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SMN is Required for Dynamic Relocalization of Methylated Nucleolar Proteins During Skeletal Muscle Differentiation

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SMN is Required for Dynamic Relocalization of Methylated Nucleolar Proteins During Skeletal Muscle Differentiation

Benoît F. Paquette

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Abstract

Deletions or loss-of-function mutations in the Survival of Motor Neuron 1 (Smn1) gene in humans is responsible for Spinal Muscular Atrophy (SMA), one of the leading genetic causes of infant mortality. The pathological hallmarks of this disease include the degeneration of lower motor neurons in the anterior horn of the spinal cord, weakness, paralysis and atrophy of the associated skeletal muscles and eventually of the entire trunk, often times causing respiratory failure and early death during disease progression. Although current knowledge supports the notion that this disease arises from the massive motoneuron loss early in development, mounting evidence suggests that intrinsic muscle defects may also contribute to the pathology. Arginine methylation is also known to be important for normal skeletal muscle differentiation. Since SMN can serve as an adaptor module for arginine methylated proteins, we speculated that SMN will perform its function in skeletal muscle by promoting methyl-dependent interactions.

This study has documented a dynamic profile of proteins containing methylated arginines during myoblast differentiation, and that this dynamic profile is dependent on the presence of SMN. Furthermore, I have identified Fibrillarin, a snoRNP component, as one of the methylated proteins that is misregulated in the absence of SMN. Finally, I have also shown that the profile of arginine methylated proteins differs between skeletal muscles from wild type mice and a mouse model of SMA. Taken together, these results represent a novel defect in SMA and provide evidence for the importance of skeletal muscle tissue in this disease.

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List of Abbreviations

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

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

ATP - Adenosine tri-phosphate

BSA - Bovine serum albumin

CARM1 - Coactivator-associated arginine methyltransferase 1

CB - Cajal Bodies

cDNA- Complementary deoxyribonucleic acid

DMEM - Dulbecco's modified Eagle's medium

EBNA2 - Epstein-Barr virus nuclear antigen2

FBS - Fetal bovine serum

GAPDH - Glycerol-3-phosphate dehydrogenase

GST - Glutathione S-transferase

hnRNP - heterogeneous nuclear Ribonucleoprotein Particle

HRP - Horseradish peroxidise

MHC - Myosin heavy chain

mRNA - messenger Ribonucleic Acid

mRNP - messenger Ribonucleoprotein Particle

NMJ - Neuromuscular junction

OD - Optical density

PBS - Phosphate buffered saline

PBST - Phosphate buffered saline and tween

PCR - Polymerase chain reaction

PRMTs - Protein arginine *N*-methyltransferases

PVDF - Polyvinylidene difluoride

RNA - Ribonucleic Acid

RNP(s) - Ribonucleoprotein Particles

SAM - S-adenosyl-L-methionine

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

snoRNP(s) - small nucleolar Ribonucleoprotein Particles

snRNP(s) - small nuclear Ribonucleoprotein Particles

WCE - Whole cell extraction

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Chapter 1. Introduction:

1.1 Spinal Muscular Atrophy

1.1.1 Epidemiology

Autosomal recessive spinal muscular atrophy (SMA) is a neuromuscular disorder that is characterized by the degeneration of lower motor neurons in the anterior horn of the spinal cord, resulting in symmetrical weakness, paralysis and atrophy of the proximal voluntary muscles of legs, arms, and eventually of the entire trunk during disease progression (Melki 1997; Wirth, Brichta et al. 2006). SMA affects approximately 1 in 10 000 live births and is considered one of the leading genetic causes of infant mortality (Pearn 1980). Additionally, there is a 1/35 carrier frequency, as determined by direct molecular genetic testing (Feldkotter, Schwarzer et al. 2002; Cusin, Clermont et al. 2003).

1.1.2 Clinical Classification

Because of the wide variability of SMA, the International SMA Consortium defined 5 clinical groups based on the age of onset and achieved motor abilities (Munsat and Davies 1992; Zerres and Rudnik-Schoneborn 1995; Briese, Esmaeili et al. 2005) (Fig. 1A).

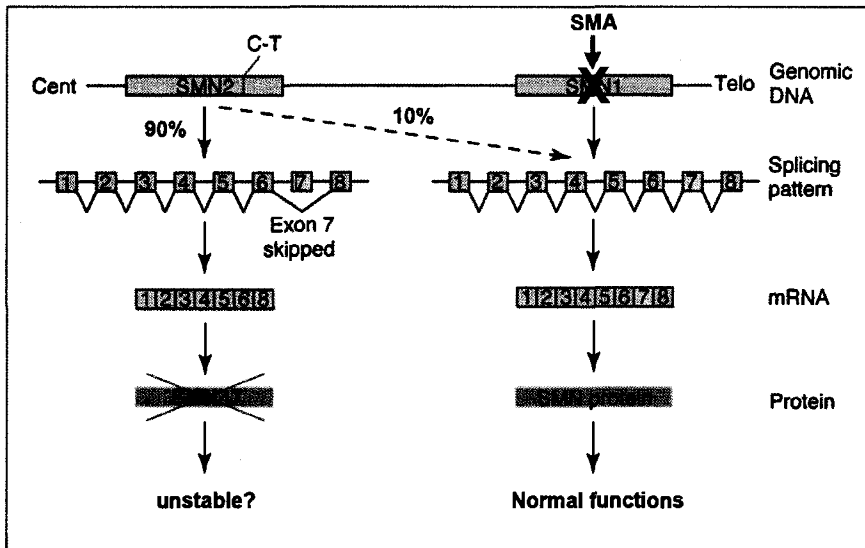
1. Type 0 SMA (prenatal onset) reduced fetal movement *in utero*; some degree of spontaneous ventilation and movement at birth (Briese, Esmaeili et al. 2005).
2. Type I SMA (acute form, Werdnig-Hoffmann disease, MIM #253300) is the most severe form with generalized muscle weakness and hypotonia ("floppy infant") and

Figure 1

A

Type	Age at onset	Motor abilities
0	Prenatal onset	Reduced fetal movements *in utero*; some degree of spontaneous ventilation and movement at birth
I	At birth or <6 months	Never able to sit
II	<18 months	Unable to stand or walk unaided
III	>18 months	Able to walk unassisted
IV	Adult onset	Mild proximal muscle weakness

B



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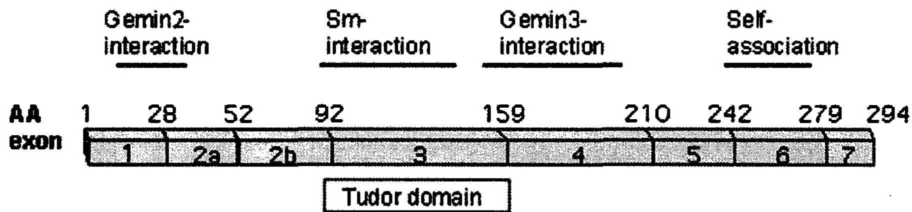


Figure 1: Spinal muscular atrophy. (A) Table showing the classification system of spinal muscular atrophy, according to age of onset and severity of the disease. (modified from Briese M. et al. 2005). (B) The SMN genomic locus in healthy individuals and in SMA Type I patiens. (modified from Khoo B. et al. 2003). (C) Full length SMN protein schematic representation.

a disease onset within the first 6 months of life. The children are never able to sit or walk and usually die within the first 2 years.

3. Type II SMA (intermediate form, MIM #253550) patients are able to sit but never able to walk unaided, usually present first symptoms after the first 6 months of life, and survive beyond 2 years.
4. Type III SMA (juvenile SMA, Kugelberg-Welander disease, MIM #253400) patients are able to sit and walk, and the lifespan is not reduced. Disease onset before the age of 3 years is classified as type IIIa, whereas an age of onset beyond 3 years is classified as type IIIb SMA. There is a marked difference in the preservation of ambulatory capacity between type IIIa and IIIb. Whereas only 44% of the type IIIa individuals are still able to walk by the age of 20 years, 90% of type IIIb patients of the same age have retained this ability (Zerres and Rudnik-Schoneborn 1995).
5. Type IV SMA (adult form, MIM #271150) patients are comparatively mildly affected with an age of onset later than 30 years; they have a normal life expectancy.

1.2 Spinal Muscular Atrophy and the Survival of Motor Neuron protein

1.2.1 Survival of Motor Neuron Gene and Transcript

Genetic analysis and physical mapping in SMA patients led to the discovery of the causative gene for this disease, which was named 'survival of motor neuron' (SMN) (Lefebvre, Burglen et al. 1995). In humans, the SMN gene is duplicated as an inverted repeat in a 500 kb region of chromosome 5 at locus 5q13 (Lefebvre, Burglen et al. 1995). SMA is a result of

deletions or loss-of-function mutations in the telomeric copy of the SMN gene (SMN1) (Lefebvre, Burglen et al. 1995). The centromeric copy (SMN2) contains a number of silent single nucleotide differences with SMN1, and one of these mutations, a C to T transition in exon 7, causes this exon to be aberrantly skipped during splicing (Lorson, Hahnen et al. 1999; Cartegni and Krainer 2002; Kashima and Manley 2003). As a consequence, the SMN2 gene mainly produces a truncated, and unstable SMN protein (SMNΔEx7) (Fig. 1B), lacking the last 16 amino acids at the carboxyl terminus (Lorson, Hahnen et al. 1999; Monani, Lorson et al. 1999; Lorson and Androphy 2000; Khoo, Akker et al. 2003). This shortened protein can compensate partially during embryonic development for the deficiency of SMN1 in most cells, but not in motor neurons (Monani, Sendtner et al. 2000; Le, Pham et al. 2005). All SMA patients retain at least one intact copy of the SMN2 gene. This implies that complete absence of SMN genes in humans (as confirmed in several other organisms) is embryonic lethal (Schrank, Gotz et al. 1997). It has also been reported that the number of SMN2 gene copy is inversely correlated with phenotype severity; that is, the more copies of SMN2 the less severe the disease (Campbell, Potter et al. 1997). Therefore, this makes the SMN2 gene a genetic modifier in SMA patients. In general, the severity of SMA inversely correlates with the total amount of full length SMN protein expressed in patient cells (Lefebvre, Burlet et al. 1997).

Interestingly, the development of animal models for SMA has yielded important insights into disease pathogenesis but also provided crucial *in vivo* systems for research. The SMN2 gene is unique to humans; other organisms only possess a single copy of the *Smn* gene. Nematode, fly and mouse models with no functional SMN protein have an early embryonic lethal phenotype. The SMA *Drosophila* model was established when several missense

mutations in the *Drosophila smn* gene were identified and resulted in elimination of the ability of the resulting protein to self-associate. The resulting progeny of these mutants could survive to the late larval stage, only because of the maternal contribution of wild-type SMN in these early stages of life. Before death, these larvae developed severe motor abnormalities associated with disorganization of the neuromuscular junction. This phenotype could only be rescued by the expression of wild-type SMN protein in both motor neuron and muscle but not in either tissue alone. Additionally, a hypomorphic zebrafish model for SMA was created by the use of morpholino antisense oligonucleotide "knockdown" technology. This technique decreases the levels of endogenous SMN protein to approximately 60% (McWhorter, Monani et al. 2003). These fish showed defects in motor neuron axonal outgrowth and pathfinding during development, without abnormalities in other types of neurons or in muscles.

To overcome the embryonic lethality of *Smn* (-/-) mice, two major approaches were taken to create a clear behavioral phenotype for SMA models in mice. The first approach was to make transgenic mice with a conditional knockout of the exon 7 of the murine *Smn* gene in specific tissues using the Cre-LoxP system (Frugier, Tiziano et al. 2000). The second strategy used to create transgenic SMA mice was to express the human SMN2 gene in the *Smn* knockout background. The phenotypes of these animals were dependent on SMN2 gene copy number; SMN null (Smn-/-) mice with one or two copies of the gene closely resembled Type I SMA, whereas the same type of mice with eight copies of the gene had a rescued phenotype (Hsieh-Li, Chang et al. 2000; Monani, Sendtner et al. 2000).

1.2.2 Survival of Motor Neuron Protein and Its Core Complex

SMN self-oligomerizes and is part of a stable multiprotein complex called the SMN complex; generally composed of SMN and at least 7 other proteins called Gemins and another component known as unrip. These Gemins include Gemin2/SIP1, Gemin3/DP103 (a DEAD-box RNA helicase), Gemin4, Gemin5/p175 (a WD repeat protein), Gemin6, Gemin7 and Gemin8. Gemins2, 3, 5 and 7 interact directly with SMN, while Gemin4 and Gemin6 are indirectly associated with SMN through their binding to Gemin3 and Gemin7, respectively (See Fig. 2 for SMN complex schematic) (Liu and Dreyfuss 1996; Charroux, Pellizzoni et al. 1999; Charroux, Pellizzoni et al. 2000; Baccon, Pellizzoni et al. 2002; Gubitz, Mourelatos et al. 2002; Pellizzoni, Baccon et al. 2002; Otter, Grimmler et al. 2007). Gemin 8 has been shown to interact with the Gemin6-Gemin7 heterodimer, and is thought to associate with the Sm proteins and play an important role in the snRNP assembly line (Carissimi, Saieva et al. 2006). The most prominent structural motifs within the amino acid sequences of the Gemins are a DEAD-box motif in Gemin3 (Charroux, Pellizzoni et al. 1999; Campbell, Hunter et al. 2000) and an array of 13 WD repeats in Gemin5 (Gubitz, Mourelatos et al. 2002). As RNA helicases have been implicated in nearly all processes related to RNA metabolism, it is likely that the helicase activity and predicted ATPase activity of Gemin3 are critical for the SMN complex function (see section 1.2.3 below for further details). It was recently shown that Gemin5 exists in a complex containing Gemin3 and 4, and is responsible for the binding of snRNA independently of the SMN complex and releases them for their assembly into small nuclear ribonucleoprotein particles (snRNPs) (see section 1.2.3 bellow for further details) to the SMN complex

Figure 2:

The SMN complex

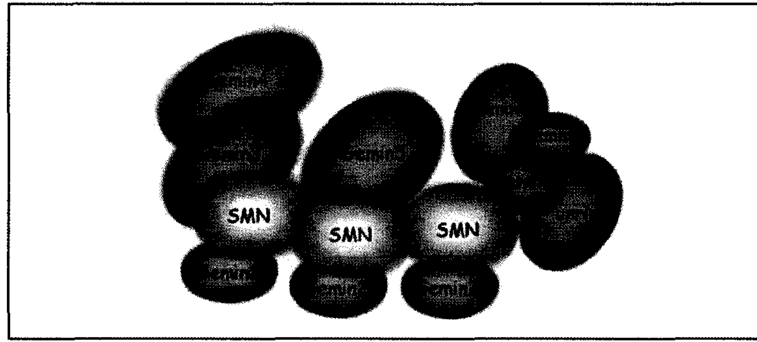


Figure 2: The role of the SMN complex and arginine methylation in snRNP biogenesis. The uridine-rich small nuclear RNAs (U snRNAs), with the exception of U6, are transcribed by RNA polymerase II (polII) as precursors and are then exported into the cytoplasm. Following export to the cytoplasm, U snRNAs and non-phosphorylated PHAX bind to the SMN complex. SmD1, SmD3 and SmB are first methylated by the methylosome, 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. This step is required for cap hypermethylation and 3'-end maturation. A properly assembled Sm core and the m3G-cap structure are prerequisite for U snRNP import into the nucleus. Back in the nucleus, the SMN complex and snRNPs then transit into Gems and/or Cajal bodies (modified from Pellizzoni, EMBO Rep., 2007).

