Identification and characterization of an arginine-methylated Survival of Motor Neuron (SMN) Interactor in Spinal Muscular Atrophy (SMA)

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AUTHORIZATION

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ABSTRACT

Spinal Muscular Atrophy (SMA) is a neuronal degenerative disease caused by the mutation or loss of the Survival Motor Neuron (SMN) gene. The cause for the specific motor neuron susceptibility in SMA has not been identified. The high axonal transport/localization demand on motor neurons may be one potentially disrupted function, more specific to these cells. We therefore used a large-scale immunoprecipitation (IP) experiment, to identify potential interactors of SMN involved in neuronal transport and localization of mRNA targets. We identified KH-type splicing regulatory protein (KSRP), a multifunctional RNA-binding protein that has been implicated in transcriptional regulation, neuro-specific alternative splicing, and mRNA decay. KSRP is closely related to chick zipcode-binding protein 2 and rat MARTA1, proteins involved in neuronal transport/localization of β-actin and microtubule-associated protein 2 mRNAs, respectively. We demonstrated that KSRP is arginine methylated, a novel SMN interactor (specifically with the SMN Tudor domain; and not with SMA-causing mutants). We also found this protein to be misregulated in the absence of SMN, resulting in increased mRNA stability of KSRP mRNA target, $p21^{cip/waf1}$. A role for SMN as an axonal chaperone of methylated RBPs could thus be key in SMA pathophysiology.

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LIST OF ABBREVIATIONS

AChE:	Acetylcholinesterase
aDMA:	Asymmetric Dimethylated Arginine
AMV RT:	(Avian Myeloblastosis Virus) Reverse Transcriptase
ANOVA:	Analysis of Variance
cAMP:	Cyclic adenosine monophosphate/ 3'-5'-cyclic adenosine monophosphate
CARM1:	Coactivator-Associated aRginine Methyltransferase 1
cDNA:	complementary DeoxyriboNucleic Acid
Cip1/Waf1:	cyclin inhibitor protein 1/wild type p53-activated factor 1
CNS:	Central Nervous System
CRM1:	Chromosome Region Maintenance 1
Ctrl:	Control
DAPI:	4', 6-diamidino-2-phenylindole
DMEM:	Dulbecco's Modified Eagle's Medium
DMSO:	Dimethylsulfoxide
FBP1:	Far upstream element binding protein 1

FBP2:	Far upstream element binding protein 2
GAP43:	Growth Associated Protein 43
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GST:	Glutathione *S*-transferase
HIV:	Human Immunodeficiency Virus
Het:	Heterozygote
hnRNP R:	heteronuclear Ribonucleoprotein R
IgG:	Immunoglobulin
IP:	Immunoprecipitation
IPTG:	Isopropyl-b-D-1-thiogalactopyranoside
IVTT:	*in vitro* coupled transcription/translation
KH:	K-Homology Domain
KSRP:	KH-Type Splicing Regulatory Protein
LC-MS/MS:	*microcapillary reverse-phase* Liquid Chromatography *coupled online with electrospray ionization tandem* Mass Spectrometry
MAP2:	Microtubule-associated protein 2
MARTA1:	Microtubule-associated protein 2-RNA trans-acting protein 1

MEFs:	Mouse Embryonic Fibroblasts
MMA:	Monomethylated Arginine
mRNP:	messenger Ribonucleoprotein particle
NCBI:	National Center for Biotechnology Information
NGF:	Nerve Growth Factor
p38 MAP kinase:	p38 mitogen-activated protein kinase
PBS:	Phosphate Buffered Saline
pre-mRNA:	pre-messenger Ribonucleic acid
PRMTs:	Protein Arginine Methyltransferases
PVDF:	Polyvinylidene fluoride
RG/PGM:	Arginine-Glycine/Proline-Glycine-Methionine
RIP140:	Receptor Interacting Protein 140
RIPA:	Radio Immuno Precipitation Assay (Buffer)
RNase A:	Ribonuclease A
RNP:	Riboucleoprotein
RT:	Room Temperature
RT-PCR:	Reverse Transcriptase-Polymerase Chain Reaction

Sam68:	Src-Associated in Mitosis of 68 kDa
sDMA:	Symmetric Dimethylated Arginine
SDS-PAGE:	Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
SEM:	Standard Error of Mean
shRNAs:	small hairpin RiboNucleic Acids
SMA:	Spinal Muscular Atrophy
SMN:	Survival of Motor Neuron
snRNAs:	small nuclear Ribonucleic Acids
snRNPs:	small nuclear Ribonucleoprotein Particles
TBST:	Tris-Buffered Saline Tween-20
UTR:	Untranslated region
VgRBP71:	[*Xenopus laevis* Vg1 mRNA] RNA Binding Protein 71
WT:	Wild Type
ZBP2:	Zipcode binding protein 2

CHAPTER 1. General Introduction

1.1 *Spinal Muscular Atrophy (SMA) Disease*

1.1.1 Definition

Spinal Muscular Atrophy (SMA) is a severe autosomal recessive neuromuscular disorder. Its principal distinguishing feature is the selective degeneration of spinal and lower bulbar motor neurons, in the anterior horn of the spinal cord (Ogino and Wilson 2004).

1.1.2 Epidemiology

SMA is the second most common fatal autosomal recessive disorder in humans, following cystic fibrosis; and the most common genetic cause of infant mortality (Ogino and Wilson 2004; Prior et al. 2010; D'Amico et al. 2011). The present estimated incidence of this disorder is 1 in 6,000 to 10,000 live births (Ogino and Wilson 2004); with 4.1 per 100,000 live births for spinal muscular atrophy type I (Mailman et al. 2002). The carrier frequency is estimated at 1:38-1:50 (Ogino and Wilson 2004).

1.1.3 Clinical Description

SMA pathophysiology includes a progressive proximal muscle weakness and eventual wasting of these voluntary limb muscles, ultimately leading to paralysis, and in the most severely afflicted cases, death due to respiratory distress by the age of 2 years (Briese et al. 2005; D'Amico et al. 2011).

1.1.4 Clinical Characteristics

SMA is divided into five clinical subtypes, categorized by age of onset and motor function, as defined by "maximal functional status achieved" (Zerres and Davies 1999; D'Amico et al. 2011; Markowitz et al. 2012):

i. Type 0 SMA – Prenatal, congenital SMA. Prenatal onset resulting in a lifespan of under six months. Decreased *in utero* fetal movement, with severe weakness and early respiratory failure at birth.

ii. Type I SMA – Werdnig-Hoffmann Disease. Most common and severe, accounting for half of diagnosed patients. Clinical signs seen by 6 months, and children never acquire the ability to sit unsupported, and do not survive beyond 2 years, without respiratory support.

iii. Type II SMA – Dubowitz / Intermediate Disease. Onsets between 7 and 18 months. Children can sit unsupported, some can even hold a standing position, but none can walk independently. Almost three-quarters are alive at 25 years of age.

iv. Type III SMA – Kugelberg-Welander Disease. Onset of proximal muscular weakness before (IIIa) or after the age of 3 years (IIIb) is determinant of ability to still be able to walk, later in life. Patients can stand and walk autonomously, and have an almost normal lifespan.

v. Type IV SMA – Adult SMA. Describes disorder with adult onset and mild disease course with minor muscle weakness. Patients do not experience respiratory or nutritional difficulties, and have a normal lifespan.

