Biol Rep 18: 79-83.

McAndrew, P., Parsons, D., et al. (1997). "Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC copy number." Am J Hum Genet 60(6): 1411-1422.

McBride, A.E. and Silver, P.A. (2001). "State of the arg: protein methylation at arginine comes of age." Cell 106: 5-8.

Meister, G., Buhler, D., et al. (2001). "A multiprotein complex mediates the ATP-dependent assembly of spliceosomal U snRNPs." Nat Cell Biol 3(11): 945-949.

Meister, G., Eggert, C. and Fischer, U. (2002). "SMN-mediated assembly of RNPs: a complex story." Trends Cell Biol 12: 472-478.

Meister, G. and Fischer, U. (2002). "Assisted RNP assembly: SMN and PRMT5 complexes cooperate in the formation of spliceosomal UsnRNPs." EMBO 21(21): 5853-5863.

Melki, J. (1997). "Spinal muscular atrophy." Curr Opin Neurol 10(5): 381-385.

Mobarak, C.D., Anderson, K.D., et al. (2000). "The RNA-binding protein HuD is required for GAP-43 mRNA stability, GAP-43 gene expression, and PKC-dependent neurite outgrowth in PC12 cells." Mol Biol Cell 11: 3191-3203.

Monani, U., Lorson, C., et al. (1999). "A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2." Hum Mol Genet 8: 1177-1183.

Monani, U., Sendtner, M., et al. (2000). "The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn (-/-) mice and results in a mouse with spinal muscular atrophy." Hum Mol Genet 9(3): 333-339.

Monani, U. (2005). "Spinal muscular atrophy: a deficiency in a ubiquitous protein; a motor neuron-specific disease." Neuron 48: 885-896.

Mowen, K., Tang, J., et al. (2001). "Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription." Cell 104(5): 731-741.

Munsat, T. and Davies, K. (1992). "International SMA consortium meeting. (26-28 June 1992, Bonn, Germany)." Neuromuscul Disord 2: 423-428.

Naeem, H., Cheng, D., et al. (2007). "The Activity and Stability of the Transcriptional Coactivator p/CIP/SRC-3 Are Regulated by CARM1-Dependent Methylation." Mol Cell Biol 27(1): 120-134.

Najbauer, J., Johnson, B.A., et al. (1993). "Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyl-transferase(s) modifying arginine in numerous proteins." J Biol Chem 268: 10501-10509.

Narayanan, U., Achsel, T., et al. (2004). "Coupled in vitro import of U snRNPs and SMN, the spinal muscular atrophy protein." Mol Cell 16(2): 223-234.

Ohkura, N., Takahashi, M., et al. (2005). "Coactivator-associated arginine methyltransferase 1, CARM1, affects pre-mRNA splicing in an isoform-specific manner." J Biol Chem 280: 28927-28935.

Okano, H.J. and Darnell, R.B. (1997). "A hierarchy of Hu RNA binding proteins in developing and adult neurons." J Neurosci 17: 3024-3037.

Olaso, R., Joshi, V., et al. (2005). "Activation of RNA metabolism related genes in mouse but not human tissues deficient in SMN." Physiol Genomics 24: 97-104.

Otter, S., Grimmler, M., et al. (2007). "A comprehensive interaction map of the human survival of motor neuron (SMN) complex." J Biol Chem 282(8): 5825-5833.

Pahlich, S., Zakaryan, R.P. and Gehring, H. (2006). "Protein arginine methylation: Cellular functions and methods of analysis." Biochimica et Biophysica Acta 1764: 1890-1903.

Pascale, A., Amadio, M., et al. (2005) "Neuronal ELAV proteins enhance mRNA stability by a PKCα-dependent pathway." Proc Natl Acad Sci USA 102(34): 12065-12070.

Pascale, A., Amadio, M. and Quattrone, A. (2008). "Defining a neuron: neuronal ELAV proteins." Cell Mol Life Sci 65: 128-140.

Paushkin, S., Gubitz, A., et al. (2002). "The SMN complex, an assemblyosome of ribonucleoproteins." Curr Opin Cell Biol 14: 305-312.

Pearn, J. (1980). "Classification of spinal muscular atrophies." Lancet 1(8174): 919-922.