(Battle, Kasim et al. 2007). A different group also demonstrated that Gemin5 mediates an essential step in the assembly of U snRNPs, most likely in the transfer of Sm proteins onto the U snRNA (Otter, Grimmler et al. 2007). In addition, it has been shown that Gemin2 stabilizes the SMN complex (Ogawa, Usui et al. 2007) and that Gemins are essential for snRNP assembly activity (Shpargel and Matera 2005). Unrip is an atypical component of the SMN core complex that binds Gemin6 and Gemin7 but does not localize in Gems or Cajal bodies, this protein can also be found to bind the cap-independent translation factor UNR. It is also thought that unrip plays a role in sequestering the SMN complex in the cytoplasm for snRNP assembly (Hunt, Hsuan et al. 1999; Carissimi, Baccon et al. 2005; Grimmler, Otter et al. 2005; Carissimi, Saieva et al. 2006). Other proteins that interact with this stable SMN "core" complex are usually referred to as "SMN complex substrates".

1.2.3 Survival of Motor Neuron Protein, Roles and Functions

SMN is a 294 amino acid protein that is generally found in the cytoplasm and nucleus of cells. While SMN is present throughout the nucleoplasm, it is found concentrated in discrete nuclear bodies called Gems (for "Gemini" of Cajal bodies (CB)) (Liu and Dreyfuss 1996). Gems have been demonstrated to often be associated with CBs and are similar in size and number (Liu and Dreyfuss 1996; Young, Le et al. 2000). Immunofluorescence microscopy studies show co-localization of Gems and CB in most cell lines and adult tissues; however, they are distinct and separated in fetal tissues and certain types of cultured cells (Paushkin, Gubitz et al. 2002). This suggests that Gems and CBs are distinct nuclear structures that could have a dynamic functional relationship (see below).

SMN has been implicated in a wide variety of cellular pathways such as the assembly and nuclear import of spliceosomal snRNPs, pre-mRNA splicing, and regulation of gene expression at the transcriptional level (Eggert, Chari et al. 2006; Pellizzoni 2007). The best-characterized function of the SMN complex is its role in the assembly of the spliceosomal snRNPs (Yong, Wan et al. 2004) (Fig. 2). It has undeniably been shown that the SMN complex is essential and sufficient to mediate the adenosine tri-phosphate (ATP)-dependent assembly of the seven-membered ring of common Sm proteins around a specific site called the Sm site, present in spliceosomal U snRNAs (Pellizzoni 2007). The SMN complex is also required along with Importin beta and Transportin for subsequent nuclear import of the newly assembled snRNPs (Narayanan, Ospina et al. 2002; Narayanan, Achsel et al. 2004) (Fig. 2). Apart from its role in snRNP biogenesis, the SMN complex may be involved in the assembly of other ribonucleoproteins particles (RNPs) in cells (Terns and Terns 2001; Paushkin, Gubitz et al. 2002; Azzouz, Pillai et al. 2005). For example, SMN was found to interact with several known components of small nucleolar ribonucleoprotein particles (snoRNPs) that have been involved in posttranscriptional processing and modification of ribosomal RNA; namely fibrillarin, GAR1 and nucleolin (Liu and Dreyfuss 1996; Jones, Gorzynski et al. 2001; Pellizzoni, Baccon et al. 2001; Lefebvre, Burlet et al. 2002). It has therefore been hypothesized that the SMN and/or SMN complex might have a role in the assembly and metabolism of snoRNP; similar to its established role in snRNP biogenesis (Fischer, Liu et al. 1997; Pellizzoni, Kataoka et al. 1998; Buhler, Raker et al. 1999; Meister, Buhler et al. 2000). There is additional evidence that SMN associates with the heterogeneous nuclear RNP (hnRNP) R protein and β-actin mRNA in neuronal processes and could contribute to the assembly of specific mRNA's into mRNP articles

as well as their targeting to the axonal transport system and their localized translation regulation in motor neurons (Rossoll, Jablonka et al. 2003).

1.2.4 Survival of Motor Neuron Protein Tudor Domain: A Methyl-Sensing Protein Module

The SMN protein harbors a few motifs that are well-conserved throughout evolution and between species, namely a YG box motif and a Tudor domain (Fig. 1C). The YG-motif is responsible for SMN's self-oligomerization properties. The Tudor domain is a ~60 amino acid conserved domain. The Tudor domain was originally found to be present in proteins that associate with nucleic acids (Ponting 1997). It is known that the Tudor domain mediates several of the interactions between SMN and its arginine-glycine rich (RG)-containing binding partners. Interestingly, methylation on the arginine residues of these motifs have been shown to increase binding to the Tudor domain (Brahms, Meheus et al. 2001; Friesen, Massenet et al. 2001; Meister, Buhler et al. 2001; Cote and Richard 2005; Cheng, Cote et al. 2007). This is the case for Sm (B, B′, D1 and D3) proteins (Friesen, Paushkin et al. 2001; Meister, Buhler et al. 2001; Meister, Eggert et al. 2001), and Sm-like proteins such as Lsm4 (Brahms, Meheus et al. 2001; Friesen, Paushkin et al. 2001), the Epstein-Barr virus nuclear antigen2 (EBNA2) (Barth, Liss et al. 2003), and p80-coilin (Boisvert, Cote et al. 2002; Hebert, Shpargel et al. 2002), as well as many other proteins (Cote and Richard 2005). A few studies suggest that the methylation state of Coilin, a marker of Cajal Bodies, can affect the recruitment of SMN and assembled snRNP to CBs. Moreover, it has been observed that in the presence of methylation inhibitors SMN accumulates in greater number of Gems, a structure devoid of Coilin and snRNPs (Boisvert, Cote et al. 2002; Hebert, Shpargel et al. 2002). Moreover, it was observed that a subset of arginine methylated proteins were mislocalized in severe Type I SMA fibroblasts (Boisvert, Cote

et al. 2002). This suggests that arginine methylation can also influence the intracellular trafficking of SMN and its binding partners. Taken together, these results strongly argue for a central role for arginine methylation in the regulation of several aspects of SMA activities.

1.3 Arginine Methylation

Arginine methylation is a posttranslational modification of proteins that can be found on the guanidino nitrogen atoms of arginine (Gary and Clarke 1998). Arginines can be dimethylated in two different ways: symmetrical dimethylated arginine ω-N^G,N^G (sDMA) and asymmetrical dimethylated arginine ω-N^G,N'^G (aDMA). This modification is carried out by protein arginine *N*-methyltransferases (PRMTs), a family comprised of nine members in higher eukaryotes, that are classified as either Type I (PRMT1, 3, 6, 8 and CARM1) or Type II (PRMT5, 7 and 9).

Arginine methylation is known to influence many cellular processes, including nuclear export (Lee, Henry et al. 1996), nuclear import (Xu, Chen et al. 2001), protein-protein interactions (Bedford, Frankel et al. 2000) and transcription (Chen, Ma et al. 1999; Yun and Fu 2000; Mowen, Tang et al. 2001; Wang, Huang et al. 2001). It is also known, as mentioned above, that many proteins harbouring sDMA modifications make better substrates for the SMN Tudor domain (Brahms, Meheus et al. 2001; Friesen, Massenet et al. 2001; Boisvert, Cote et al. 2002; Barth, Liss et al. 2003; Cote and Richard 2005).

These sDMA-containing proteins include spliceosomal snRNP Sm proteins. Indeed, it is PRMT5 that methylates and drives the transfer of the Sm proteins D1, D3 and B/B' to the SMN complex during snRNP biogenesis (Friesen et al., MCB, 2001; Meister et al., Curr. Biol., 2001). Moreover, a recent study reported that Sm proteins can also be methylated by CARM1, a type I

PRMT (Cheng, Cote et al. 2007)(Boulanger et al., Biochem. J., 2004). Interestingly, this modification did not influence their interaction with the Tudor domain of SMN, although CARM1 methylation of another protein, CA150, did promote binding (Cheng, Cote et al. 2007). This suggests that for the same protein, methylation by different PRMTs, can result in distinct binding affinity for the Tudor domain of SMN, let alone, could even possibly change the function of that protein (Cheng, Cote et al. 2007). Hence it is becoming clear that several PRMTs may play an important role in the regulation of SMN protein-protein interactions.

1.3.1 Arginine Methylation and Cellular Differentiation

The requirement of arginine methylation has been documented for differentiation of various cell types including neuronal differentiation (Cimato, Ettinger et al. 1997) and erythroid differentiation (Bakker, Blazquez-Domingo et al. 2004). Chen and colleagues, in 2002, showed the importance of arginine methylation for proper skeletal muscle development and differentiation by using a general methylation inhibitor called AdOx that completely ablated morphological differentiation of proliferating myoblasts into multinucleated myotubes (Chen, Loffler et al. 2002). This research also went one step further and showed that CARM1 is necessary for skeletal muscle differentiation, likely through its gene expression regulatory activity (e.g. through methylation of histones and cofactors), although they do not rule out the contribution of other mechanisms. Since this report, an increasing amount of results support the importance of arginine methylation during skeletal muscle differentiation. For example, in a recent paper, scientists showed the involvement of PRMT5 during skeletal muscle development. They state that PRMT5 has an important physiological role during the induction

of skeletal muscle differentiation because it facilitates ATP-dependent chromatin remodelling at myogenic loci through interactions with other proteins and via its role in the methylation of histone 3 and/or 4, which then lead to gene expression involved in myogenesis (Dacwag, Ohkawa et al. 2007). The increasing evidence of the crucial role that arginine methylation and PRMTs play during muscle differentiation led us to investigate how arginine methylation and PRMTs profile change during normal myoblast differentiation, and how this profile is misregulated in SMN-deficient cells.

1.4 A Specialized Role for SMN in the Neuromuscular System?

Recent studies found SMN in dendrites and axons of spinal cord motor neurons, as part of RNP granules associated with cytoskeletal filaments (Pagliardini, Giavazzi et al. 2000; Rossoll, Kroning et al. 2002; Zhang, Pan et al. 2003). Moreover, SMN has also been localized at the neuromuscular junction (NMJ) and in "small dot-like" patterns in the cytoplasm of skeletal muscles (Burlet, Huber et al. 1998; Young, Le et al. 2001). The idea that SMN might have a muscle-specific function is not a new one. Previous researchers have shown cocultures of SMA type I and type II muscles with wild-type motorneurons failed to sustain innervations, whereas muscles from control groups or SMA type III patients maintained stable connections, suggesting a muscle specific requirement for SMN (Braun, Croizat et al. 1995; Guettier-Sigrist, Hugel et al. 2002). These observations have been corroborated by two studies in mouse and Drosophila model systems. In the mouse study, a deletion of SMN exon 7 directed to skeletal muscle led to a severe muscular dystrophy phenotype (Cifuentes-Diaz, Frugier et al. 2001). It has also recently been reported using an *in vitro* model that mouse skeletal muscle differentiation is

impaired in the presence of low levels of SMN (Shafey, Cote et al. 2005). In the fly, zygotic SMN mutants showed abnormal motor behaviour related to NMJ defects, and remarkably, restoration of SMN gene activity in both neurons and muscles were required to improve the phenotype (Chan, Miguel-Aliaga et al. 2003). Additionally, it has recently been shown that the depletion of drosophila SMN results in a loss of Act88F, an actin isoform. Furthermore it was shown that drosophila SMN forms a complex with α-actinin and that SMN is a sarcomeric protein (Rajendra, Gonsalvez et al. 2007). Collectively, these studies show that relatively high levels of SMN are required in muscles, although the reason for this requirement is unclear. It can therefore be hypothesized that SMN may have a specific and distinct role in skeletal muscles and that this tissue may have a more direct contribution than initially thought to the motor defects seen in SMA patients.

1.5 Rationale, Hypothesis, and Specific Objectives

1.5.1 Specific Role for SMN and Arginine Methylation in Skeletal Muscle

Despite a large amount of work on SMN function, the molecular mechanism leading to motor neuron degeneration and development of SMA remains to be elucidated. Although initially considered a purely motor neuron disease, recent studies have provided evidence for an additional and specific role of SMN in skeletal muscles. Thus, suggesting that this tissue might have a more direct contribution to motor defects found in SMA patients apart from the documented muscle denervation caused by motor neuron degeneration. Indeed, reports have demonstrated that a disruption of SMN directed to skeletal muscles leads to a severe muscular dystrophy phenotype in mice (Cifuentes-Diaz, Frugier et al. 2001). Moreover, zygotic *smn* mutants in Drosophila showed abnormal motor behavior related to defects of the neuromuscular junction, and strikingly, *smn* gene activity needed to be restored in both neurons and muscle to alleviate the phenotype (Chan, Miguel-Aliaga et al. 2003). Finally, a study using hypomorphic Smn knockdown C2C12 myoblasts has revealed intrinsic defects in myoblast fusion and myotube morphology (Shafey, Cote et al. 2005).

Interestingly, arginine methylation is also known to be important for skeletal muscle differentiation (Chen, Loffler et al. 2002; Dacwag, Ohkawa et al. 2007). Since SMN can serve as an adaptor module for arginine methylated proteins (through its Tudor domain), we speculate that SMN will perform its function in skeletal muscles by promoting methyl-dependent interactions. Furthermore, we speculate that proteins interacting with SMN will be misregulated when this protein is present in low quantities, as we and others have observed previously for other SMN binding partners (Helmken, Hofmann et al. 2003; Tadesse, Deschenes-

Furry et al. 2007). Thus, identification of SMN interactors in skeletal muscles may in turn provide insights into its specific function in this tissue.

1.5.2 Hypothesis

I hypothesize that SMN will have a specific and distinct role in skeletal muscles, through interactions with methylated proteins. Hence, I also predict that methylated proteins that normally interact with SMN will be misregulated in the absence of functional levels of SMN, such as in SMA skeletal muscle tissues. Finally, I speculate that muscle defects will contribute to the SMA phenotype.

1.5.3 Specific Objectives

(i) Assess the profiles of PRMTs and arginine methylated proteins during skeletal muscle differentiation.

(ii) Determine how this profile is affected in the absence of functional levels of SMN.

(iii) Identify the arginine methylated proteins that are misregulated in SMA skeletal muscles and initiate mechanistic studies.