1.2 *Molecular Genetics of Spinal Muscular Atrophy (SMA)*

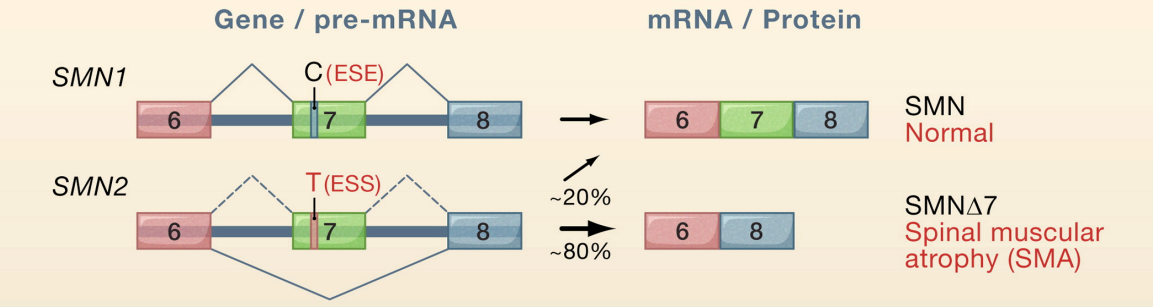
1.2.1 The Survival of Motor Neuron gene in SMA

All forms of spinal muscular atrophy described above have been mapped by linkage analysis studies to chromosome 5q11.1-13.3 (Lefebvre et al. 1995). Lefebvre et al. identified the *Smn* (survival of motor neuron) gene within this region as being deleted or mutated in 98.6% of the SMA patients in their cohort. This chromosomal region is a large inverted repeat of 500 kb, at locus 5q13, containing both the spinal muscular atrophy determining telomeric *Smn1*, and the centromeric *Smn2* gene. The *Smn2* gene differs from *Smn1* by only five centromeric nucleotides, with the single most important but still translationally silent being (840C > T) - which results in the alternative splicing of exon 7 (Lorson et al. 1999; Monani et al. 1999). This mutation disrupts the AG-rich exonic splice enhancer (ESE) region (in the center of SMN exon 7), required for inclusion of this exon (Lorson and Androphy 2000). Therefore abrogating splicing factor ASF/SF2 association (Cartegni and Krainer 2002), and creating an exonic splicing silencer (ESS) motif, that instead serves as binding site for hnRNPA1 (Kashima and Manley 2003), a known repressor protein.

1.2.2 The Survival of Motor Neuron protein in SMA

Smn2 thus primarily encodes exon 7 lacking transcripts that generate a variable amount of truncated and unstable SMNΔ7 protein and only ~20-percent of full-length SMN protein (SMN-FL), compared to *Smn1* (Lorson and Androphy 2000) (**Figure 1A**). During homozygous disruption or loss-of-function mutations in *Smn1*, as is the case in 95

A.



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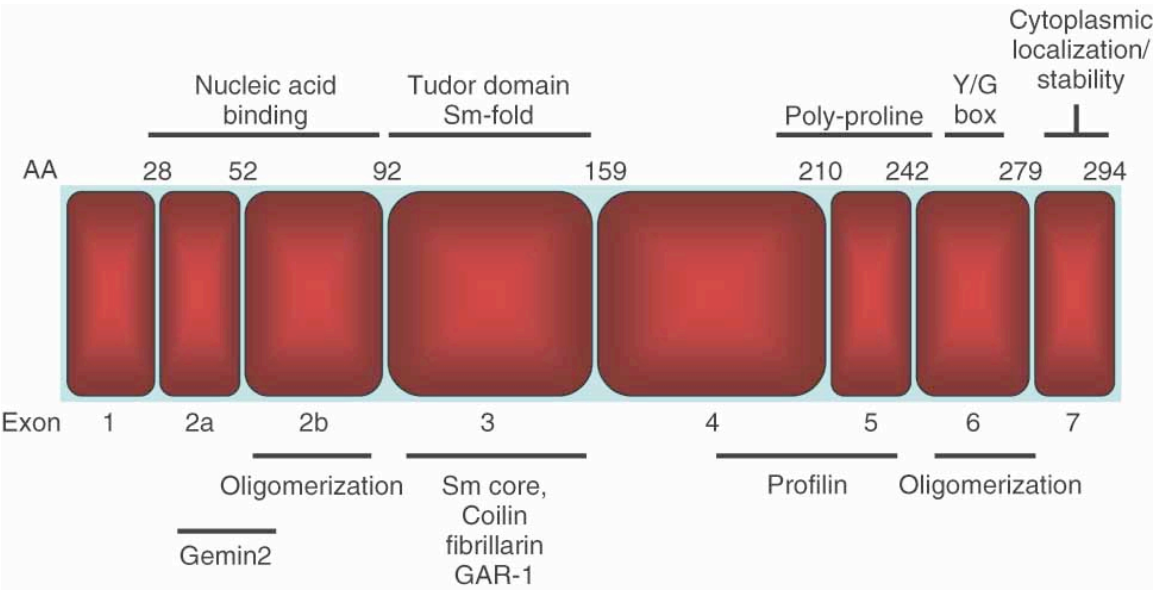


Figure 1. Spinal Muscular Atrophy (SMA) Disease and the Survival of Motor Neuron (SMN) Protein. (A) Deletion of the SMN1 gene leaves SMN2 pre-mRNA that produces mostly Δ7exon mRNA, resulting in an unstable SMNΔ7 protein. (Modified from (Cooper et al. 2009). **(B)** SMN protein sub-domains (Coady and Lorson 2011).