Pellizzoni, L., Baccon, J., et al. (2002). "Purification of native survival of motor neurons complexes and identification of Gemin6 as a novel component." J Biol Chem 277: 7540-7545.

Pellizzoni, L., Charroux, B., et al. (1999). "SMN mutants of spinal muscular atrophy patients are defective in binding to snRNP proteins." Proc Natl Acad Sci USA 96: 11167-11172.

Pellizzoni, L., Kataoka, N., et al. (1998). "A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing." Cell 95: 615-624.

Pellizzoni, L. (2007). "Chaperoning ribonucleoprotein biogenesis in health and disease." EMBO Rep 8(4): 340-345.

Perrone-Bizzozero, N. and Bolognani, F. (2002). "Role of HuD and Other RNA-Binding Proteins in Neural Development and Plasticity." J Neurosci Res 68: 121-126.

Ponting, C.P. (1997). "Tudor domains in proteins that interact with RNA." Trends Biochem Sci 22: 51-52.

Robinow, S., Campos, A.R., et al. (1988). "The elav gene product of Drosophila, required in neurons, has three RNP consensus motifs." Science 242: 1570-1572.

Rossoll, W., Kroning, A.K., et al. (2002). "Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons?" Hum Mol Genet 11: 93-105.

Rossoll, W., Jablonka, S., et al. (2003). "Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons." J Cell Biol 163(4): 801-812.

Rouget, R., Vigneault, F., et al. (2005). "Characterization of the survival motor neuron (SMN) promoter provides evidence for complex combinatorial regulation in undifferentiated and differentiated P19 cells." Biochem J 385: 433-443.

Sakai, K., Gofuku, M., et al. (1994). "A hippocampal protein associated with paraneoplastic neurologic syndrome and small cell lung carcinoma." Biochem Biophys Res Comm 199: 1200-1208.

Schenkel, J. (1988). "RNA-binding properties of hnRNP proteins." Euro J Biochem 171: 565-569.

Schurter, B.T., Koh, S.S., et al. (2001). "Methylation of histone H3 by coactivator-associated arginine methyltransferase 1." Biochem 40: 5747-5756.

Scorilas, A., Black, M.H., et al. (2000). "Genomic organization, physical mapping, and expression analysis of the human protein arginine methyltransferase 1 gene." Biochem Biophys Res Comm 278: 349-359.

Selenko, P., Sprangers, R., et al. (2001). "SMN Tudor domain structure and its interaction with the Sm proteins." Nat Struct Biol 8: 27-31.

Shafey, D., MacKenzie, A.E. and Kothary, R. (2008). "Neurodevelopmental abnormalities in neurosphere-derived neural stem cells from SMN-depleted mice." J Neurosci Res 86(13): 2839-2847.

Shaw, G. and Kamen, R. (1986). "A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation." Cell 46: 659-667.

Smith, C.L., Afroz, R., et al. (2004). "GAP-43 mRNA in growth cones is associated with HuD and ribosomes." J Neurobiol 61: 222-235.

Smith, W.A., Schurter, B.T., et al. (2004). "Arginine methylation of RNA helicase a determines its subcellular localization." J Biol Chem 279: 22795-22798.

Soreq, H. and Seidman, S. (2001). "Acetylcholinesterase-new roles for an old actor." Nat Rev Neurosci 2: 294-302.

Stallcup, M.R. (2001). "Role of protein methylation in chromatin remodeling and transcriptional regulation." Oncogene 20(24): 3014-3020.

Steller, U., Kohls, S., et al. (1996). "The RNA binding protein HuD: rat cDNA and analysis of the alternatively spliced mRNA in neuronal differentiation cell lines P19 and PC12." Brain Res Mol Brain Res 35: 285-296.

Szabo, A., Dalmau, J., et al. (1991). "HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal." Cell 67: 325-333.

Tadesse, H., Deschênes-Furry, J., et al. (2008). "KH-Type Splicing Regulatory Protein Interacts with Survival Motor Neuron Protein and is Misregulated in Spinal Muscular Atrophy." Hum Mol Genet 17(4): 506-524.

Talbot, K., Miguel-Aliaga, I., et al. (1998). "Characterization of a gene encoding Survival Motor Neuron (SMN)-related protein, a constituent of the spliceosome complex." Hum Mol Genet 7: 2149-2156.