Chapter 2. Material and Methods:

2.1 Cell Culture

C2C12 mice myoblast cells were a gift from Dr. Kothary's laboratory and maintained in Dulbecco's modified Eagle's medium (DMEM; Multicell, Wisent) growth media containing L-glutamine, supplemented with 10% fetal bovine serum (FBS; Multicell, Wisent), 2% penicillin streptomycin (Multicell, Wisent) and 1% sodium pyruvate (Multicell, Wisent). Cells were incubated in 5% CO_2 at 37°C. Cells were passed at 25% confluency and plated in 100 mm dishes for protein extractions, in 6 well plates for RNA isolation, and in 24 well plates on cover slips for immunofluorescence experiments. For the myogenic differentiation experiments, cells were allowed to grow to confluency before the media was replaced with low-serum differentiation media containing 2% donor horse serum (Gibco, Invitrogen). The differentiation media was replaced every day for the entire differentiation time course; usually up to 5 days.

Hela and PRMT1 +/+ and -/- ES cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Multicell, Wisent) growth media containing L-glutamine, supplemented with 10% fetal calf serum (FCS; Multicell, Wisent), 2% penicillin streptomycin (Multicell, Wisent) and 1% sodium pyruvate (Multicell, Wisent). Cells were incubated in 5% CO_2 at 37°C. Cells were passed at 25% confluency and plated in 100 mm dishes for protein extractions, in 6 well plates for RNA isolation, and in 24 well plates on cover slips for immunofluorescence experiments.

2.2 Immunofluorescence

Cells grown on cover slips were rinsed twice in PBS and fixed in 4% paraformaldehyde for 10 min at room temperature then washed in PBS three subsequent times. Cells on cover slips were then rendered permeable by incubation in 0.5% triton X-100 in PBS for 10 min at room temperature and washed three times in PBS. The cells were then incubated with a specific primary antibody for 1 hour and subsequently washed once with 0.1% triton X-100 in phosphate buffered saline (PBS) containing 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4, 0.24 g of KH2PO4 in 1 L of distilled H2O and was adjusted to pH 7.4., then twice with PBS. Following the washes, the cover slips with cells were incubated for 1 hour at room temperature in the dark with a specific secondary antibody. A final wash with 0.1% triton X-100 in PBS, then twice with PBS was done before mounting the cover slips with Vectashield mounting medium with DAPI (Vector laboratories Inc.) onto glass slides. Microscopy was performed with a Zeiss Axio Imager.Z1 instrument. Cells were observed through a Zeiss 40X objective and image acquisition was done with the AxioCam HRm camera. Images captured through different fluorescent filters where then merged using Adobe Photoshop CS2 (Adobe).

2.3 Protein Extractions, SDS-Page and Western Blot Analysis

Protein extracts were obtained by lysing cells from appropriate 100 mm dishes. Cells were washed once with PBS, the cells were then harvested with whole cell extraction (WCE) buffer containing 10% glycerol, 50 mM Tris HCl pH7.5, 400 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF and 1% NP-40. The protein samples where left on ice 30 min in the presence of the whole cell extraction buffer, spun down to remove insoluble matter; supernatants were kept at

-20°C for subsequent use in western blot analysis. Protein concentration was determined using the Bradford method. 1 µl of WCE from samples was added to 1 ml of protein dye reagent (Bio-Rad). As a standard, specific quantities of bovine serum albumin (BSA) were added to 1 ml of Bradford dye. Optical densities (OD) of samples were measured with a spectrophotometer at 595 nm wavelength, and protein concentrations were extrapolated from the OD obtained from the standard curve. Equal amount of proteins were then added to Laemmli reducing buffer containing 25% glycerol, 125 mM Tris-HCL pH 6.8, 4% sodium dodecyl sulfate (SDS), 700 mM β-mercaptoethanol, bromophenol blue. Proteins were resolved on 12% SDS-polyacrylamide gel (SDS-PAGE) and transferred overnight to an Immobilon-P Polyvinylidene Difluoride (PVDF) membrane (Millipore) for western analysis. Membranes were blocked with 5% non-fat milk in PBS with 0.05% tween-20 (PBST) for one hour at room temperature, and then incubated with a specific primary antibody in 2% non-fat dry milk in PBST and 0.02% sodium azide for one hour at room temperature or overnight at 4°C. Membranes were then washed 3 times for 10 min in PBS-T, and then incubated with a specific secondary horseradish peroxidise (HRP) conjugated antibody in 2% non fat milk in PBS-T for one hour at room temperature. Membranes were washed 3 times for 10 minutes in PBS-T prior to Chemiluminescent HRP substrate detection (Millipore).

2.4 Immunoprecipitation

HA-fibrillarin and HA-SmB were transfected into Hela cells according to manufacturer's protocol with Lipofectamine 2000 (Invitrogen). Cells were harvested from appropriate 100 mm dishes, washed once with PBS then incubated with whole cell extraction buffer as described

above. Cell lysates were then incubated with 1-2 μg of anti-HA antibody or Asym25 antibody accordingly for 1 hour at 4°C with occasional gentle agitation. Then 50 μl of a 50% protein A-Sepharose (Sigma) slurry was added and incubated at 4°C for 30 min with constant end-over-end mixing. The beads were washed twice with lysis buffer and once with PBS. Laemmli reducing buffer was then added to the beads and the mixture was boiled at 95°C for 5 mins. Immunobloting was performed as described above.

2.5 Mice Muscles Cross- and Longitudinal-Sections

Skeletal muscles from mice hind legs were embeded in OTC and were cryosectioned at 10 μm thick and mounted on positive charged slides (superfrost/plus ; Fisher). Slides and tissu sections were subjected to a heat treatment of 37°C for 30 min to adhere to the slide. Tissues where then fixed and immunostained as described in the immunofluorescence section above.

2.6 Semi-Quantitative polymerase chain reaction (PCR) Amplification Analysis

Total RNA from different time points during myoblast differentiation were extracted in TRIzol reagent (Invitrogen) according to manufacturer's protocol. Complementary deoxyribonucleic acid (cDNA) was synthesized from 1.5 μg of RNA from each time point using an AMV reverse transcriptase reaction from Promega according to the manufacturer's protocol with the addition of 20 units of RNAguard (Amersham Biosciences) and 10 mM of dNTPs per reaction. 10 μM of oligo dT (18) per reaction was added before RT reactions and incubated at

65°C for 10 min. Total volume of cDNA from RT reaction was kept in 20 µl at -80°C until used for subsequent PCR reactions.

Oligonucleotides were designed against mouse Glycerol-3-phosphate dehydrogenase (GAPDH) and mouse PRMT1. Primer sequences for GAPDH (F) 5'-ACCACAGTCCATGCCATCAC-3', GAPDH (R) 5'-TCCACCACCCTGTTGCTGTA-3', for PRMT1 (F) 5'-CCAGCTGTGGGCTGAGCTAGA-3', PRMT1 (R) 5'-GTTGGCTTTGACAATCTTCAC-3'. Semi-quantitative PCR was carried out in PCR clear, flat top Thermowell tubes (Corning Incorporated), using GoTaq Green mixes (Promega) according to manufacturer's instructions. These samples were processed on a BioRad Dyad Disciple Peltier Thermal Cycler. The PCR conditions used for GAPDH were 95°C for 3 min, followed by 22 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, ending with 5 min at 72°C. The PCR conditions used for PRMT1 were 95°C for 3 min, followed by 35 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, ending with 10 min at 72°C. To separate PRMT1 v1 from v2 transcripts, PCR products were ran on a 2.5% agarose gel for 4 hours.

Chapter 3. Results:

3.1 Assessments of the Profiles of PRMTs and Arginine Methylated Proteins During Skeletal Muscle Differentiation.

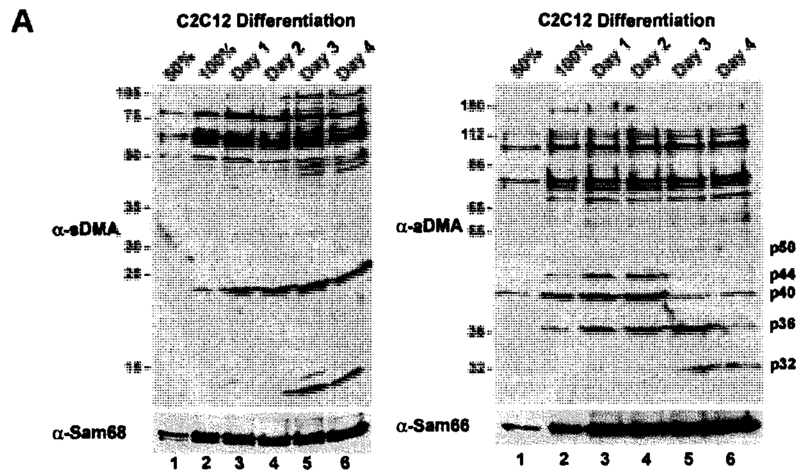
As previously demonstrated by Chen and colleagues, while using a general methylation inhibitor called AdOx, methylation is a crucial element in skeletal muscle differentiation. Moreover, it has been shown that arginine methylation by both CARM1, a type I methyltransferase, and PRMT5, a type II methyltransferase, play important roles in skeletal muscle differentiation; and for this reason we wanted to characterize the arginine methylation profiles of proteins during skeletal muscle differentiation. Using an *in vitro* approach we were able to induce C2C12 myoblasts into differentiation. This protocol provided us with myotubes as a terminal differentiation after 5 days of differentiation. Through a generous gift, we also obtained two C2C12 myoblast SMN knockdown stable cell lines, which have been extensively characterized, from D. Shafey and Dr. R. Kothary (Shafey, Cote et al. 2005). These hypomorph cell lines have increasing differentiation defects directly related to the functional SMN levels found within each line.

3.1.1 Dynamic Protein Expression Profile of Arginine Methylated Proteins During Myoblast Differentiation Is Dependent On the Presence of SMN

To investigate the changes in arginine methylated protein profiles during myoblast differentiation, we performed total protein extractions at different time points throughout differentiation from C2C12 and SMN knockdown 2A2 (65% knowkdown of SMN) myoblast cell lines and subsequently used them in a Western blot analysis of sDMA- and aDMA-containing proteins. Using antibodies produced from our laboratory that are specific for different subsets of aDMA (ASYM series of antibody) and sDMA (SYM series of antibodies) containing proteins we were able to analyses specific groups of arginine methylated proteins. Interestingly the banding pattern observed in C2C12 cells with the aDMA antibodies changed throughout differentiation (Fig. 3A; e.g. see indicated band at ~32kDa, ~36kDa, ~40kDa and ~44kDa in the right panel). Interestingly, while looking at proteins extractions from the SMN knockdown 2A2 myoblast during differentiation, the changes occurring in the normal C2C12 myoblast did not follow the same pattern. For example, in C2C12 myoblast differentiation the intensity of the band at 44kDa decreases at later time points, whereas in SMN-knockdown 2A2 myoblast differentiation, the intensity of p36 remains constant throughout the time course (Fig. 3B). Although the sDMA protein profile was also dynamic, we were surprised to see that the patterns observed with the sDMA antibodies had little difference throughout differentiation between normal C2C12 myoblasts and the SMN knockdown 2A2 myoblast cell line.

Figure 3

A



B

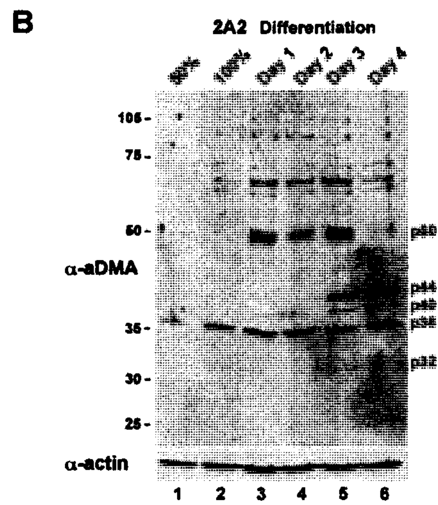


Figure 3: aDMA protein profile is dynamic during myoblast differentiation and differs from its profile in cells with low levels of SMN protein. A) Undifferentiated C2C12 cells (myoblasts) were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Total cell lysate was done with Whole Cell Extraction Buffer kept on ice for 30 min, span down, and the supernatant were separated by SDS-PAGE, transferred to an Immobilon-P membrane and immunobloted with sDMA (left panel) or aDMA (right panel) antibody. Loading control was done by immunobloting with anti-Sam68. n=3 B) Undifferentiated 2A2 SMN knockdown myoblast (65% knockdown) clone were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Total cell lysate was done with Whole Cell Extraction Buffer kept on ice for 30 min, span down, and the supernatant were separated by SDS-PAGE, transferred to an Immobilon-P membrane and immunobloted with aDMA antibodies. Loading control was done by immunobloting with anti-actin. n=3

3.1.2 Dynamic Intra-Cellular Distribution of Arginine Methylated Proteins During Skeletal Muscle Differentiation Is Dependent On the Presence of SMN

The differentiation time course enabled us to take snap shots, by indirect-immunostaining microscopy, of the localization of aDMA and sDMA containing proteins at regular time intervals during differentiation. Cells were fixed and then used for indirect-immunofluorescence using sDMA and aDMA-specific anibodies, and a monoclonal antibody (MF20) directed against embryonic and postnatal isoforms of myosin heavy chain (MHC) used as a marker of differentiation. Indirect immunofluorescence staining performed with sDMA-specific antibodies, Sym10 (Fig. 4) and Sym11 (Fig. 5) yielded similar patterns: Both antibodies recognized proteins mostly in the nucleus,with no signal in nucleoli and a weak cytoplasmic staining (Fig. 4, 75% through day 5 and Fig. 5, 75% through day 5, respectively). Staining patterns stayed the same throughout differentiation from myoblasts to myotubes for both antibodies.