-98% of spinal muscular atrophy patients, the *Smn2* gene product is still however able to rescue embryonic lethality and compensate during embryonic development for this *Smn1* deficiency in all cells, with the exception of motor neurons. Although *Smn1* loss is essential to SMA pathology, the number of *Smn2* copies determines its severity; as most SMA type I patients have (2 copies of *Smn2*), type II (3 copies), whereas type III and VI (3 or 4 copies) (Feldkotter et al. 2002; Mailman et al. 2002). *Smn2* copy number/phenotype correlation is actually even closer than the inverse correlation between SMN protein level and disease severity (Sumner et al. 2006). Rodents only possess one copy of the SMN gene that resembles the human SMN1 gene (Bergin et al. 1997). The SMN protein is 294 amino acids, with a molecular weight of 38kDa (**Figure 1B**) (Lefebvre et al. 1997); it is ubiquitous, yet particularly abundant in spinal cord motor neurons, with a primarily cytoplasmic localization (Coovert et al. 1997). In developing rats or mice, it is present at varying levels in most tissues, with the highest expression being detected throughout embryonic development (Bergin et al. 1997; La Bella et al. 1998).

1.3 *The Survival of Motor Neuron Protein Function*

1.3.1 The Core Survival of Motor Neuron Complex

SMN is nuclearly localized into two discrete bodies, known as "gems" ("Gemini", twin of Cajal Bodies) and Cajal bodies (CBs) (Liu and Dreyfuss 1996; Carvalho et al. 1999; Young et al. 2001) - that are similar in size and number. CBs were first referred to as nucleolar "accessory" bodies, by Santiago Ramón y Cajal, when he discovered them

more than a century ago (Cajal 1903). They are coiled body nuclear structures, conserved from plants to animals and enriched in snRNAs (Jablonka et al. 2000). The SMN macro-

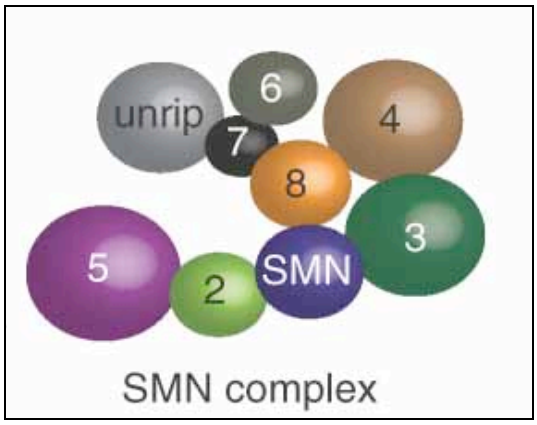


Figure 2. The SMN/Gemins/Unrip Core Complex. The core SMN macromolecular complex consists of SMN, Gemins2-8 and Unrip (modified from (Fischer et al. 2011).

molecular complex comprises of SMN and seven other proteins known as "gemins" (**Figure 2**): Gemin2/SIP1, Gemin3/dp103, Gemin4/GIP1 (Charroux et al. 1999; Charroux et al. 2000; Grundhoff et al. 1999; Meister et al. 2000), Gemin5/p175 (Gubitz et al. 2002), Gemin6 (Pellizzoni et al. 2002), Gemin7 (Baccon et al. 2002), and Gemin8 (Carissimi et al. 2006). Gemins2, 3, 5 and 7 directly interact with SMN, while Gemin4 indirectly associates through 3 and Gemin6 through 7. Gemin8 interacts with a heterodimer of Gemin6 and 7 (Otter et al. 2007). Another component of this complex, named unrip has also been identified (Carissimi et al. 2005).

1.3.2 Survival of Motor Neuron complex Assembly of SnRNPs

This SMN complex, composed of SMN, Gemins, and Unrip is essential for proper assemblage of Smith class (Sm) core proteins of the uridine-rich snRNPs (UsnRNPs).

The chaperone function of the SMN complex facilitates the assembly of these snRNPs, major components of spliceosomes, cellular elements that carry out pre-mRNA splicing (**Figure 3**). In the cytoplasm, the SMN complex interacts with newly arginine-methylated Sm proteins. There, the SMN complex facilitates the specific binding of this Sm complex - as a stable heptameric ring - onto the Sm site, a highly conserved sequence motif found on newly exported spliceosomal uridine-rich small nuclear RNAs (UsnRNAs) (Meister et al. 2001; Meister et al. 2002; Pellizzoni et al. 2002). Nuclear import subsequently follows this SMN-dependent formation of small nuclear ribonucleoprotein particles (snRNPs).

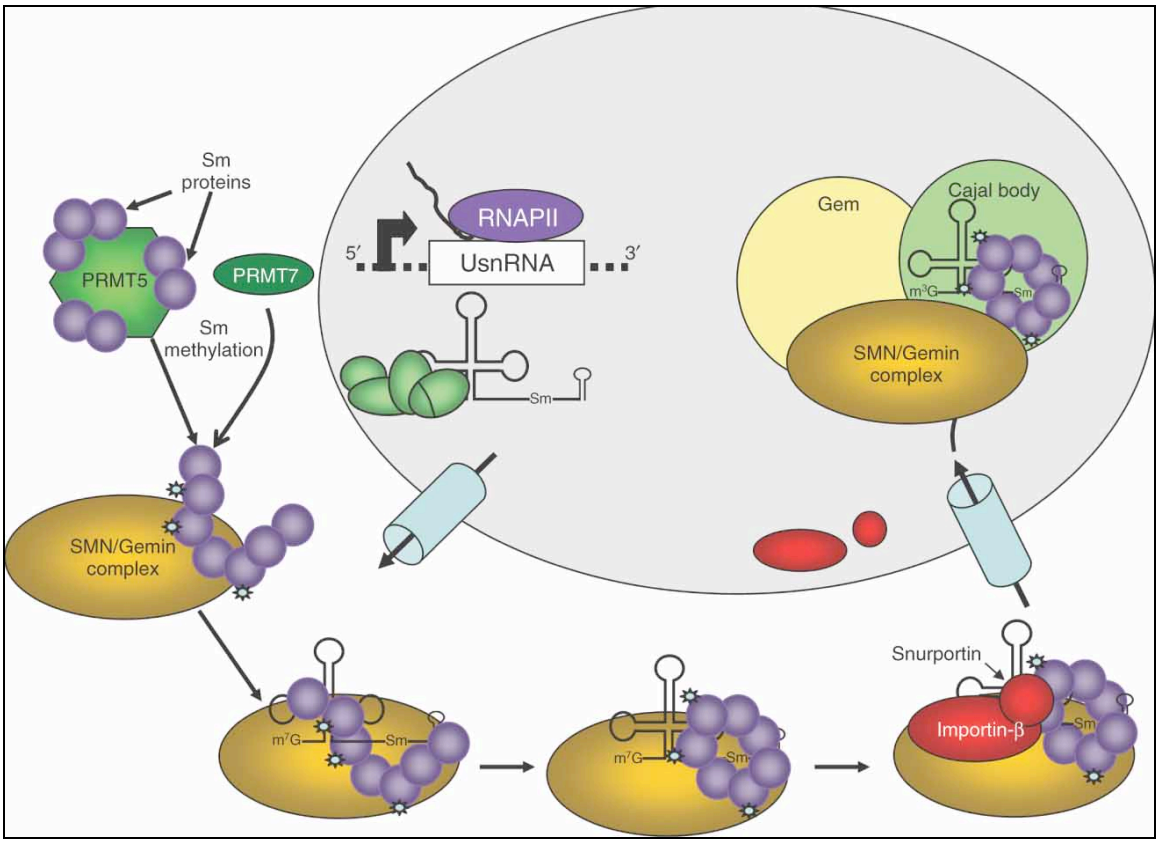


Figure 3. The SMN SnRNPs Assembly Pathway. (i) Uridine-rich small nuclear RNAs (UsnRNAs) are transcribed via RNA polymerase II (RNAPII)‐dependent expression, and exported to the cytoplasm by a *nuclear export complex* [consisting of PHAX, XPO1/CRM1, RAN and the Cap-Binding Complex (CBC)]. (ii) Within the cytoplasm, PRMT5 and 7 specifically methylate the C-terminal tails of SmB, SmD1, and SmD3;