Tora, M., Barbera, V.M. and Real, F.X. (2000). "Detection of HuD transcripts by means of reverse transcriptase and polymerase chain reaction: implications for the detection of miminal residual disease in patients with small cell lung cancer." Cancer Lett 161: 157-164.

Vanderklish, P.W. and Edelman, G.M. (2005). "Differential translation and fragile X syndrome." Genes Brain Behav 4: 360-384.

Vitte, J., Fassier, C., et al. (2007). "Refined characterization of the expression and stability of the SMN gene products." Am J Pathol 171: 1269-1280.

Voegel, J.J., Heine, M.J., et al. (1998). "The coactivator TIF2 contains three nuclear receptor binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways." EMBO 17: 507-519.

Wang, H., Huang, Z., et al. (2001). "Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor." Science 293(5531): 853-857.

Wein, G. and Rossler, M. (2003). "The 3'-UTR of the mRNA coding for the major protein kinase C substrate MARCKS contains a novel CU-rich element interacting with the mRNA stabilizing factors HuD and HuR." Eur J Biochem 270: 350-365.

Will, C.L. and Luhrmann, R. (2001). "Spliceosomal UsnRNP biogenesis, structure and function." Curr Opin Cell Biol 13: 290-301.

Wolffe, A.P. and Guschin, D. (2000). "Review: chromatin structural features and targets that regulate transcription." J Struct Biol 129: 102-122.

Wu, Q., Bruce A.W., et al. (2009). "CARM1 is required in embryonic stem cells to maintain pluripotency and resist differentiation." Stem Cells 27(11): 2637-2645.

Xie, B., Invernizzi, C.F., et al. (2007). "Arginine Methylation of the Human Immuno-deficiency Virus Type 1 Tat Protein by PRMT6 Negatively Affects Tat Interactions with both Cyclin T1 and the Tat Transactivation Region." J Virol 81(8): 4226-4234.

Xu, C. and Henry, M.F. (2004). "Nuclear export of hnRNP Hrp1p and nuclear export of hnRNP Npl3p are linked and influenced by the methylation state of Npl3p." Mol Cell Biol 24: 10742-10756.

Xu, W., Chen, H., et al. (2001). "A transcriptional switch mediated by cofactor methylation." Science 294: 2507-2511.

Yadav, N., Lee, J., et al. (2003). "Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice." Proc Natl Acad Sci USA 100: 6464-6468.

Yan, G.Z. and Ziff, E.B. (1995). "NGF regulates the PC12 cell cycle machinery through specific inhibition of the Cdk kinases and induction of cyclin D1." J Neurosci 15(9): 6200-6212.

Yan, G.Z. and Ziff, E.B. (1997). "Nerve growth factor induces transcription of the p21 WAF1/CIP1 and cyclin D1 genes in PC12 cells by activating the Sp1 transcription factor." J Neurosci 17(16): 6122-6132.

Yano, M., Okano, H.J. and Okano, H. (2005). "Involvement of Hu and heterogeneous nuclear ribonucleoprotein K in neuronal differentiation through p21 mRNA post-transcriptional regulation." J Biol Chem 280(13): 12690-12699.

Yao, K.M., Samson, M.L., et al. (1993). "Gene elav of Drosophila melanogaster: a prototype for neuronal-specific RNA binding protein gene family that is conserved in flies and humans." J Neurobiol 24:723–739.

Yong, J., Golembe, T., et al. (2004). "snRNAs contain specific SMN-binding domains that are essential for snRNP assembly." Mol Cell Biol 24: 2747-2756.