Figure 4

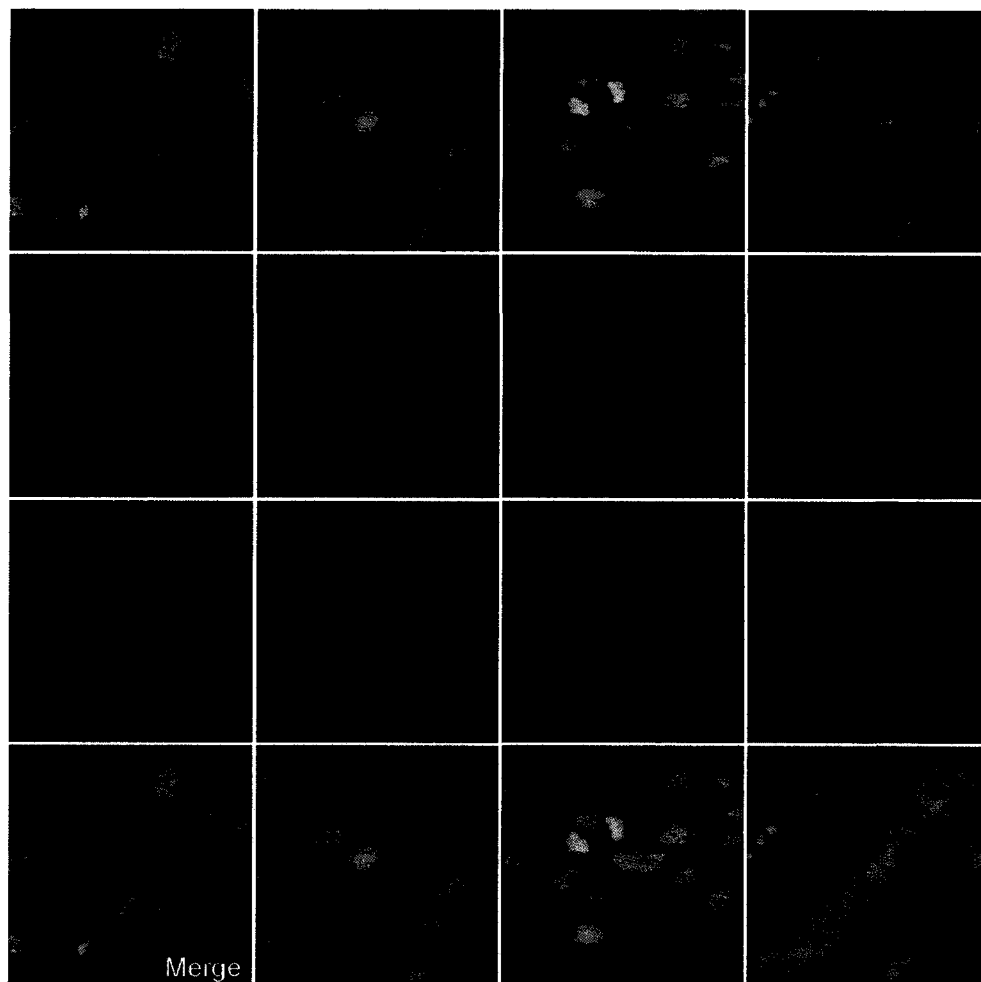


Figure 4: sDMA-specific Sym10 antibody staining patterns in C2C12 cell differentiation. Undifferentiated C2C12 cells (myoblasts) were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with anti-sDMA antibodie Sym10. Anti-Myosin Heavy Chain (anti-MHC) antibody was used to demonstrate the terminal differentiated myotubes. Secondary antibodies used were conjugated to Alexa 594 (red: MHC) and Alexa 488 (green: Sym10). n=4

Figure 5

C2C12

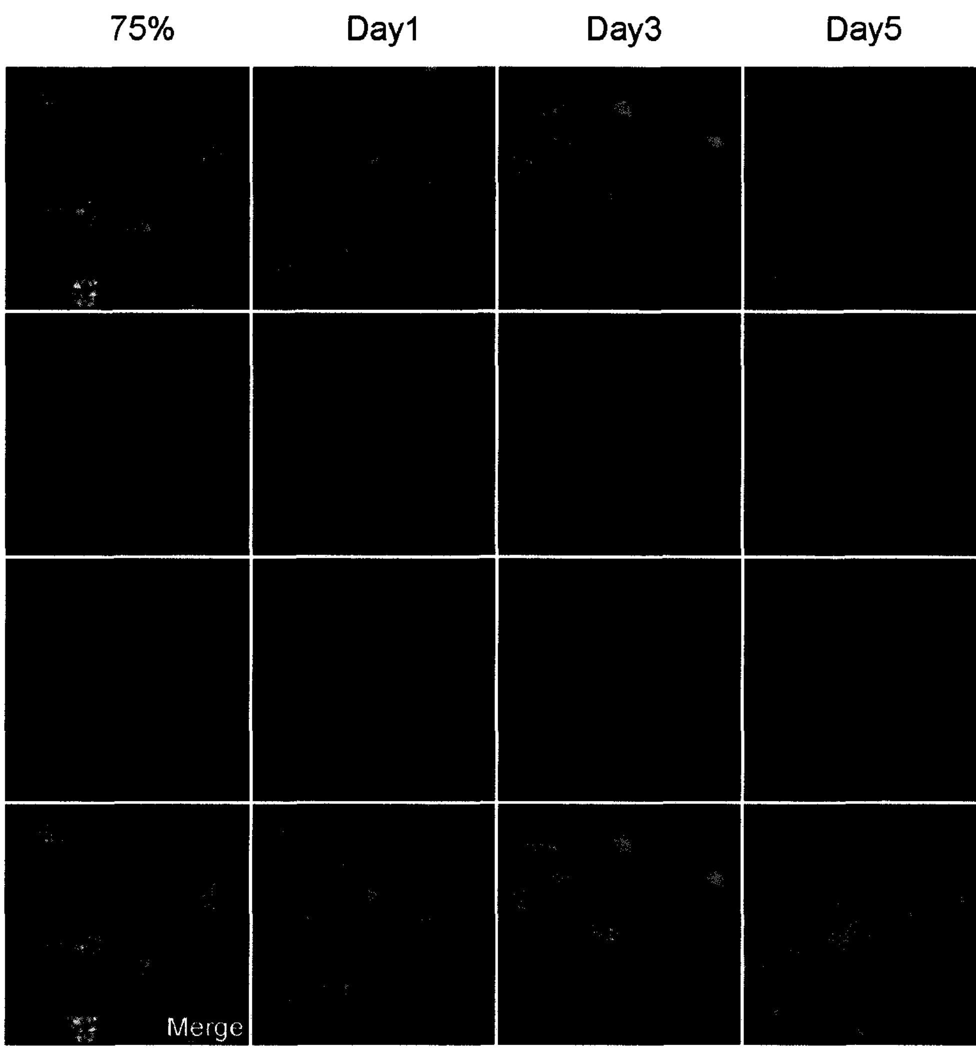


Figure 5: sDMA-specific Sym11 antibody staining patterns in C2C12 cell differentiation. Undifferentiated C2C12 cells (myoblasts) were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with anti-sDMA antibodie Sym11. Anti-Myosin Heavy Chain (anti-MHC) antibody was used to demonstrate the terminal differentiated myotubes. Secondary antibodies used were conjugated to Alexa 594 (red: MHC) and Alexa 488 (green: Sym11). n=4

Interestingly, indirect immunofluorescence staining performed with the two aDMA-specific antibodies Asym24 (Fig. 6) and Asym25 (Fig. 7) gave distinct patterns. Asym24-reactive proteins localized primarily in the nucleus, again excluded from nucleoli (Fig. 6, 75% through day 5). This profile remained unchanged throughout differentiation with the appearance of a very weak cytoplasmic signal in myotubes. In contrast, proteins reacting with the Asym25 antibody had a predominantly nucleolar staining (Fig. 7, 75%), and changed during differentiation to a more diffused signal within the nucleoplasm and clear cytoplasmic staining observed at later time points of differentiation (Fig. 7, 75% through day 5).

Arginine methylated protein profiles revealed that proteins reacting with the Asym 25 antibody are dynamic during myoblast differentiation *in vitro*. Our establishment of the existence of a dynamic profile of arginine methylated proteins during myoblast differentiation is suggestive of a regulatory mechanism that could involve SMN.

Figure 6

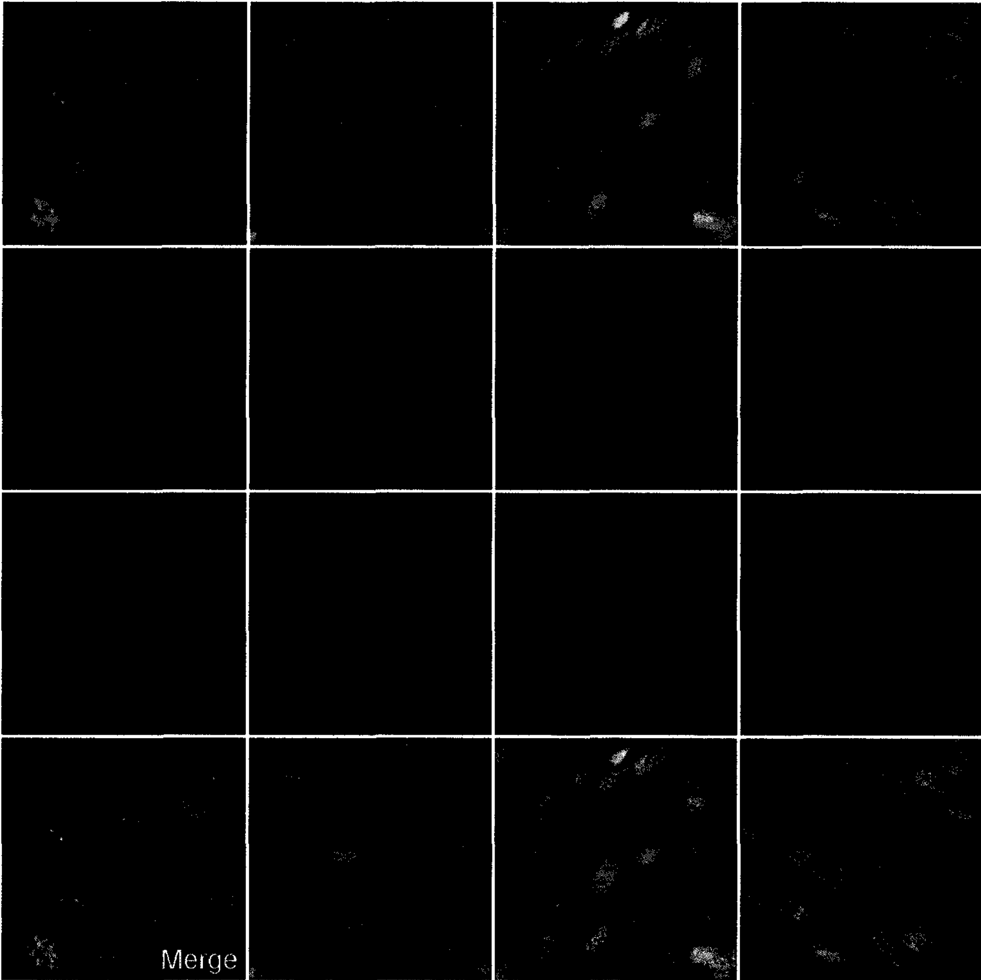


Figure 6: aDMA-specific Asym24 antibody staining patterns in C2C12 cell differentiation. Undifferentiated C2C12 cells (myoblasts) were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with anti-aDMA antibodie (Asym24). Anti-Myosin Heavy Chain (anti-MHC) antibody was used to demonstrate the terminal differentiated myotubes. Secondary antibodies used were conjugated to Alexa 594 (red: MHC) and Alexa 488 (green: Asym24). n=4

Figure 7

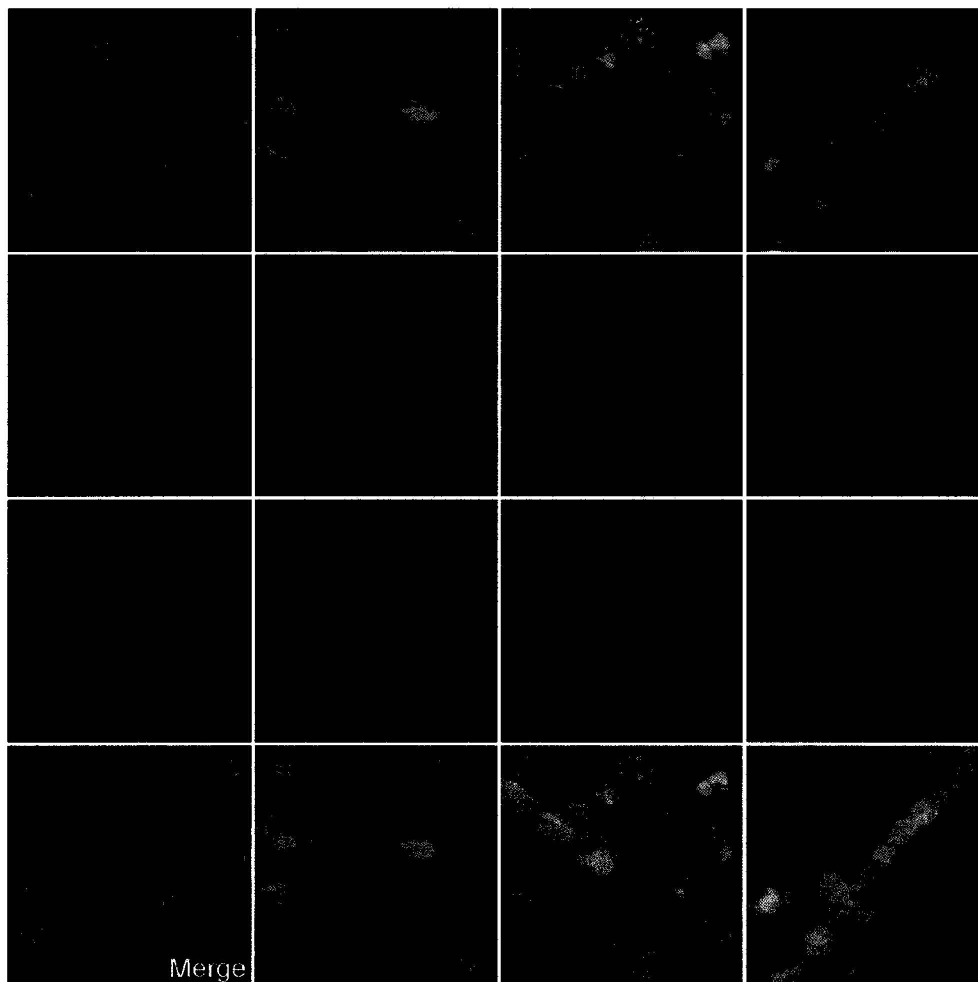


Figure 7: aDMA-specific Asym25 antibody staining patterns in C2C12 cell differentiation. Undifferentiated C2C12 cells (myoblasts) were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with anti-aDMA antibodie (Asym25). Anti-Myosin Heavy Chain (anti-MHC) antibody was used to demonstrate the terminal differentiated myotubes. Secondary antibodies used were conjugated to Alexa 594 (red: MHC) and Alexa 488 (green: Asym25). n=4

3.1.3 Asymmetric Dimethylated Arginine-Containing Proteins Are Misregulated In SMN-Deficient Myoblasts During Differentiation

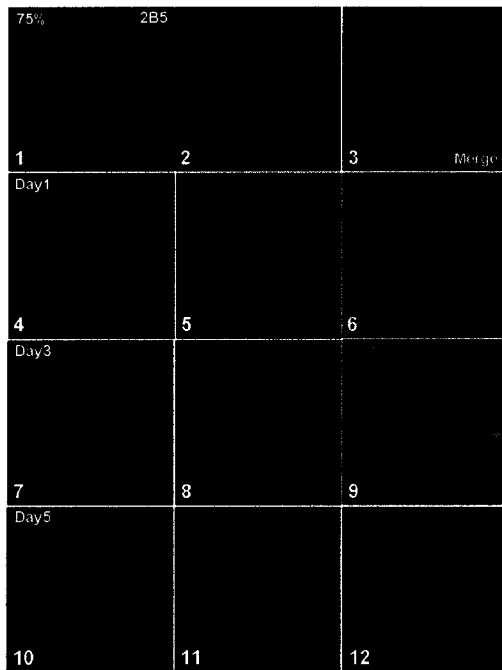
Since SMN has been involved in skeletal muscle differentiation (Shafey, Cote et al. 2005) and knowing that methylation plays an important role in regulating SMN's protein-protein interactions; we wanted to see if there would be any differences in the localization and overall arginine methylation profiles of proteins during skeletal muscle differentiation in SMN-deficient myoblasts. Using the same experimental setup as in the previous section we performed indirect immunofluorescence microscopy with our panel of methyl-dependent antibodies (Fig. 8).