enhancing affinity for the SMN complex, of these partially assembled snRNA-free Sm proteins (SmD1/SmD2; SmB/B′/SmD3; SmE/SmF/SmG). (iii) This methylated Sm complex binds the SMN/Gemin/unrip complex - allowing the specific recruitment of snRNAs - and is guided by gemin5 onto the snRNA's Sm-binding site, where it forms a heptameric ring (iv). (v) The entire complex is then bound and transported into the nucleus by the *nuclear import complex* [consisting of snurportin and importin-β]. (vi) Upon nuclear entry, the import complex is released, whereas the SMN complex and snRNPs begin their transition through the Cajal Body (CB) (Modified from (Coady and Lorson 2011).

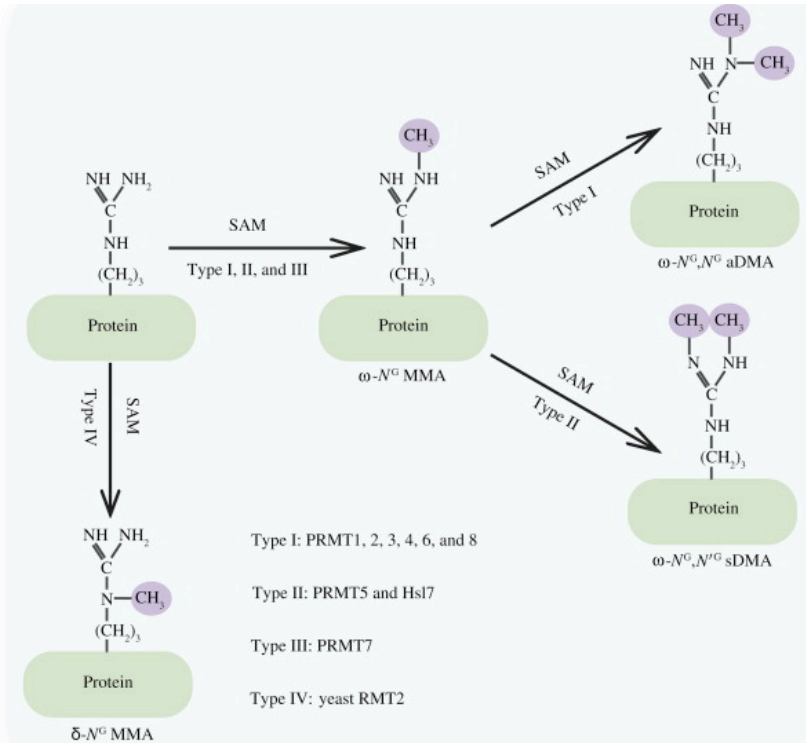
Although snRNP assembly is the main function ascribed to the SMN complex, it has also been implicated in the regulation of gene expression, pre-mRNA splicing, and the assembly and metabolism of snoRNPs (Liu and Dreyfuss 1996; Meister et al. 2000; Pellizzoni et al. 2001). For our purpose here, we will focus on the arginine methylation related functions of the SMN complex.

1.4 *Survival of Motor Neuron and Arginine Methylation*

1.4.1 Arginine Methylation and Protein Arginine Methyltransferases (PRMTs)

Arginine methylation is a post-translational modification in which proteins are covalently marked by addition of methyl groups to their arginine residues (Gary and Clarke 1998). Methyl groups are transferred from the methyl donor S-adenosyl methionine (AdoMet or SAM), and placed on one of the terminal nitrogen atoms of the guanidino group in arginine (Paik and Kim 1980). In most cases, glycine residues are often found flanking these methylated arginine residues (Gary and Clarke 1998). Three distinct forms of methylation exist in mammalian cells: the most common, ADMA, asymmetric dimethylarginine derivative, where two methyl groups are on one of the ter-

A.



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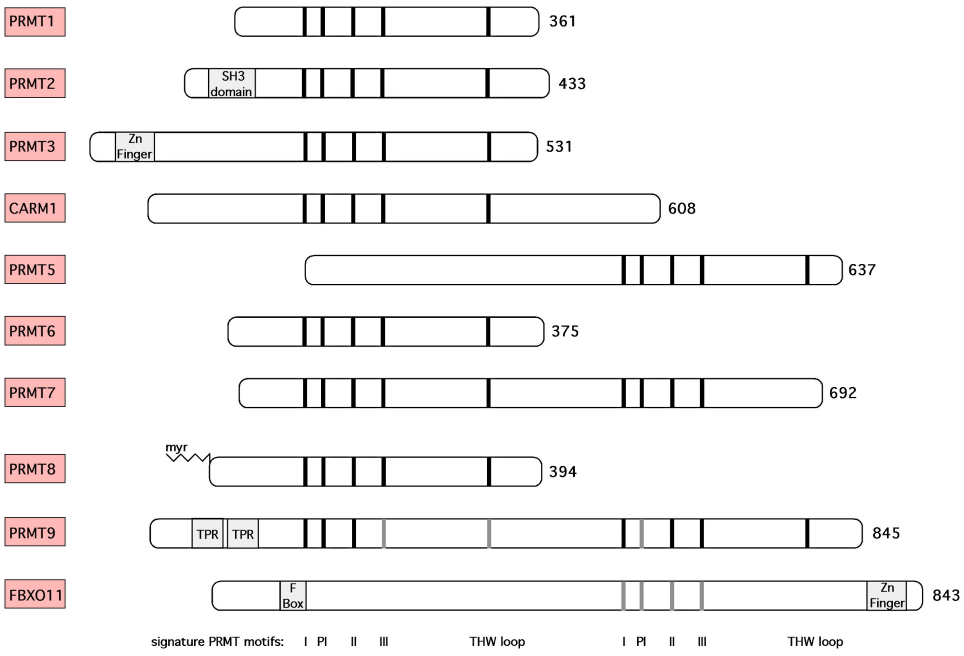