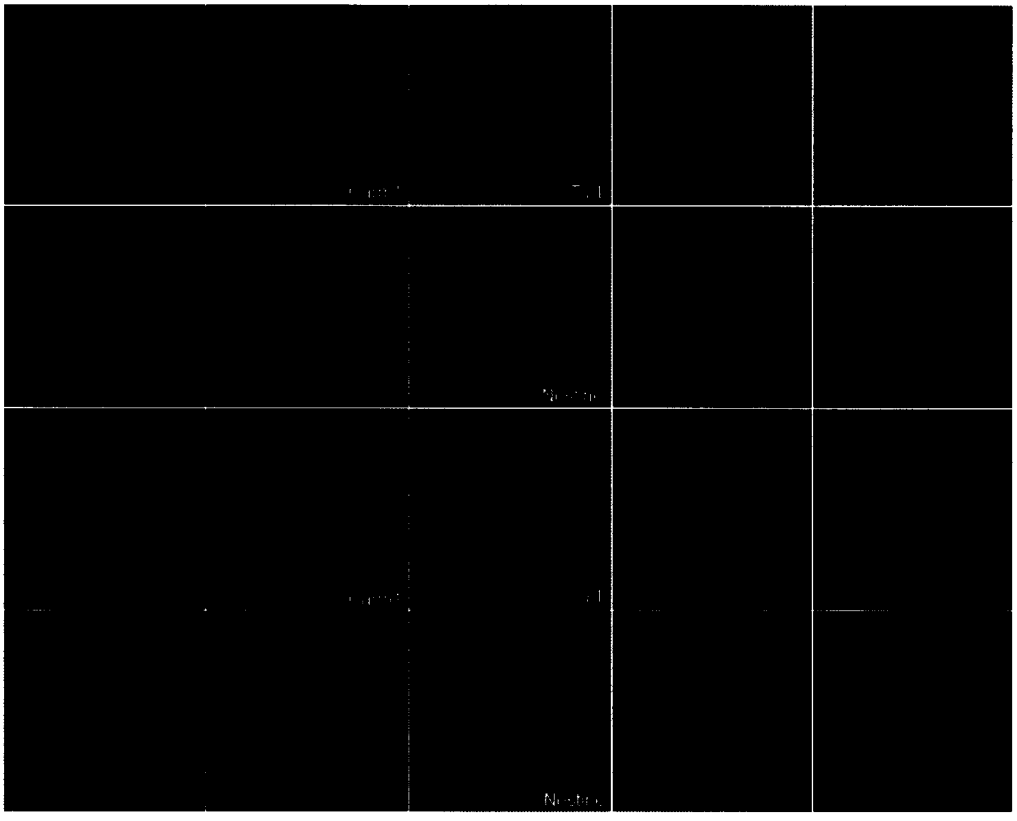
Yu, M.C., Bachand, F., et al. (2004). "Arginine methyltransferase affects interactions and recruitment of mRNA processing and export factors." Genes Dev 18: 2024-2035.

Yun, C. and Fu, X. (2000). "Conserved SR protein kinase functions in nuclear import and its action is counteracted by arginine methylation in Saccharomyces cerevisiae." J Cell Biol 150(4): 707-718.

Zerres, K. and Rudnik-Schoneborn, S. (1995). "Natural history in proximal spinal muscular atrophy. Clinical analysis of 445 patients and suggestions for a modification of existing classification." Arch Neurol 52(5): 518-523.

Zhang, H., Pan, F., et al. (2003). "Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization." J Neurosci 23: 6627-6637.

Zhang, Z., Lotti, F., et al. (2008). "SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing." Cell 133(4): 585-600.


0hr.
0hr.
48hr.
48hr.

Appendix 1: Supplementary Data

Figure 15. Immunofluorescence of differentiation markers in MN-1 cells. Attempts to identify changes in the localization of predicted markers for different stages of motor neuron differentiation were unsuccessful (n=2). Immunoblotting to monitor protein levels using the same antibodies as well as additional proteins were also unsuccessful (data not shown).