Staining throughout differentiation on the 2B5 SMN knockdown line (55% knockdown of SMN) revealed that sDMA-specific antibodies (Sym10 and Sym11) as well as Asym24 aDMA-specific antibody signals remained similar to that seen during normal C2C12 myoblasts differentiation, with little differences in localization throughout the time course. Strikingly, indirect immunofluorescence staining performed with aDMA-specific antibody Asym25 did not follow the profile observed in normal C2C12 myoblasts differentiation. In 2B5 cells, staining with the Asym25 antibody also started in the nucleolus, but remained in the nucleolus during differentiation (Fig. 8A). The difference in localization of aDMA-containing proteins specifically reacting with the Asym25 antibody during myoblast differentiation likely results from the loss of a specific function of SMN in this process.

Figure 8

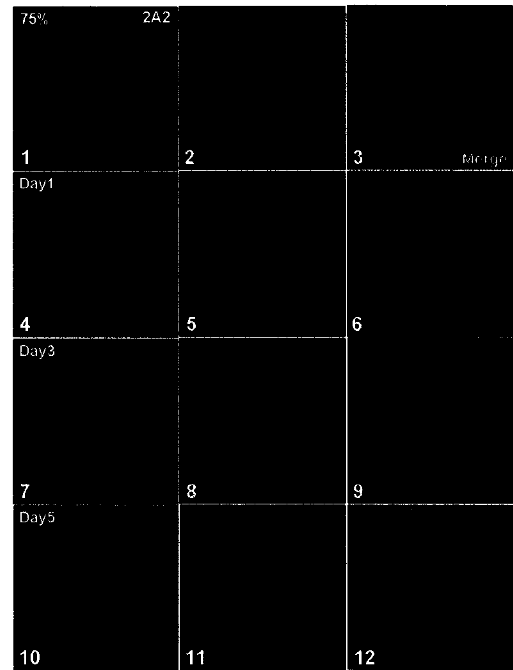
A

2B5 myoblast-myotube differentiation



B

2A2 myoblast-myotube differentiation



C

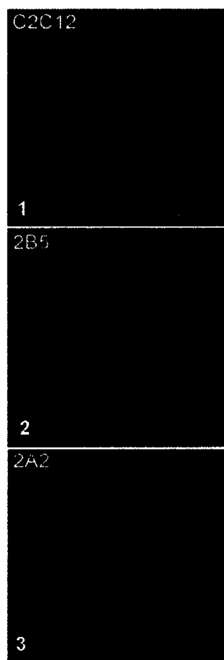


Figure 8: Asym25 staining pattern differs in myoblast with low SMN protein levels. A) Undifferentiated 2B5 SMN knockdown myoblast (55% knockdown) clone were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with Asym25 (aDMA-specific) antibody. Anti-Myosin Heavy Chain (anti-MHC) antibody was used to demonstrate the terminal differentiated myotubes. Secondary antibodies used were conjugated to Alexa 594 (red: MHC) and Alexa 488 (green: Asym25). n=4 B) Undifferentiated 2A2 SMN knockdown myoblast (65% knockdown) clone were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with Asym25 (aDMA-specific) antibody. Anti-Myosin Heavy Chain (anti-MHC) antibody was used to demonstrate the terminal differentiated myotubes. Secondary antibodies used were conjugated to Alexa 594 (red: MHC) and Alexa 488 (green: Asym25). n=4 C) Zoom in on Immunostaining from Asym25 antibody done on terminally differentiated myotubes from C2C12, 2B5 and 2A2 cells on day 5 of differentiation. n=4

Although some cytoplasmic staining occurred in certain 2B5 myotubes (see Fig. 8A, panel 10 and Fig. 8C, panel 2) it was significantly reduced as compared to the cytoplasmic staining observed in normal C2C12 myotubes (Fig. 4, 75% through day 5).

To further investigate this phenomenon and strengthen the link with SMN, we proceeded to do the same staining on the 2A2 SMN clone (65% knockdown of SMN). We observed the same patterns in the 2A2 cells (Fig. 8B). Although the staining with Asym25 was similar to the one obtained with 2B5 cells, there was extremely little or no staining in the cytoplasm of 2A2 myotubes (see Fig. 8B, panel 10 and Fig. 8C, panel 3). These observations strongly suggest an involvement of SMN in the resulting misregulation of aDMA-containing proteins. Furthermore, it appears that a more severe lack in SMN proteins results in a more severe mislocalisation of Asym25-specific proteins during myoblast differentiation. This effect is not a result of a specific cell line since we used two different SMN knockdown myoblast clones to confirm these observations.

The series of observations from the above sections (3.1.1 - 3.1.3) sparked an interest as to which PRMTs would be involve in such a dynamic methylation of arginine within specific proteins during myoblast differentiation (will be addressed in section 3.4).

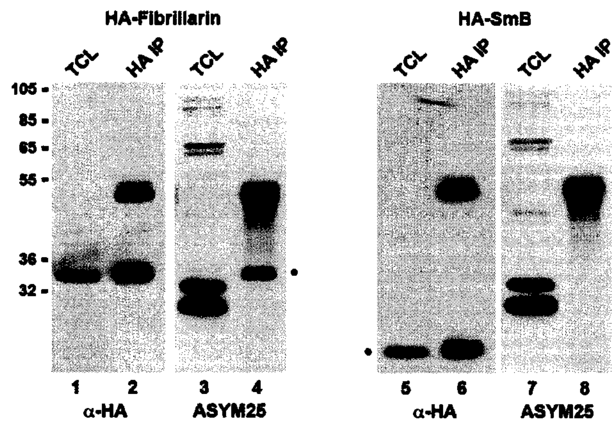
3.2 Fibrillarin Is Misregulated In the Absence of Functional Levels of SMN

3.2.1 Fibrillarin Is Recognized Directly by the ASYM25 Antisera

As previously mentioned, Asym25 reacted with proteins residing within the nucleolus of myoblasts. To further characterize the possible proteins Asym25 might be reacting with, we tested various nucleolar proteins that are known to be methylated asymmetrically, by western blot analysis (Fig. 9A), and indirect immunofluorescence staining (Fig. 9B). Using this candidate approach we identified one of the nucleolar proteins that may be reacting with the Asym25 antibody as fibrillarin, a box C/D snoRNP component. To confirm immunoreactivity with Asym25, HA-tagged fibrillarin was expressed in HeLa cells, immunoprecipitated with an HA antibody and subjected to Western blot analysis with Asym25. As expected, anti-HA antibody recognized HA-fibrillarin in total cell lysate, and in the HA-immunoprecipitate (IP) (Fig. 9A, lanes 1 & 2). The aDMA-specific Asym25 antibody recognized the same molecular weight band in the total cell lysate and in the HA-IP (Fig. 9A, lanes 3 & 4), therefore showing that the Asym25 antibody recognized directly fibrillarin. As a negative control, SmB, a known sDMA containing protein, was expressed in HeLa cells with an HA-tag, and subjected to the same treatment. Anti-HA antibodies recognized HA-SmB in total cell lysate, and in the HA-IP (Fig. 9A, lanes 5 & 6). However, the aDMA-specific Asym25 antibody failed to recognize the same molecular weight band in the total cell lysate and HA-IP corresponding to SmB (Fig. 9A, lanes 7 & 8), therefore demonstrating the specificity that the Asym25 antibody has towards aDMA-containing proteins, including fibrillarin. To further verify that Asym25 can recognize endogenous fibrillarin, we performed indirect immunofluorescence in HeLa cells (Fig. 9B). As

Figure 9

A



B

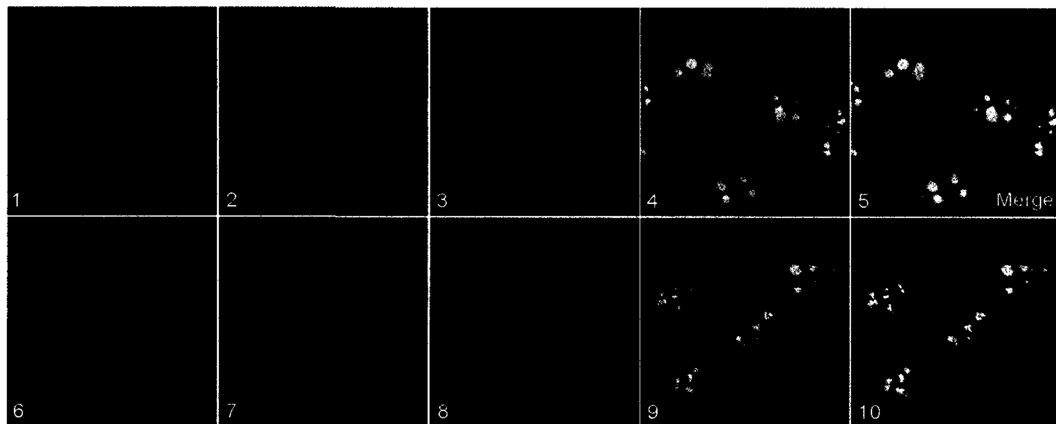


Figure 9: Fibrillarin is one of the aDMA containing protein recognized by Asym25 antibody. A) HeLa cells were kept in DMEM with 10% Fetal Bovine Serum. Prior to transfection the medium was changed to DMEM without Fetal Bovine Serum and without antibiotics. Transfection was performed with lipofectamine according to protocol with HA-Fibrillarin or HA-SmB. Three hours after transfection a final concentration of 10% Fetal Bovine Serum was added to the plates. Total cell lysate was done with Whole Cell Extraction Buffer kept on ice for 30 min, span down, and the supernatant were separated by SDS-PAGE, transferred to an Immobilon-P membrane and immunobloted with anti-HA or Asym25 antibody. Migration of fibrillarin and SmB are indicated with asterisk. n=1 B) Endogenous Fibrillarin partially co-localizes with the Asym25 signal in the fibrillar portion of the nucleolus in HeLa cells. HeLa cells were kept in DMEM with 10% Fetal Bovine Serum. Cells were then fixed and immunostained with Asym25 antibody and anti-fibrillarin, followed by secondary antibody conjugated to Alexa 594 (red: fibrillarin) and Alexa 488 (green: Asym25). n=3

observed in C2C12 cells, aDMA-containing proteins reacting with Asym25 were seen throughout the nucleus and in concentrated aggregates in the nucleolus (Fig. 9B, panels 2 and 7). Fibrillarin was primarily found in ring-like structures in the nucleolus (Fig. 9B, panels 3 and 8). The overlay of Asym25-recognized aDMA-containing proteins and fibrillarin showed a nice co-localization in the nucleolus (Fig. 9B, 4 and 9); once again providing evidence that fibrillarin is one of the proteins recognized by the aDMA-specific Asym25 antibody.

3.2.2 Fibrillarin Dynamic Relocalization During Skeletal Muscle Differentiation Is Dependent on SMN

Having identified fibrillarin as a nucleolar protein that reacts with the Asym25 antibody, we wanted to assess if fibrillarin followed the same dynamic profile as Asym25 during wild type and SMN-deficient myoblast differentiation using indirect immunofluorescence microscropy. Using a monoclonal anti-fibrillarin antibody 72B9, aDMA-specific Asym25, and a monoclonal anti-MF20 against myosin heavy chain, a terminal differentiation marker for myotubes, we observed similar profiles between Asym25 and fibrillarin staining in C2C12 myoblasts (Fig. 10A). As expected, both fibrillarin and Asym25 antibodies stained nucleoli in undifferentiated C2C12 myoblasts (Fig. 10A, panels 1-8), and as differentiation progressed, both stainings became diffused to the nucleoplasm, with weak cytoplasmic signal (Fig. 10A, panels 9-16).

Using the same approach in the SMN knockdown cell line 2A2 (65% knockdown) (Fig. 10B), fibrillarin and Asym25 staining were seen in the nucleolus of undifferentiated 2A2 cells, and unchanged in differentiated myotubes along with barely detectable cytoplasmic staining (Fig. 10B, panels 1-16). The same observations are applicable to the 2B5 SMN knockdown cell

Figure 10

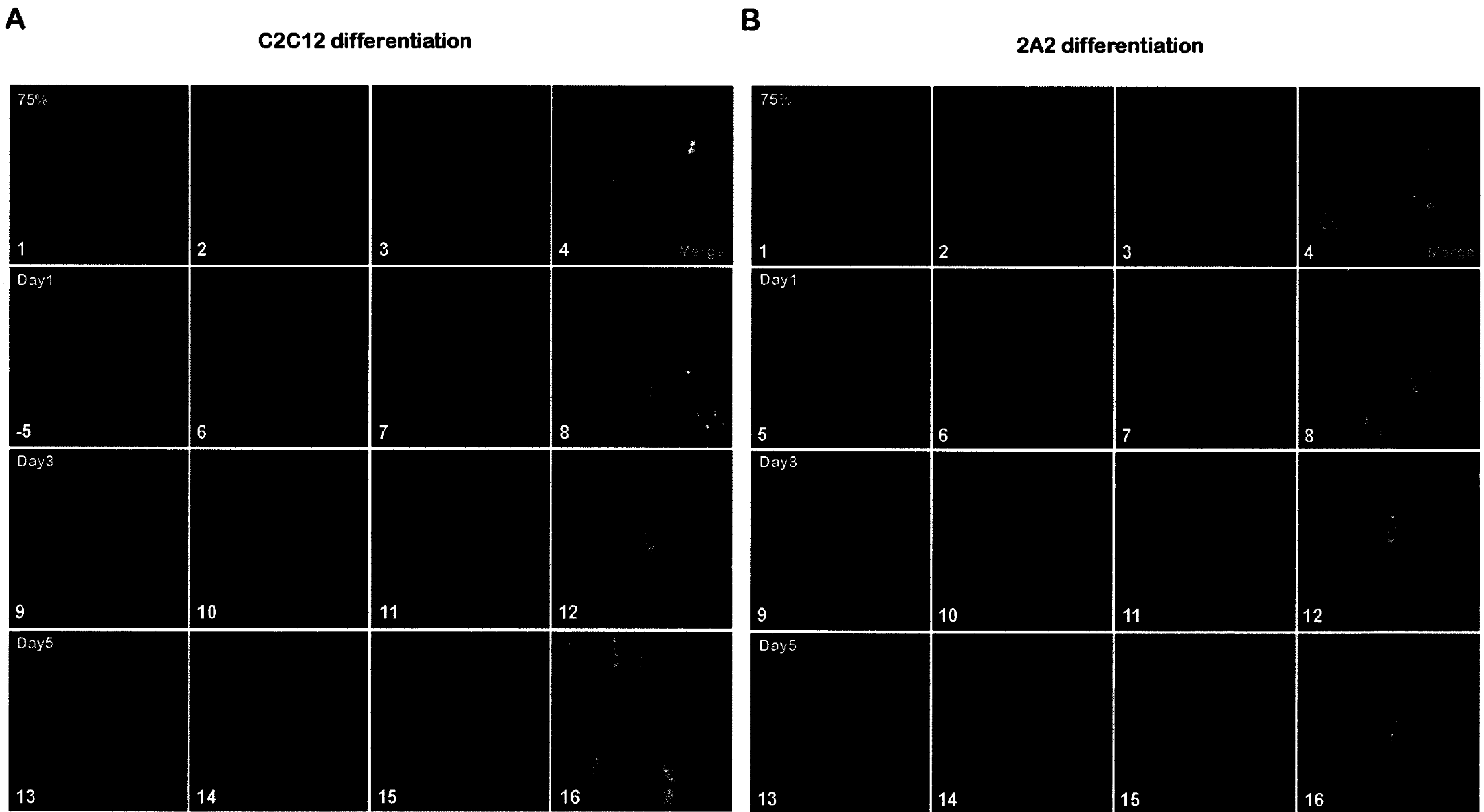


Figure 10: Endogenous Fibrillarin follows Asym25 staining patterns during myoblast differentiation. A) Undifferentiated C2C12 cells (myoblasts) were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with Asym25, anti-fibrillarin. Anti-Myosin Heavy Chain (anti-MHC) antibody was used to demonstrate the terminal differentiated myotubes. Secondary antibodies used were conjugated to Alexa 594 (red: fibrillarin), Alexa 488 (green: Asym25) and Alexa 647 (far red: MHC). n=3 B) Undifferentiated 2A2 SMN knockdown myoblast (65% knockdown) clone were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with Asym25, anti-fibrillarin. Anti-Myosin Heavy Chain (anti-MHC) antibody was used to demonstrate the terminal differentiated myotubes. Secondary antibodies used were conjugated to Alexa 594 (red: fibrillarin), Alexa 488 (green: Asym25) and Alexa 647 (far red: MHC). n=3