Figure 4. Arginine methylation and PRMTs. (**A)** PRMTs catalyze the transfer of methyl groups from AdoMet/SAM to the arginines in substrate proteins. Type I PRMTs

catalyze the formation of ω-N^G-monomethylarginine (MMA) and asymmetric ω-N^G,N^G-dimethylarginine (aDMA). Type II PRMTs catalyze the formation of MMA and symmetric ω-N^G,N'^G-dimethylarginine (sDMA). Type III PRMTs catalyze the formation of only MMA. Type IV enzymes catalyze the formation of δ-N^G-monomethylarginine (modified from (Ahmad and Cao 2012). **(B)** The mammalian PRMT family currently contains nine highly related members; all harboring signature motifs I, post-I, II, and III and the conserved THW loop (in black bars). FBXO11 may have arginine methyltransferase activity, but is not structurally related to the other PRMTs (see text) (modified from (Bedford and Clarke 2009).

minal nitrogen atoms of the guanidino group; MMA, monomethylated derivative, with a single methyl group on terminal nitrogen; and SDMA, symmetric dimethylarginine derivative, one methyl group on each of the terminal guanidine arginines (**Figure 4A**). Nine highly related members now compose the mammalian PRMT family. All members harbor signature motifs, and a conserved THW loop (**Figure 4B**). All types I, II, and III PRMTs can generate the MMA, but only type I enzymes (PRMT1, 2, 3, 4, 6, and 8) subsequently generate ADMAs and type IIs (PRMT 5, 7 and 9), SDMAs. Type III PRMTs can only form MMA derivatives (PRMT7 being the only candidate, thus far), whereas Type IVs perform monomethylation on the δ-nitrogen (as reviewed in (Blackwell and Ceman 2012). Although some amino acid similarities between F-box only proteins and the PRMT family have been identified; the arginine methyltransferase activity attributed to FBXO11, may only be the result of residual contamination from co-purified PRMT5 (Bedford and Clarke 2009).

Even though arginine methylation has largely been shown as a negative regulator of protein-protein interactions (Mostaqul Huq et al. 2006; Bedford and Clarke 2009); methylation of arginine residues is actually key to protein interactions with the so-called 'Tudor' domains (Cote and Richard 2005).

1.4.2 The Tudor Domain of SMN

The Tudor domain was first identified on proteins that interact with RNA (Ponting 1997), and is one of several motifs found on the SMN protein (**Figure 1B**). It is a roughly 60 amino acid domain, around the center of the protein, that mediates interaction with a set of specific binding partners. The latter possess glycine/arginine-rich (GAR) motifs, upon which, the methylation of the arginine residues, increases their affinity, and thus, binding, to the Tudor domain (Meister et al. 2001; Friesen et al. 2001; Brahms et al. 2001; Cote and Richard 2005). These include, Sm proteins, Sm B, B' (Brahms et al. 2001), D1 and D3 (Friesen et al. 2001; Meister et al. 2001), as well as Sm-like protein LSm4 (Brahms et al. 2001). This phenomenon is not limited to these spliceosomal snRNPs components however, as it also applies to Cajal Body marker p80-coilin, Epstein-Barr virus nuclear antigen 2 (EBNA2) (as reviewed in (Cote and Richard 2005); and even more importantly, in this context, SMN uses its Tudor domain to bind arginine methylated RNA Binding Proteins (RBPs).

1.4.3 Arginine Methylated RNA Binding Proteins (RBPs)

The considerable distance from cell body to neuronal synapse creates a complex challenge for most neurons, of averting the synthesis of proteins needed at the synapse, during mRNA transport (Liu-Yesucevitz et al. 2011). A local RNA translation mechanism is thus needed to circumvent this unique requirement of these specialized cells. One key mechanism for this local regulation of mRNA translation and synthesis of proteins is facilitated partly by mRNA binding proteins (RBPs). RBPs mediate the assembly of RNA-protein complexes that form so called RNA granules, where

translational repression occurs (Kiebler and Bassell 2006). Moreover, an increasing number of mutations linked to neurological conditions are being found within some of these RBPs that are part of neuronal RNP granules. For instance, TAR DNA binding protein-43 (TDP-43) and fused in sarcoma (FUS) protein mutations are amongst the principal causes of amyotrophic lateral sclerosis (ALS) (Lagier-Tourenne et al. 2010); trinucleotide repeat expansions on Fragile X Mental Retardation Protein (FMRP) that of fragile X mental retardation syndrome (Darnell et al. 2011). As SMN, through its Tudor domain, associates with methylated RBPs in these neuronal RNP granules; a misregulation of RBPs is also highly probable in SMA (upon loss or mutation of SMN). We used a large-scale immunoprecipitation assay, detailed in the following manuscript, to select such a candidate neuronal RBP and characterized it in an SMA context.

1.4.4 RNA Binding Protein KH-type Splicing Regulatory Protein (KSRP)

KH-type splicing regulatory protein (KSRP) is a multi-KH (K homology motif, named for its recurrence in heterogeneous nuclear ribonucleoprotein K, hnrnp K protein) domain single stranded DNA- and RNA-binding protein. It is a homologue of *Xenopus laevis* VgRBP71 (Vg1 mRNA Binding Protein), rat MARTA1 (MAP2-RNA trans-acting 1), a protein responsible for the nucleocytoplasmic targeting of MAP2 mRNAs, through interaction with its dendritic targeting element (Rehbein et al. 2002), and of chick zipcode-binding protein 2 (ZBP2), a protein involved in the neuronal localization of β-actin (Gu et al. 2002). KSRP was first identified as *c-myc* transcriptional regulator FBP2 (FUSE-Binding Protein 2) (FUSE; far upstream element originally identified upstream of

c-myc) - that along with other FBP family members FBP1 and 3, can bind this element and upregulate *c-myc* transcription (Davis-Smyth et al. 1996). It was subsequently identi-