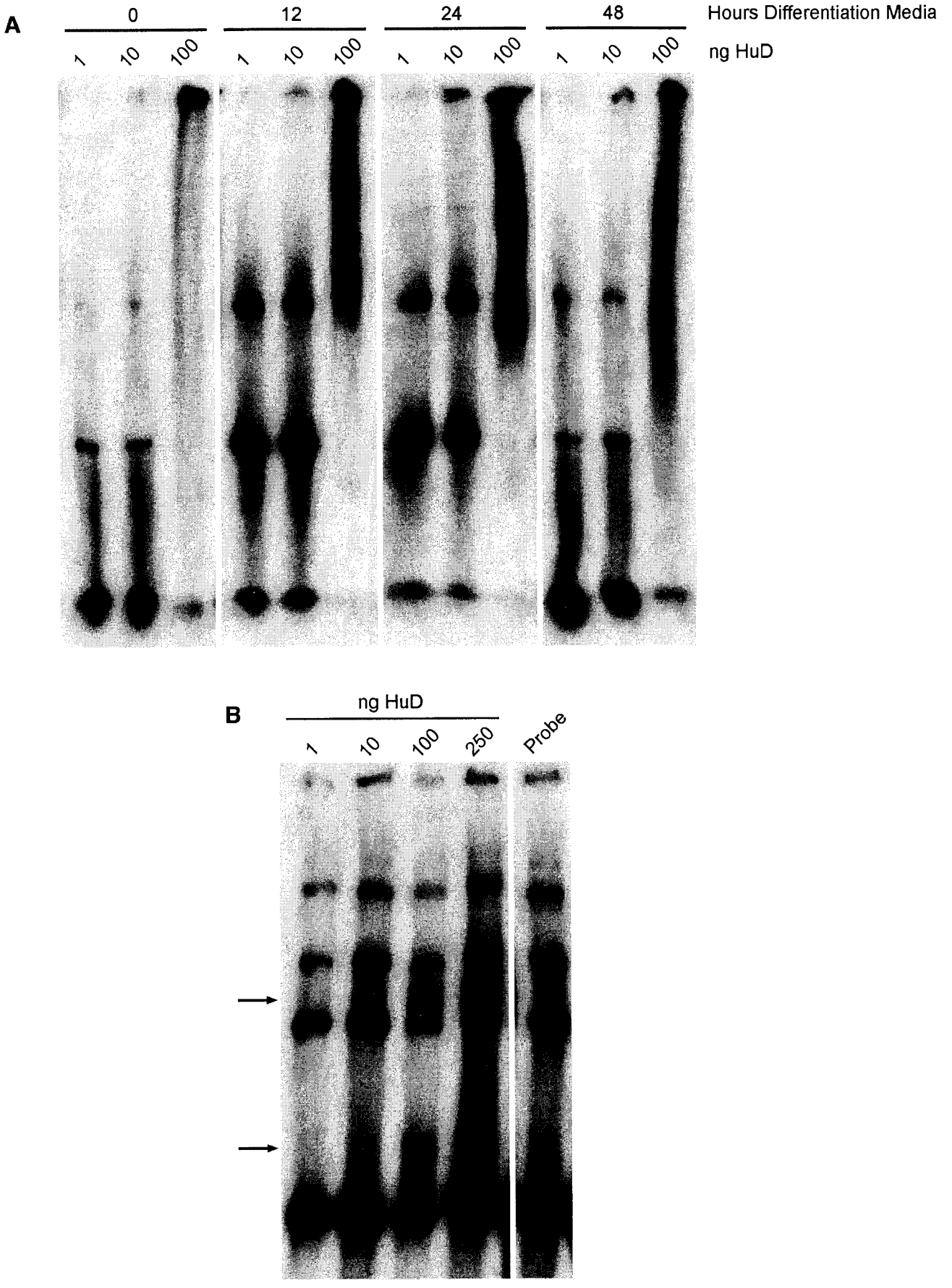

A
0
12
24
48
Hours Differentiation Media
1
10
100
ng HuD
B
ng HuD
1
10
100
250
Probe

Figure 16. Additional attempts to determine an interaction of p21 mRNA with HuD in MN-1 cells and its regulation by CARM1. A, Methylation assays were performed using GST-HuD incubated with protein extracts taken from differentiated MN-1 cells at the indicated times. GST-HuD pulldowns followed by gel shift assays with radioactive p21 indicate that HuD has a higher affinity for p21 mRNA when incubated with extracts in early differentiation in comparison to undifferentiated cells. **B,** Assays using only MN-1 extracts and p21 indicate that proteins within the extract are capable of binding p21 mRNA. Attempts to supershift the bands using the HuD antibody were unsuccessful (n=3).