line (55% knockdown), with the exception of having a slightly more cytoplasmic staining than the 2A2 cell line (Fig. 8B and C). These observations followed the patterns observed with Asym25 as described above. Namely, there seems to be a correlation between functional SMN levels and the extent of mislocalization of fibrillarin; the more severe the lack of functional SMN, the more fibrillarin seems to be confided within the nucleolus. The colocalization of proteins reacting with the 72B9 and Asym25 antibodies in this experiment provided further evidence that Asym25 can recognize fibrillarin in mouse muscle cells. It also confirmed that fibrillarin is one of the proteins mislocalized or misregulated in the presence of low levels of SMN, therefore providing more evidence of the wide/broad impact of the roles and functions that SMN has in skeletal muscle and how they may be affected in SMA patients.

3.2.3 B23 Nucleolar Protein has a Similar Dynamic Relocalization During Skeletal Muscle Differentiation, which Is also Dependent on SMN.

To further investigate the dynamic relocalization of fibrillarin we used a candidate approach to test other ribosomal nucleolar proteins. B23 is a non ribosomal protein and interacts with SMN, although it does not participate in snoRNP biogenesis. In normal C2C12 myoblasts B23 can be seen within the nucleolus, but as differentiation takes effect it slowly diffuses to the nucleoplasm and the strong nucleoli staining becomes diffused (Fig. 11A). Interestingly, in the SMN-knockdown cell lines 2B5 (Fig. 11B) and 2A2 (Fig. 11C) we clearly see the nucleoli staining throughout the differentiation. The interesting link between fibrillarin and B23 apart from the similar staining during muscle differentiation in normal C2C12 and SMN-knockdown cell lines

2B5 and 2A2 is that both proteins are known to interact with SMN, providing further evidence that functional SMN levels in muscle cells is critical for proper development.

Figure 11

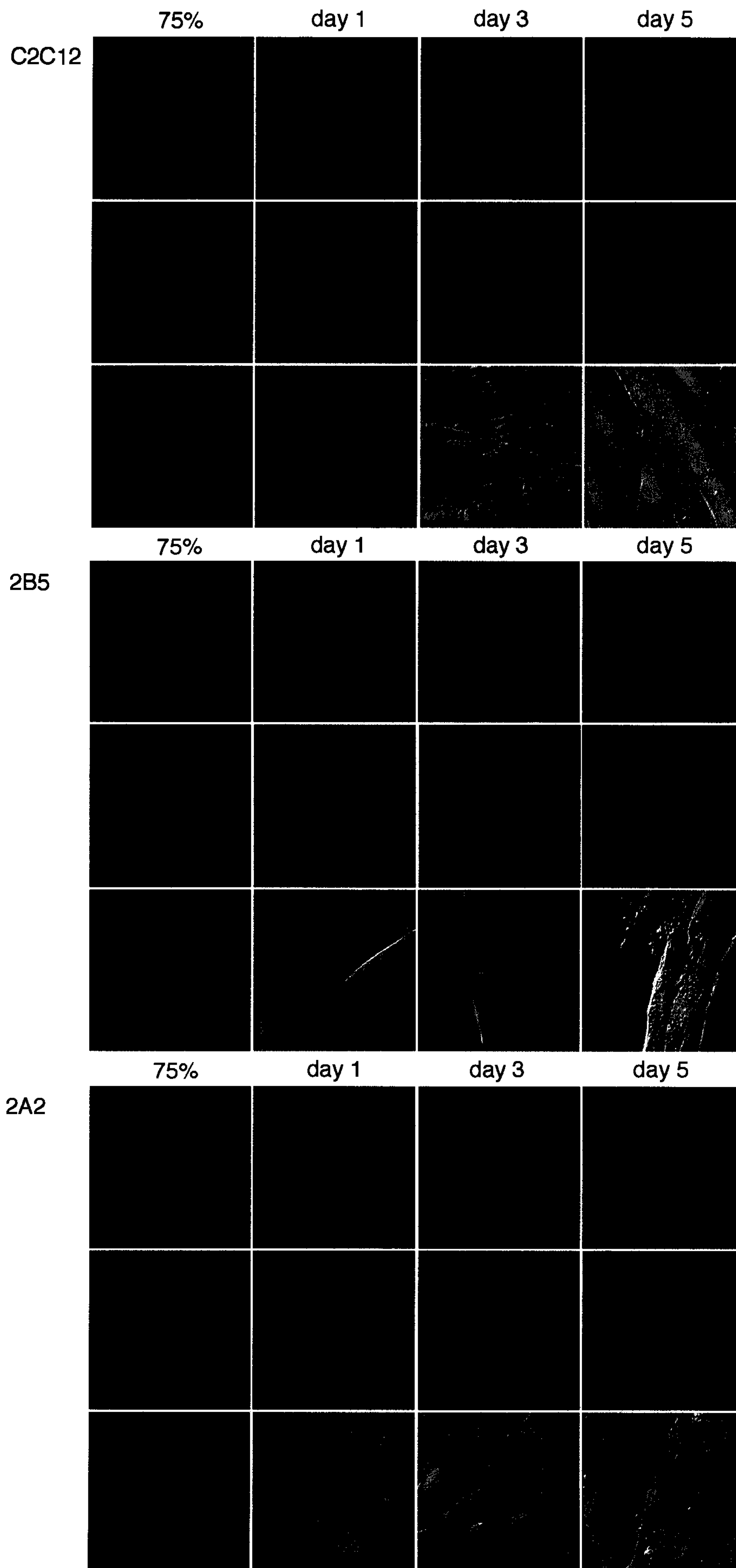


Figure 11: Endogenous B23 follows Asym25 and fibrillarin staining patterns during myoblast differentiation. A) Time point differentiation from myoblast to myotube of C2C12 cells. Undifferentiated cells were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with anti-B23 antibody. DIC immaging was used to demonstrate differentiated myotubes. Secondary antibodie used was conjugated to Alexa 488 (green: B23), n=1. B) Time point differentiation from myoblast to myotube of 2B5 SMN knockdown cells. Undifferentiated cells were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with anti-B23 antibody. DIC immaging was used to demonstrate differentiated myotubes. Secondary antibodie used was conjugated to Alexa 488 (green: B23), n=1. C) Time point differentiation from myoblast to myotube of 2A2 SMN knockdown cells. Undifferentiated cells were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Cells were fixed and immunostained with anti-B23 antibody. DIC immaging was used to demonstrate differentiated myotubes. Secondary antibodie used was conjugated to Alexa 488 (green: B23), n=1.

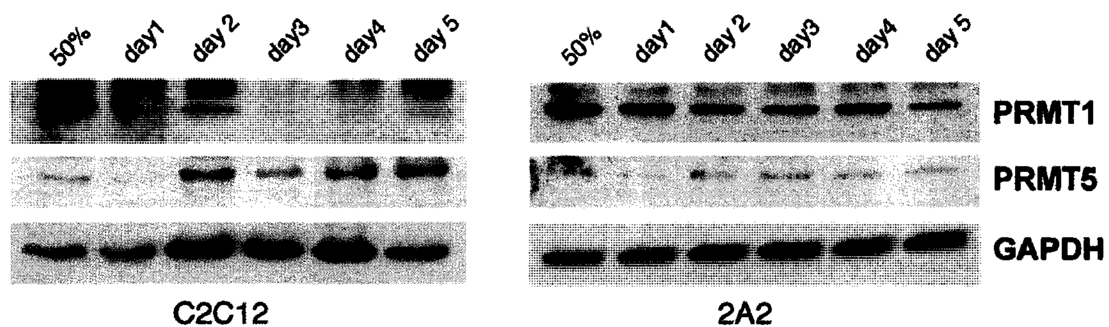
3.3 Expression Profile of PRMT1 and PRMT5 During Skeletal Muscle Differentiation

The arginine methylation profiles seen in earlier sections prompted us to investigate the levels and profiles of different PRMTs responsible for aDMA and sDMA modifications. Specifically, we assessed the expression profiles of PRMT1 and PRMT5, since they have both been reported to interact with and methylate fibrillarin (Yanagida, Hayano et al. 2004). Knowing that these interactions usually occur in normal *in vitro* models, we wanted to investigate PRMT1 and PRMT5 protein profiles during normal myoblast differentiation into myotubes compared to the profiles seen in SMN knockdown myoblast cell line 2A2. Overall PRMT5 levels remained unchanged in C2C12 and 2A2 myoblast cell lines throughout differentiation (Fig. 12A). In contrast, PRMT1 was present in early time points of normal C2C12 myoblast differentiation, but drastically decreased during mid to late time points of differentiation (Fig. 12A). Strikingly, this downregulation in PRMT1 expression was not observedin the SMN knockdown cell line 2A2, where PRMT1 levels remained constant throughout differentiation (Fig. 12A). Observation of a decrease in PRMT1 protein levels during myoblast differentiation and the loss of this profile in SMN knockdown myoblast cell line led us to investigate the PRMT1 mRNA transcript profile throughout differentiation of C2C12 and SMN knockdown 2A2 myoblast cell lines. Little to no difference is seen between PRMT1 RNA expression in C2C12 and 2A2 myoblast cell lines throughout differentiation. Interestingly, inversely to what is seen with PRMT1 protein levels, there is a very small increase of PRMT1 transcript in the mid to late time points of differentiation in the normal C2C12, although this

Figure 12

A

Protein profiles of PRMTs during myoblast differentiation



B

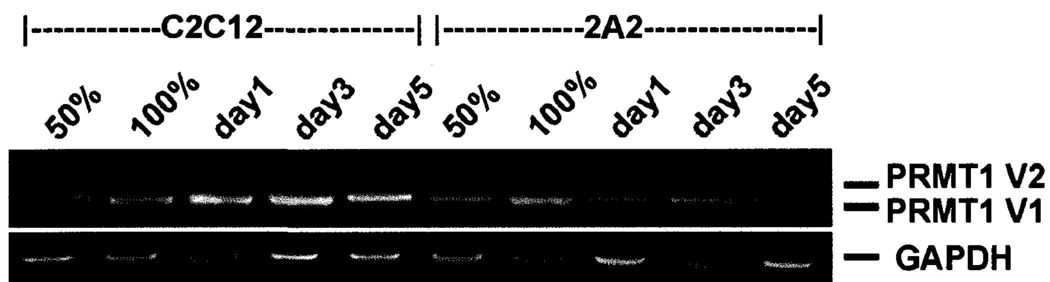


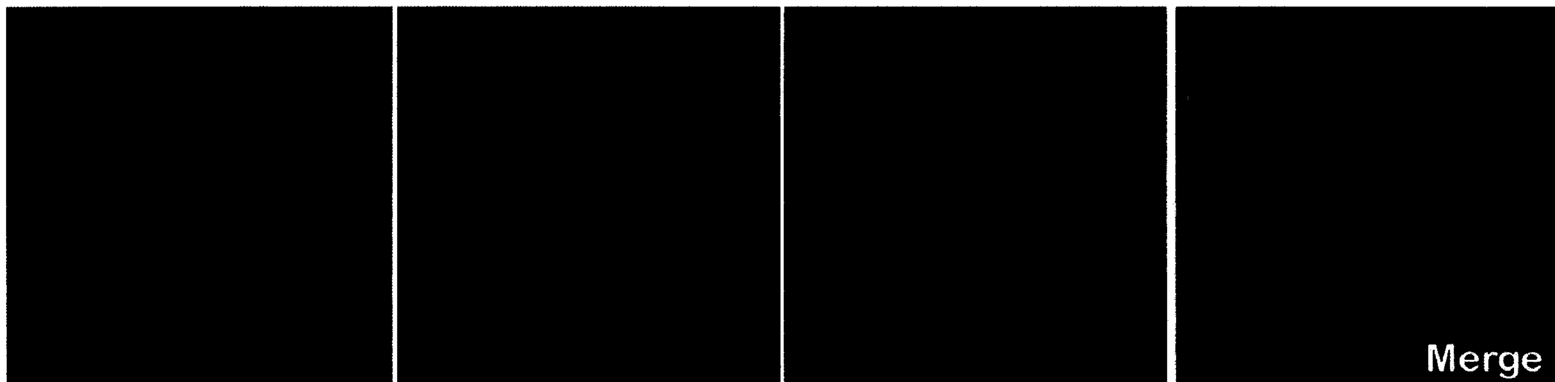
Figure 12: PRMT1 expression decreases during normal myoblast differentiation, but fails to decrease in SMN knockdown clone cells. A) C2C12 and 2A2 SMN knockdown myoblasts (65% knockdown) were kept in DMEM with 10% Fetal Bovine Serum. Differentiation was done over the course of 5 days in differentiation medium DMEM with 2% Horse Serum. Total cell lysate was done with Whole Cell Extraction Buffer kept on ice for 30 min, span down, and the supernatant were separated by SDS-PAGE, transferred to an Immobilon-P membrane and immunobloted with anti-PRMT1 and anti-PRMT5. Loading control was done by immunobloting with anti-GAPDH. n=3 B) PRMT1 variant 1 and 2 transcript levels were compared by RT-PCR. Total cDNA from C2C12 and 2A2 SMN knockdown myoblasts were isolated before and during differentiation, where amplified by PCR using isoform specific olligonucleotides. GAPDH transcript was amplified as a positive control and to show that an equal amount of cDNA was used for each reaction. PCR products were resolved on a 2.5% agarose gel and visualized by ethidium bromide staining. n=1 duplicate

difference in transcripts is likely not significant (Fig. 12B). There is no discernable difference in PRMT1 transcript in the SMN knockdown 2A2 myoblast cell line, as was expected from the unchanged PRMT1 protein levels seen earlier in this line (Fig. 12B). The discrepancy between PRMT1 mRNA and protein profiles during C2C12 differentiation suggest PRMT1 expression is likely regulated at the level of translation and/or protein turnover during this process. Moreover, this regulation seems to be dependent on the presence of functional levels of SMN. Further experiments will be required to determine the mechanism(s) by which SMN may regulate the expression level of PRMT1.