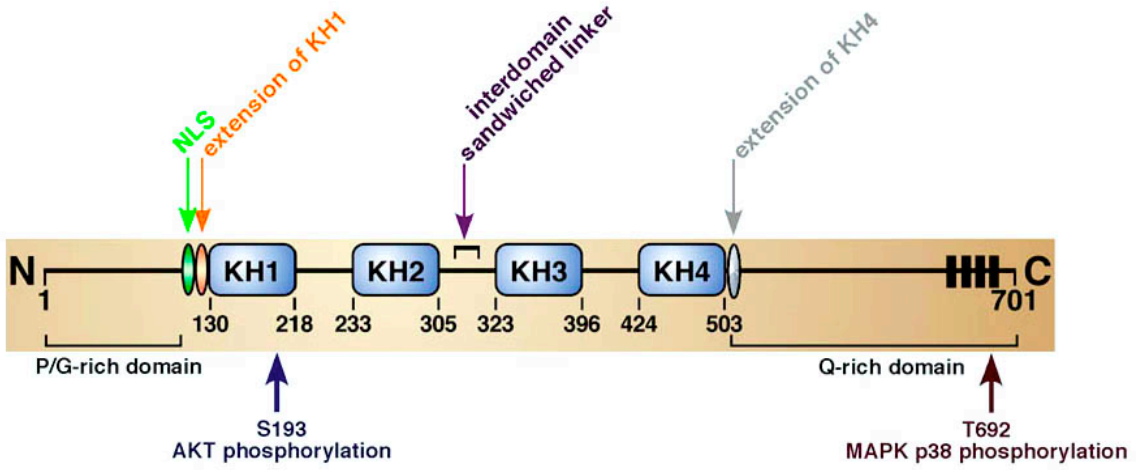

Figure 5. KH-type Splicing Regulatory Protein. Schematic representation of the primary structure of human KSRP: KH1-4, K-Homology motifs; NLS, nuclear localization signal; S193, AKT1/2 phosphorylation site; T692, MAPK p38 phosphorylation site; and C-terminal Y-rich repeats are indicated as vertical bars (modified from (Gherzi et al. 2010; Briata et al. 2011).

fied as part of a multiprotein complex that specifically binds to an intronic splicing enhancer element, downstream of the neuron-specific N1 exon of the *c-src* pre-mRNA, resulting in its alternative splicing (Min et al. 1997). KSRP has also been shown to be an ARE-BP (an AU-rich element Binding Protein) that binds to ARE-containing mRNAs, regulating their stability (Chen et al. 2001; Gherzi et al. 2004). KSRP uses its KH-motifs to recognize AREs, and subsequently recruits a multi-subunit protein complex with 3'-5' endonucleolytic activity, the exosome, for mRNA degradation (Gherzi et al. 2010; Briata et al. 2011). AU-rich elements (AREs) are cis-acting elements found in the 3'UTRs of short-lived transcripts - such as transcription factors, cytokines and chemokines - that

regulate their rapid mRNA decay rates (Khabar 2005). AREs are bound by ARE-BPs, of which some are mRNA decay-promoting factors like KSRP, while others could be stabilizing factors.

As illustrated above, KSRP is thus implicated in a variety of RNA metabolism processes: transcription, alternative splicing, localization and stability. KSRP is a 75 KDa protein expressed at much higher levels in neuronal cells, but that is still present in non-neuronal cells (Min et al. 1997). It is a mostly nuclear protein, primarily due to a nuclear localization signal (NLS) located at its amino-terminus, which also contains a proline-glycine (PG)-rich region, and a putative alpha-helix, involved in protein-protein recognition (**Figure 5**). Its four KH-domains comprise its central region, followed by four tyrosine-rich repeats at its C-terminal, which are integral for its function in *c-myc* transcription upregulation (Gherzi et al. 2010; Briata et al. 2011).

1.5 *Research Rationale, Hypothesis and Specific Objectives*

1.5.1 Rationale

The proposition that SMA defects occur exclusively due to splicing deficiency brought on by the disruption of the snRNPs assembly pathway, seems too one-dimensional, in light of the various RNP granules that contain and require SMN function (Liu-Yesucevitz et al. 2011). The disruption of another SMN function, perhaps even an axonal one, or a defect related to an SMN interactor or co-factor in these neuronal RNP granules, leading to SMA, is thus highly probable. The Tudor domain of SMN serves as an adaptor module for arginine methylated proteins. We will use this domain and/or arginine methylation specific antibodies to identify neuronal specific arginine methylated interactors that associate with SMN in these complexes. These identified partners will assist in further defining the role and function of SMN in neuronal RNP granules.

1.5.2 Hypothesis

I hypothesize that SMN has a definite role in neuronal RNP granules, through functional interactions with its arginine methylated partners. I therefore predict that in SMA conditions, a misregulation of these SMN interacting partners would take place.

1.5.3 Specific Objectives

I. Identify arginine methylated neuronal SMN protein interactors.

II. Characterize identified SMN interactor, in SMA conditions.

III. Investigate functional involvement of SMN interacting protein in SMA.

CHAPTER 2

KH-type splicing regulatory protein interacts with survival motor neuron protein and is misregulated in spinal muscular atrophy

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CONTRIBUTION OF CO-AUTHORS

All experiments in this manuscript were performed by H. Tadesse; with the exceptions of Fig. 6 [assisted by L. Hubers (see acknowledgements)], Fig. 7B-D, and Fig 8D by J. Deschênes-Furry, and Fig. 1D-G by S. Boisvenue. Figures were created and manuscript written mainly by J. Côté, along with H. Tadesse.

ABSTRACT

KH-type splicing regulatory protein (KSRP) is closely related to chick zipcode-binding protein 2 and rat MARTA1, which are involved in neuronal transport and localization of β-actin and microtubule-associated protein 2 mRNAs, respectively. KSRP is a multifunctional RNA-binding protein that has been implicated in transcriptional regulation, neuro-specific alternative splicing and mRNA decay. More specifically, KSRP is an essential factor for targeting AU-rich element-containing mRNAs to the exosome. We report here that KSRP is arginine methylated and interacts with the Tudor domain of SMN, the causative gene for spinal muscular atrophy (SMA), in a CARM1 methylation-dependent fashion. These two proteins colocalize in granule-like foci in the neurites of differentiating neuronal cells and the methyltransferase CARM1 is required for normal localization of KSRP in neuronal cells. Strikingly, this interaction is abrogated by naturally-occurring Tudor domain mutations found in human patients affected with severe Type I SMA, a strong indication of its functional significance to the etiology of the disease. We also report for the first time that Q136E and I116F Tudor mutations behave similarly to the previously characterized E134K mutation, and cause loss of Tudor interactions with several cellular methylated proteins. Finally, we show that KSRP is misregulated in the absence of SMN, and this correlated with increased mRNA stability of its mRNA target, $p21^{cip1/waf1}$, in spinal cord of mild SMA model mice. Our results suggest SMN can act as a molecular chaperone for methylated proteins involved in RNA metabolism and provide new insights into the pathophysiology of SMA.