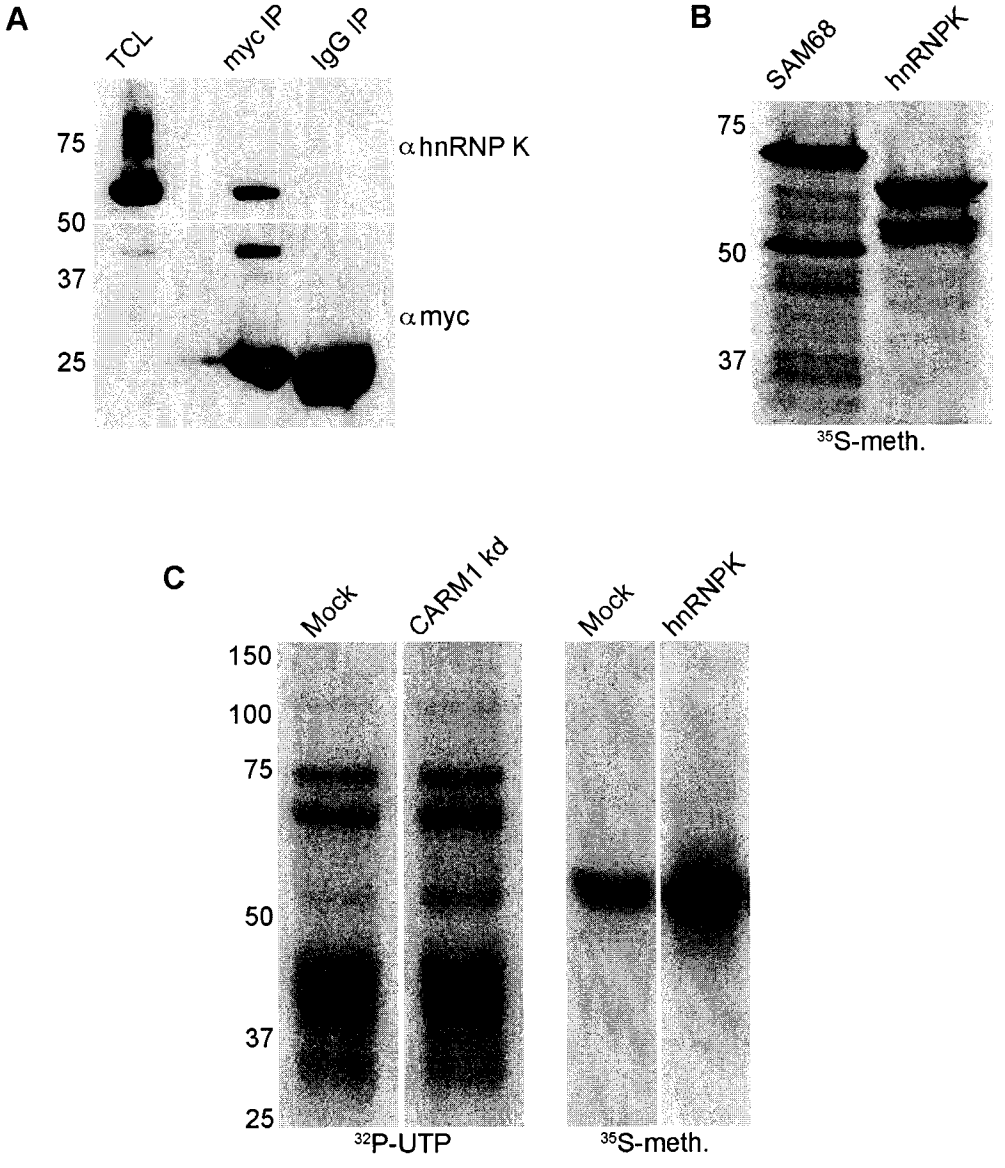

A
TCL
myc IP
IgG IP
75
50
37
25
αhnRNP K
αmyc
B
SAM68
hnRNPK
75
50
37
35S-meth.
C
Mock
CARM1 kd
Mock
hnRNPK
150
100
75
50
37
25
32P-UTP
35S-meth.

Figure 17. The interaction of hnRNP K with HuD and p21 mRNA. A, hnRNP K co-immunoprecipitates with HuD following myc-HuD transfection in MN-1 cells. **B,** hnRNP K protein was successfully produced in a transcription and translation reaction. **C,** Crosslinking of mock and hnRNP K in these reactions with ^{32}P-p21 shows that hnRNP K binds p21 mRNA, and corresponds to the molecular weight of a protein whose RNA-binding properties are influenced by CARM1 (n=3).