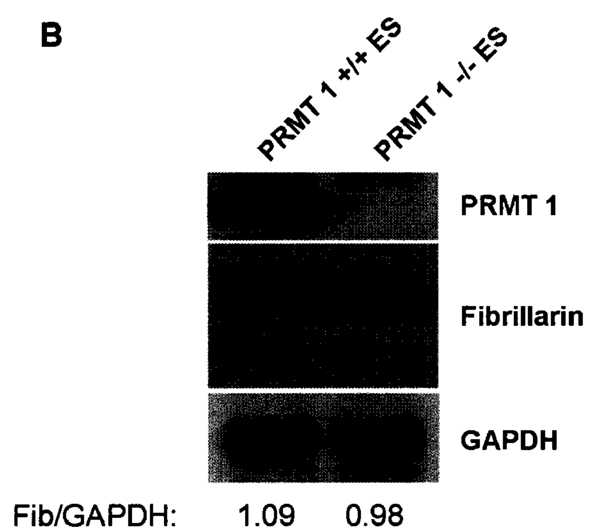
We next wanted to investigate the possibility that PRMT1 may be involved in the mechanism regulating fibrillarin relocalization during skeletal muscle differentiation. To initiate these experiments, we first assessed the intracellular localization of fibrillarin in the presence or absence of PRMT1 using knock-out ES cells generated through retroviral insertion (Pawlak et al. 2000). Similar localization was observed in PRMT1$^{+/+}$ and PRMT1$^{-/-}$ ES cell lines using the monoclonal anti-fibrillarin antibody 72B9 for indirect immunofluorescence mircroscopy (Fig. 13A). We next determined the amount of fibrillarin in PRMT1$^{+/+}$ and PRMT1$^{-/-}$ ES cell lines and similar levels of fibrillarin were observed between the two cell lines, using GAPDH levels as loading control (Fig. 13B).

Figure 13

A



B



Fib/GAPDH: 1.09 0.98

Figure 13: PRMT1 protein levels do not alter fibrillarins localization of expression. A) PRMT1$^{+/+}$ and PRMT1$^{-/-}$ ES cells were kept as previously described (Pawlak 2000). Cells were fixed and immunostained with aDMA-specific Asym25 and 72B9 (anti-fibrillarin) antibodies. Secondary antibodies used were conjugated to Alexa 594 (red: 72B9) and Alexa 488 (green: Asym25). n=1 duplicate B) Total cell lysate on PRMT1$^{+/+}$ and$^{-/-}$ ES cells was done with Whole Cell Extraction Buffer kept on ice for 30 min, span down, and the supernatant were separated by SDS-PAGE, transferred to an Immobilon-P membrane and immunobloted with anti-PRMT1 and anti-fibrillarin. Loading control was done by immunobloting with anti-GAPDH. n=1 duplicate

3.4 Aberrant Arginine Methylation Profiles in Skeletal Muscle Tissues from SMA Model Mice

3.4.1 General Methylation Profiles by Western Blots Shows Differences in Both sDMA and aDMA-Containing Proteins

Having observed specific patterns of arginine methylated proteins during myoblast differentiation, we wanted to investigate any similar profile changes in skeletal muscle tissues from WT and SMA mice. Hence, we compared hind leg muscle total protein extractions from wild type and SMA mice. Results from Western blot analysis with sDMA and aDMA antibodies revealed both quantitative and qualitative differences for several methylated proteins between wild type and SMA model mice (Fig. 14). This also included a protein found in the aDMA blot at 36 kDa, previously observed in our *in vitro* models of C2C12 and SMN knockdown 2A2 myoblast cell lines during differentiation. These preliminary observations suggest that methylated proteins may also be misregulated in *in vivo* SMA-model mice. Further experiments using different development time point in WT and SMA-model mice will be required to confirm and expand the understanding of these observations.

Figure 14

Profiles of R methylated proteins in SMA muscles

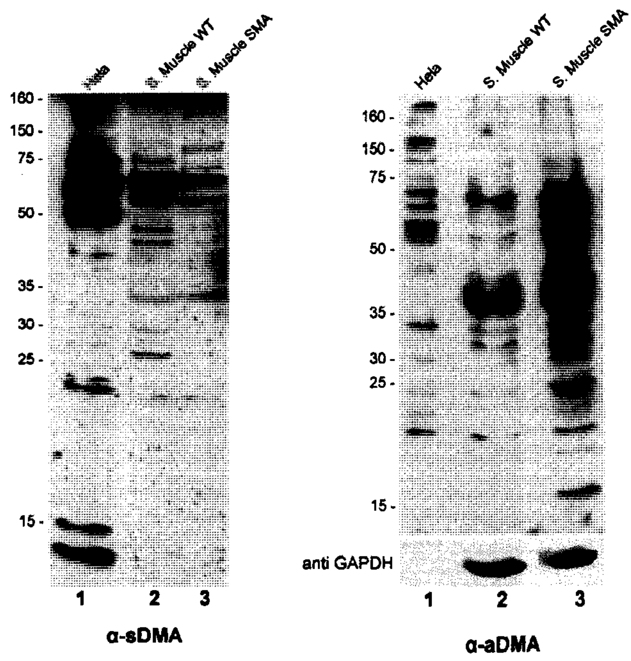


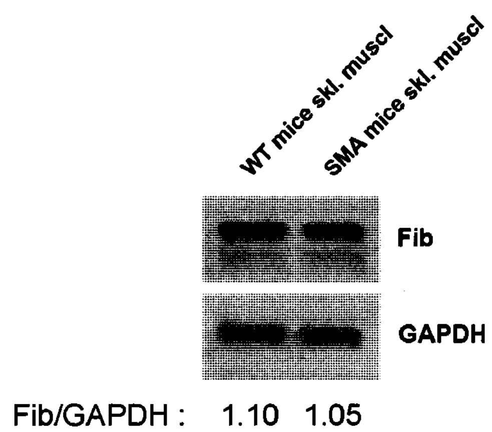
Figure 14: sDMA- and aDMA-containing protein patterns change between WT mice muscles and SMA mice muscles. Muscles were taken from post-natal 3 month old mice. Muscle protein extraction was performed with tissues frozen and crushed in liquid nitrogen, then incubated with Whole Cell Extraction Buffer kept on ice for 30 min, span down, and the supernatant were separated by SDS-PAGE, transferred to an Immobilon-P membrane and immunobloted with sDMA (left panel) or aDMA (right panel) antibody. Loading control was done by immunobloting with anti-GAPDH. HeLa cell extraction was run as a means of comparison. n=1

3.4.2 Western Blots Shows Fibrillarin Protein Levels Remain Unaffected in Presence of Low Amounts of SMN in WT and SMA Model Mice.

Knowing that low levels of SMN affected the localization of fibrillarin, we investigated whether the amount of SMN protein in skeletal muscles affected the levels of fibrillarin protein, as was observed in previous research for certain SMN interactors (Helmken, Hofmann et al. 2003; Tadesse, Deschenes-Furry et al. 2007). We investigated the amount of fibrillarin in total skeletal muscle protein extracts from WT and SMA mice (heterozygote mice containing only one functional allele of mouse *Smn*). Similar levels of fibrillarin were observed between the two, using GAPDH levels as loading control (Fig. 15A). These data suggest that fibrillarin protein levels are not dependent on levels of SMN protein in these animals, but that the misregulation of fibrillarin seems to solely be at the level of localization during skeletal muscle differentiation as was observed in C2C12 and SMN-knockdown myoblast and in preliminary skeletal muscles sections immunofluorescence (Fig. 15B and C). In these muscle sections we see that fibrillarin and Asym25 have a small amount of colocalization in wild type mice muscle sections (Fig. 15B). Noticeably, there is stronger collocalization in the heterozygous mice muscle sections (Fig. 15C).

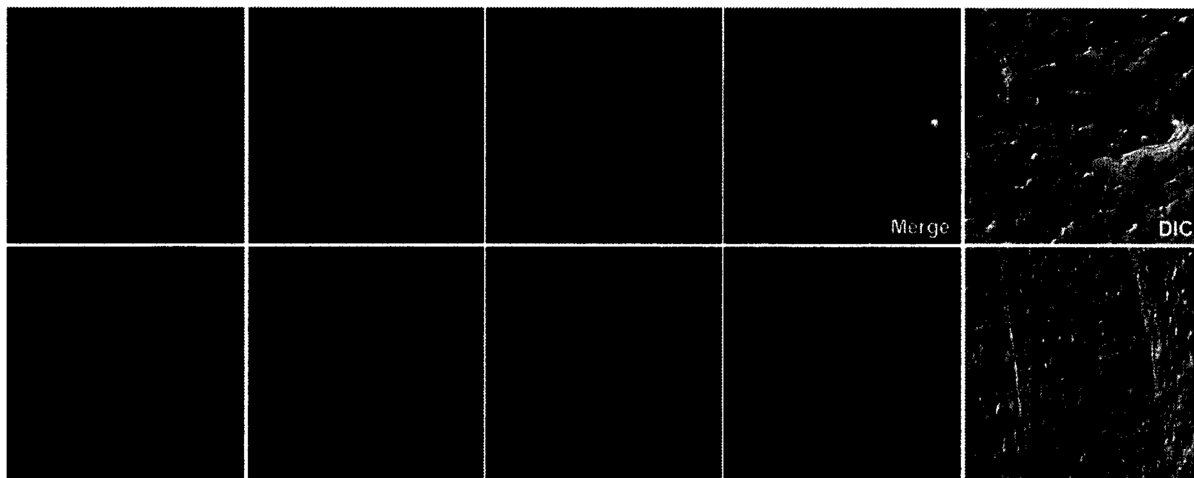
Figure 15

A



B

WT mice muscle section



C

Het mice muscle section

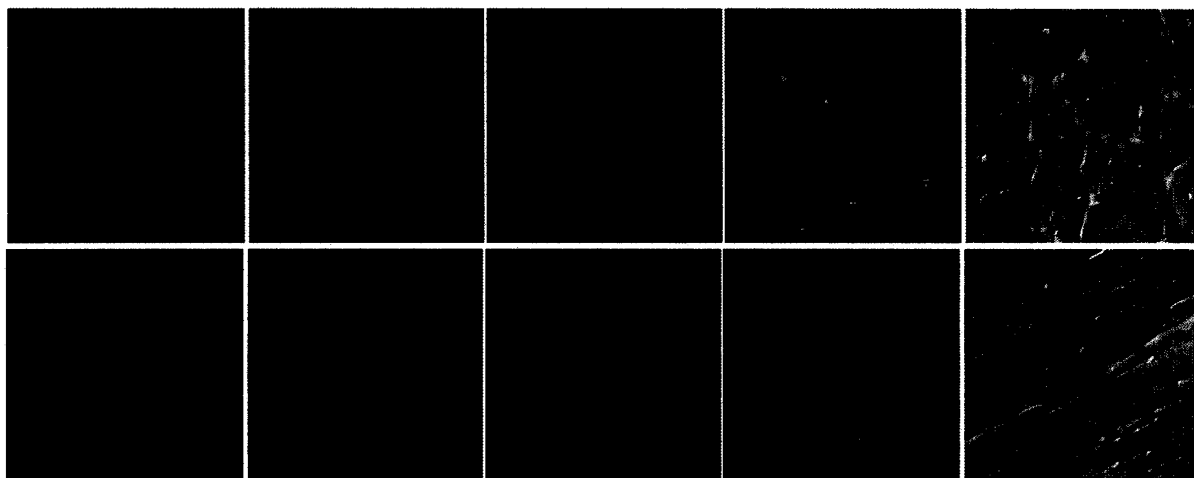


Figure 15: Fibrillarin protein levels are not affected by low levels of SMN protein. A)Muscles were taken from post-natal 3 month old mice. Muscle protein extraction was performed with tissuesfrozen and crushed in liquid nitrogen, then incubated with Whole Cell Extraction Buffer kept on ice for 30 min, span down, and the supernatant were separated by SDS-PAGE, transferred to an Immobilon-P membrane and immunobloted with anti-fibrillarin antibody. Loading control was done by immunobloting with anti-GAPDH. Cross sections of WT and SMN Heterozygous +/- mice reveils partial co-localizatoin and seperated localizatoin between Asym25 and fibrillarin. B) Cross-sections and longitudinal sections on Wild type mice were done from hind leg muscles imbeded in OCT and sliced on a cryostat at 10 microns eache. Slices were fixed and immunostained with Asym25 and anti fibrillarin antibodies. DIC immaging was used to demonstrate myotubes, n=1. C) Cross-sections and longitudinal sections on SMN heterozigous (+/-) mice were done from hind leg muscles imbeded in OCT and sliced on a cryostat at 10 microns eache. Slices were fixed and immunostained with Asym25 and anti fibrillarin antibodies. DIC immaging was used to demonstrate myotubes, n=1.

Chapter 4. Discussion:

4.1 Study Summary

This study has documented a dynamic profile of proteins containing methylated arginines during myoblast differentiation, and that this dynamic profile is dependent on the presence of functional levels of SMN. Furthermore, I have identified Fibrillarin, a snoRNP component, as one of the methylated proteins that is misregulated in the absence of SMN. Finally, I have also showed that the profile of arginine methylated proteins differs between skeletal muscles from wild type and SMA model mice. Taken together, these results represent a novel defect in SMA and provide evidence for the importance of skeletal muscle tissue in this disease.