INTRODUCTION

Proximal spinal muscular atrophy (SMA) is a common autosomal recessive disease characterized by the selective degeneration of α-motoneurons in the anterior horn of the spinal cord. With a prevalence of ~1 in 6000 live births and a carrier frequency of ~1 in 50, SMA is amongst the leading genetic causes of infant deaths (Ogino and Wilson 2004). Typical clinical characteristics are symmetrical muscular weakness, ultimately leading to muscle atrophy, paralysis and death from respiratory distress within ~2 years of age (Melki 1997). Based on time of onset of the disease and its severity, SMA can be divided into five types, with Type 0 and Type I (Werdnig-Hoffman syndrome) being the most severe forms (Briese et al. 2005). SMA is caused by disruption of the *Smn1* gene (Lefebvre et al. 1995) and the level of functional SMN protein in patients correlates with severity of the disease (Coovert et al. 1997; Lefebvre et al. 1997). In humans, a second copy of the *Smn* gene exists, but naturally harbors a non-polymorphic C→T transition that interferes with the normal splicing of exon 7, resulting in the expression of a truncated, and unstable, form of the protein (Lorson et al. 1999; Monani et al. 1999). *Smn* is highly conserved throughout evolution and its function is essential for cell viability. Motoneuron degeneration and SMA-like phenotypes have been observed in mouse models with reduced *Smn* gene dosage or in which human *Smn2* is expressed at low levels on an *Smn*-null background. These studies suggest, that, in humans, the *Smn2* gene products compensate for *Smn1* deficiency during embryonic development, in all cells except motoneurons (reviewed in (Monani 2005).

SMN exists in the cell as part of a stable macromolecular complex consisting of at least 8 tightly associated components that include Gemins2-8 and unrip (reviewed in (Pellizzoni 2007). The SMN complex is involved in the cytoplasmic assembly of Sm proteins and U snRNAs into small nuclear ribonucleoprotein particles (snRNPs), the core components of the pre-mRNA splicing machinery (Pellizzoni 2007; Eggert et al. 2006; Paushkin et al. 2002). Although not much is known about the role of individual Gemins, their presence is required for the integrity of the SMN complex and its function in snRNP assembly (reviewed in (Pellizzoni 2007). After additional cytoplasmic remodeling steps, the mature snRNPs, along with the SMN complex, are imported into the nucleus, where they transit into discrete nuclear foci termed Cajal bodies for further maturation before ultimately functioning in splicing (reviewed in (Matera and Shpargel 2006). Hence, at steady state, SMN is present in both the cytoplasm and nucleus, where it specifically accumulates in Cajal bodies, with the exception of fetal tissues and a small subset of cell lines, in which SMN also localizes to Cajal body-related structures known as Gems (Liu and Dreyfuss 1996; Carvalho et al. 1999; Young et al. 2001). Besides the stably associated Gemins core, SMN can also interact with a plethora of different binding partners (Paushkin et al. 2002; Meister et al. 2002; Yong et al. 2004). Interestingly, many SMN interactors are constituents of various RNP complexes and it is thought that SMN might also promote the assembly of these complexes, although this remains to be determined experimentally in most cases (Matera and Shpargel 2006; Azzouz et al. 2005; Terns and Terns 2001).

Arginine methylation has been implicated in several cellular processes, including

protein subcellular localization, transcription, RNA processing, and signal transduction (Bedford and Richard 2005). At the molecular level, arginine methylation acts mainly by regulating protein-protein interactions (Boisvert et al. 2005). Protein arginine *N*-methyltransferases (PRMTs), a family now comprised of nine members in higher eukaryotes, are classified as either Type I (PRMT1, 3, 6, 8 and CARM1) or Type II (PRMT5, 7 and 9) based on their capacity to catalyze the formation of asymmetric (aDMA) or symmetric dimethylated arginines (sDMA), respectively (Bedford and Richard 2005; Gary and Clarke 1998). PRMT5 is part of a 20S cytoplasmic complex named the 'methylosome'. This complex methylates an arginine/glycine (RG)-rich motif in the C-terminus of Sm proteins, which increases their affinity for the Tudor domain of SMN, hence providing the driving force for the snRNP assembly process (Meister et al. 2000; Friesen et al. 2001; Brahms et al. 2001; Cote and Richard 2005). The Tudor domain is a conserved motif that was initially found in proteins interacting with RNA but is now considered to be a structural module that can specifically recognize methylated residues (arginines and/or lysines) in proteins (Cote and Richard 2005; Sprangers et al. 2003; Maurer-Stroh et al. 2003; Kim et al. 2006). The Tudor domain of SMN, encoded by exon 3, was shown to contact directly RG-rich motifs in many proteins and arginine methylation can regulate many of these interactions (Brahms et al. 2001; Cote and Richard 2005; Sprangers et al. 2003; Boisvert et al. 2002; Hebert et al. 2002; Meister et al. 2001; Barth et al. 2003; Cheng et al. 2007). Strikingly, single point mutations in the Tudor domain that are sufficient to abrogate interactions with methylated proteins (Cote and Richard 2005) are also associated with severe forms of SMA (Cusco et al. 2004; Lefebvre et al. 1998; Sun et al. 2005), underscoring a functional relevance for this

conserved domain in the etiology of the disease. Taken together, these observations strongly suggest an important role for arginine methylation in the regulation of SMN activities.

Despite a large body of work toward clarifying SMN functions, it remains unclear how a ubiquitous function for SMN in snRNP assembly can lead to a neuronal phenotype characterized by a neurodegenerative process. The neuromuscular system may be particularly vulnerable to defects in RNA processing (Pellizzoni 2007; Eggert et al. 2006; Anderson and Talbot 2003; Wan et al. 2005), and it was postulated that reduced levels of available spliceosomal snRNPs in SMA might lead to alternative splicing defects selectively more detrimental to motoneurons (Faustino and Cooper 2003). However, SMN can also be found in axons of motoneurons, at the neuromuscular junction, as well as in the cytoplasm of skeletal muscles and thus, might have distinct activities in the neuromuscular system (Fan and Simard 2002; Bechade et al. 1999; Giavazzi et al. 2006; Jablonka et al. 2006; Rossoll et al. 2003). Finally, it was recently shown that SMN does not interact with Sm proteins in motoneuron axons (Sharma et al. 2005; Zhang et al. 2006), suggesting its Tudor domain might interact with another subset of methylated proteins in axonal processes, thus providing a molecular basis for the neuro-specific activities of SMN.