4.2 Arginine Methylation and Skeletal Muscle Differentiation

Previous studies demonstrating skeletal muscle regulation or defect in differentiation by arginine methylation focused on the regulatory role of CARM1 and PRMT5 on gene expression through their influence on chromatin accessibility (Chen, Loffler et al. 2002; Dacwag, Ohkawa et al. 2007). Our methyl-specific antibodies provided immunoblots showing a wide range of methylated proteins from differentiating myoblasts and from wild type and SMA model mice suggests that arginine methylation is likely to act at other levels. Since arginine methylation often regulates sub-cellular localization and/or activity of proteins (see Introduction), a change in the methylation status of specific proteins important for skeletal muscle differentiation may affect their role(s) in this process. In order to divulge more specific roles and functions on the

arginine methylated proteins affecting skeletal muscle differentiation we will need to identify the proteins that differ between wild type and SMA mice model, as well as in normal and SMN knockdown myoblasts. This would help us determine whether the amount of methylation is changing or if it is merely the level of the methylated proteins increasing or decreasing. Identification of these proteins would help us understand how the level of methylation and the interactions between these proteins and SMN may affect critical and necessary steps in skeletal muscle development and how they are affected in SMA patients. Furthermore, this knowledge may benefit research involved in the differentiation of other cell types.

Since arginine methylation seems increasingly important for a number of cellular functions, including muscle differentiation, we asked ourselves whether there was a correlation between the dynamic profile of methylated proteins and PRMT expression during skeletal muscle differentiation. To shed light on this question we looked at two of the nine PRMTs that are responsible for sDMA and aDMA modification. Our results showed a decreasing amount of one of the PRMTs (PRMT1) that is responsible for aDMA-modifications, and a stable and equal amount of one of the PRMTs (PRMT5) that is responsible of sDMA-modifications. The expression profile of PRMT1 is somewhat consistent with the immunoblots showing a dynamic profile for aDMA-containing proteins; which showed a decrease at later time points for several bands (Fig. 3A right panel). Similarly, this observation is consistent with the fact that we detected a dynamic relocalization only for a subset of aDMA-containing proteins during C2C12 differentiation. In contrast the increase in the level of some sDMA-containing proteins does not correlate with the observed PRMT5 level suggesting that maybe other Type II PRMT are involved (PRMT7 and/or PRMT9). Nevertheless, these experiments do not take into account

PRMT activity regulation. For example, PRMT activity may be regulated by post-translational modification, interaction with co-factors, or sub-cellular localization. Although, a few studies documenting various mechanisms of PRMT regulation are starting to emerge, this remains a fairly novel field of investigation and it will be interesting to revisit PRMT regulation during skeletal muscle differentiation as our understanding of this topic progresses.

As mentioned earlier, only a specific subset of arginine methylated proteins residing in the nucleolus at steady state showed a dynamic relocalization during skeletal muscle differentiation. What causes this relocalization, and what is its effect on protein function or cell function still remains to be elucidated. This phenomenon has not been previously reported in skeletal muscle differentiation, and its implementation *in vivo* would suggest a regulatory mechanism, a check point, or a differentiation step that is necessary during skeletal muscle differentiation (as discussed in the section below).

4.3 Misregulation of Methylated Nucleolar Proteins in the Absence of SMN

To address our hypothesis and to provide more evidence that SMN has a specific and distinct role in skeletal muscles we repeated our methyl-dependent immunoblots and immunofluorescnece on SMN knockdown myoblasts. Our immunoblots suggested that there were differences in arginine methylated protein profiles for aDMA-containing proteins. In addition, our immunofluorescence showed us that a subset of aDMA-containing nucleolar proteins failed to relocalize out of the nucleolus in the absence of SMN. These results could be suggestive of SMN having a novel role in skeletal muscle, although it would be interesting to

investigate if SMN also regulates the dynamic profile of arginine methylated proteins in other cell types. It strengthens our belief that the level of functional SMN is important in skeletal muscle for the proper regulation and/or localization of methylated proteins. It has been eloquently demonstrated (Shafey, Cote et al. 2005) that SMN is important for some aspects of skeletal muscle differentiation and the findings presented in this thesis could provide a mechanism to explain the previous observations. It would be a worthwhile investigation to find if the nucleolar proteins that are misregulated in the SMN knockdown myoblasts share a common feature, like for example: (i) interaction with SMN, (ii) implication in the same function such as the assembly of snoRNP complexes, (iii) whether they are components of the same type of snoRNP (box CD vs H/ACA), or whether it is the similar state of arginine methylation in these nucleolar proteins. In fact, some of our preliminary results show that two different non ribosomal nucleolar proteins, namely B23 and fibrillarin, would be misregulated because of their interactions with SMN. B23 is known to interact with SMN (Lefebvre, Burlet et al. 2002), but is a non ribosomal protein that is not involved in the biogenesis of snoRNP; fibrillarin is also known to interact with SMN, but is a non ribosomal protein that is involved in snoRNP biogenesis. Taking these results into consideration, one could reach the conclusion that the interaction with SMN is common between these two proteins. However, there is no literature on B23's methylation status and we therefore cannot assess its potential contribution here. Further experiments will be needed to determine other possibilities and other types of proteins (as enumerated above).

Relying on the fact that SMN is involved in many different RNP processes, one could also ask whether the snoRNP machinery is affected in the face of low amounts of functional SMN, as

was proposed previously by Terns and Terns (Terns and Terns 2001). One way of testing if SMN levels influence snoRNP biogenesis would be to use radioactive phosphor to label RNA (an approach termed "PcP labelling") collected from a fibrillarin immunoprecipitation. Since fibrillarin is known to be part of the snoRNP complex, one would assume that the snoRNA attached to this complex would precipitate with it. This would be done in cells with and without (or low levels) of SMN. Running the labelled RNA on a gel would then separate the different snoRNAs by size and therefore provide us with an insight as to whether snoRNP biogenesis is altered in the presence of low levels of SMN. This experiment could be done in myoblast cells that are undergoing muscle differentiation, and would therefore provide even more evidence as to what happens to snoRNP biogenesis during muscle differentiation. In fact, some preliminary results showed a constant level of snoRNP biogenesis during normal myoblast differentiation. We would now need to investigate this experimental setup in SMN knockdown myoblasts.

In addition to snoRNP biogenesis, nucleolar proteins can be involved in other processes, such as rRNA and tRNA maturation and modification. Once we are aware of the mechanisms that are affected by the low levels of functional SMN, it would be a very interesting investigation to know if the same phenomenon is seen *in vivo* and what its implication would be. In light of our previous results and to identify specific proteins that are misregulated because of its dependence on functional levels of SMN we used a candidate approach to identify such a protein.

4.4 Fibrillarin's Dynamic Relocalization During Skeletal Muscle Differentiation is Misregulated in the Absence of Functional SMN

Using Western blot analysis and immunofluorescence we were able to identify an arginine methylated nucleolar protein that relocalized outside of nucleoli during myoblast differentiation, and failed to do so in the presence of low levels of SMN. This protein was fibrillarin, the most abundant protein found in the fibrillar domains of the nucleolus, where rRNA transcription and early pre-ribosomal RNA (pre-rRNA) processing takes place (Warner 1990; Eichler and Craig 1994). Fibrillarin is also part of a snoRNP complex that is responsible for 2'-*O*-methylation of pre-rRNA. More specifically, it has been shown that fibrillarin is the methyltransferase responsible for this 2'-*O*-methylation (Omer, Ziesche et al. 2002). It has also been proposed that fibrillarin may coordinate both RNA and protein methylation in the nucleolus, based on its physical association with protein arginine methyltransferases PRMT1 and PRMT5 (Yanagida, Hayano et al. 2004).

Although not much is known about Box C/D snoRNP biogenesis, there can be many speculations as to how this process is initiated, carried out and terminated. SMN is known to interact with fibrillarin and GAR1; two proteins involved in the posttranscriptional processing and modification of ribosomal RNA. These two proteins have a salt stable and direct interaction with SMN (Jones, Gorzynski et al. 2001; Pellizzoni, Baccon et al. 2001) and fibrillarin is misregulated when SMN is present at low levels (this thesis). All these facts could imply a regulatory role for SMN in snoRNP biogenesis. Whether SMN is there for the release of snoRNP, the export of fibrillarin to the cytoplasm or nucleoplasm, or the import of fibrillarin into the nucleus or nucleolus or any other step there might be would be a worthwhile

investigation. Indeed, a recent study has suggested the involvement of SMN in H/ACA and box C/D snoRNP biogenesis, although experiments are still needed to verify this possibility (Darzacq, Kittur et al. 2006). SMN has already been involved in spliceosomal snRNP biogenesis, which occurs both within the nucleus and the cytoplasm. The SMN complex is responsible for binding with the Sm protein, which interacts with the newly exported spliceosomal snRNAs, and mediates the ATP-dependent assembly of the Sm core (Pellizzoni 2007) (refer to Fig. 2 for further details). It has been established that the SMN complex is the macromolecular machine used by cells for snRNP assembly, and similarly, it could also assist in any of the steps involved in snoRNP biogenesis. A recent report states that part of the snoRNP assembly and maturation process is thought to take place in CBs, where it is known that the SMN complex can be found (Matera and Shpargel 2006). Hence, it is possible that SMN may contribute to snoRNP maturation in CBs and/or regulate the trafficking of snoRNP components between CBs and the nucleolus.

Interestingly, fibrillarin and SMN co-localize in primary neurons (Jones, Gorzynski et al. 2001; Wehner, Ayala et al. 2002), in fact, a direct interaction has been demonstrated, although no functional basis for this interaction has been established, including any involvement of fibrillarin in the pathogenesis of spinal muscular atrophy. In addition, no data can be found about fibrillarin and its role in skeletal muscle differentiation. Nevertheless, fibrillarin has been shown to co-localize with SMN within the nucleoli of adult pig skeletal muscles and human foetal skeletal muscles (Young, Le et al. 2001).

It has long been known that fibrillarin is an arginine methylated protein, although the effect of methylation on its function remains unknown. There are known functions outside of the skeletal muscle differentiation paradigm, as you have seen in the above section. For example, it is known that fibrillarin is itself methylated by PRMT1, at least *in vitro* (Goulet, Gauvin et al. 2007). Whether this methylation affects its localization or function still remains unclear. Additionally, some of our preliminary results show fibrillarin still concentrated within the nucleolus in PRMT1$^{-/-}$ cells, although the methylation state of fibrillarin was not determined thoroughly in these experiments (Fig. 13A and B). Further experiments will be needed to answer the above question more clearly. It is known that fibrillarin interacts with SMN, PRMT1 and PRMT5 (Yanagida, Hayano et al. 2004; Jones, Gorzynski et al. 2001) (Wehner, Ayala et al. 2002). PRMT1 protein levels decrease during normal myoblast differentiation, where fibrillarin diffuses into the nucleoplasm and the cytoplasm. In contrast, in SMN knockdown myoblast differentiation fibrillarin resides within the nucleolus and PRMT1 is expressed constantly and equally. Taking a step back from these observations, one could ask whether it is the physical association of fibrillarin with SMN that causes its misregulation, or if it could be the physical interaction with PRMT1 (independent of its activity)? Then again would the methylation state of fibrillarin come into play during skeletal muscle differentiation, and would it alter fibrillarin function and/or localization? Does fibrillarin have an effect or involvement in the pathogenesis of SMA? Further research is necessary to answer these questions, which are all equally interesting.

4.5 Relevance of SMN's Function in Skeletal Muscles to SMA Etiology

It has been shown that SMN is the causative gene of SMA, and that SMA is characterized by loss of motoneurons from the anterior horn of the spinal cord and progressive muscular atrophy in the limbs and trunk, usually culminating in respiratory failure (Lefebvre, Burglen et al. 1995; Ogino and Wilson 2004). Along with the earlier studies done by Braun, Guettier-Sigrist, Cifuentes-Diaz and Chan (Braun, Croizat et al. 1995; Cifuentes-Diaz, Frugier et al. 2001; Guettier-Sigrist, Hugel et al. 2002; Chan, Miguel-Aliaga et al. 2003), recent research has shown that drosophila SMN is required for Actin88F expression, a flight muscle-specific actin isoform (Rajendra, Gonsalvez et al. 2007). Moreover, drosophila SMN interacts and forms a complex with α-actinin *in vivo* (Rajendra, Gonsalvez et al. 2007). Drosophila SMN has also been seen to localize to indirect flight muscles (IFMs) myofibrils and its localization to sarcomeres is evolutionarily conserved (Rajendra, Gonsalvez et al. 2007). Preliminary results from our laboratory also saw the localization of SMN to sarcomeres in WT mouse skeletal muscle sections. Additionaly, our skeletal muscle section preliminary results along with our *in vitro* data suggest there might be a shuttling of fibrillarin during skeletal muscle differentiation to the cytoplasm, although it may cycle back to the nucleolus after differentiation during normal embryonic development. Although these results are preliminary, further research will be required using mice skeletal muscle to elucidate the mechanism behind the dynamic relocalization of fibrillarin during muscle differentiation.

In a previous study, when the experiments were done with a drosophila *Smn*-null mutant, researchers found a severe muscular atrophy and acute neuromuscular dysfunction (Rajendra, Gonsalvez et al. 2007). Increasing amounts of data are providing evidence of SMN's

specific function in skeletal muscle. Furthermore, the muscle defects seen in these studies suggest the contribution of this tissue to SMA phenotype and pathology.

This study has provided further evidence that SMN has a specific and distinct role in skeletal muscles, potentially through its interaction with one or more specific nucleolar methylated proteins, including fibrillarin. We show that fibrillarin is misregulated during SMN knockdown myoblast differentiation, thus linking SMN to yet another possible skeletal muscle function, and potentially to the snoRNP assembly machinery. It was recently demonstrated that snRNP assembly activity is reduced in fibroblasts from SMA patients (Wan, Battle et al. 2005) and during myogenic differentiation (Gabanella, Carissimi et al. 2005). Similarly, it would be interesting to develop such an assay to determine if snoRNP assembly activity is reduced in normal and SMN deficient myoblasts during differentiation. We provided confirmation of a specific defect in skeletal muscles, phenotypic data correlation to myoblast differentiation defects and mechanistic evidence suggesting that muscles in SMA patients may not be fully functional regardless of innervations. Multiple studies and research on replacement therapies are concentrating on ES cells and stem cells differentiation into motor neurons as a treatment for SMA patients. It would be wise to consider skeletal muscle defects and involvement in SMA etiology, since it is unsure whether SMN-deficient muscles could even sustain innervation from stem cell or ES cell-derived motor neurons. Furthermore, we show aberrant profiles of proteins containing arginine methylation in skeletal muscles from WT and SMA model mice. This evidence furthers our belief that SMN has a specific and distinct role in skeletal muscles and how in SMN-deficient environments many different pathways may potentially be affected.

Chapter 5. Conclusions:

We have demonstrated a novel defect in hypomorphic Smn knockdown C2C12 myoblasts that should be considered relevant to the SMA research field as it demonstrates the importance of SMN in skeletal muscles and potentially implicates SMN in an important cellular process; snoRNP assembly and/or function. Moreover, we have identified a methylated protein that interacts with SMN and is misregulated during myoblast differentiation in SMN-deficient cells. The data presented in this thesis support our initial hypothesis and fulfills the specific objectives stated for this project. By demonstrating these findings we hope to help understand and develop how skeletal muscles are affected in SMA patients and how important it is to consider this tissue in research and treatment of this deadly disease.

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