We have previously reported a proteomic analysis of arginine-methylated protein complexes (Boisvert et al. 2003). This work described the use of four methyl-specific antibodies to immuno-purify protein complexes followed by LC-MS/MS mass

spectrometry analysis and resulted in the identification of over 200 new proteins that are putatively arginine methylated. These proteins included components required for pre-mRNA splicing, polyadenylation, transcription, signal transduction as well as DNA repair and many of these proteins have now been confirmed to harbor methylated arginines *in vivo* by independent studies. Since the methylated epitopes recognized by the methyl-specific antibodies also potentially constitute high-affinity binding sites for the Tudor domain of SMN, we reasoned that we may have identified novel SMN interactors in that screen. Although these proteins were purified from HeLa cells, we searched the list of identified proteins for candidates that could have important functions in neuronal cells. Among these proteins was KH-type Splicing Regulatory Protein (KSRP), a multi-KH domain RNA-binding protein implicated in a variety of cellular processes, including transcription, alternative pre-mRNA splicing, editing as well as mRNA localization and stability (Davis-Smyth et al. 1996; Lellek et al. 2000; Snee et al. 2002; Min et al. 1997; Gherzi et al. 2004). We report here that KSRP is a novel arginine methylated protein *in vivo* and that the methyltransferase CARM1 is required for its interaction with the Tudor domain of SMN. KSRP and SMN were also both found in granule-like foci in neurites of differentiating neuronal cells. Finally, KSRP protein levels are downregulated in spinal cord tissues from mild SMA model mice, and this correlates with increased $p21^{cip1/waf1}$ mRNA levels. Our results provide evidence for a novel mechanism, involving aberrant stabilization of specific KSRP mRNA targets, in the etiology of SMA.

RESULTS

KSRP interacts directly with the tudor domain of SMN

KSRP, also known as FUSE-binding protein 2 (FBP2), is the human homologue of chick zipcode-binding protein 2 (ZBP2) and rat MARTA1 (Fig. 1), which are involved in neuronal localization of β-actin and microtubule-associated protein 2 (MAP2) mRNAs, respectively (Gu et al. 2002; Rehbein et al. 2002). Hence it represents a good candidate to cooperate with SMN in its motoneuron-specific role. Moreover, KSRP harbors several RG-rich domains, often flanked by PGM motifs that are conserved among its various homologues (Fig. 1), and both of these features are indicative of potential interaction with the Tudor domain of SMN (Cheng et al. 2007). Based on these observations, we first investigated a potential interaction of KSRP with SMN. Endogenous KSRP was immunoprecipitated from N2a cell extracts, and the retained proteins were resolved by SDS-PAGE and analyzed by western blotting for the presence of SMN. Endogenous SMN was detected in the KSRP immunoprecipitate, but not in control IgG mock immunoprecipitations (Fig. 2A). Since both SMN and KSRP are RNA-binding proteins, and are known components of RNP complexes, we next wanted to assess if the co-immunoprecipitation we observed was dependent on RNA. To do so, the co-immunoprecipitations were carried out from N2a cell extracts that had been either pre-incubated in the presence of RNase A or mock-treated (Chen et al. 1997) and the presence of SMN was assessed by immunoblotting (Fig. 2B). Under both conditions, an equivalent amount of SMN was detected in the KSRP immunoprecipitate (Fig. 2B, compare lanes 3 and 6), indicating that the interaction is likely not mediated via an RNA

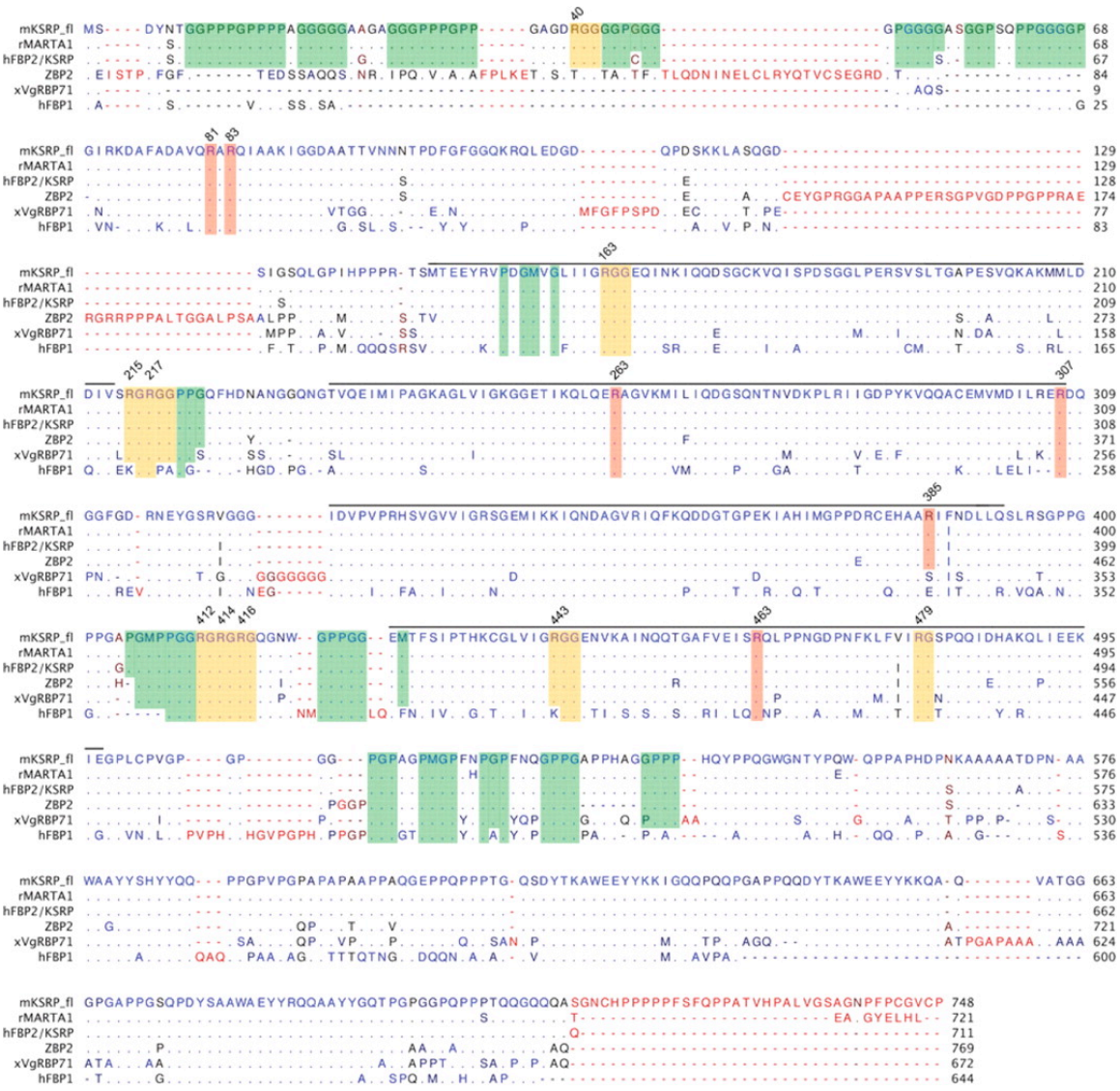


Figure 1. RG-rich and PGM motifs are conserved between KSRP and its close homologs. A multiple sequence alignment was performed using CLC Free Workbench v3.2.1, with protein sequences from mouse (Q3U0V1) and human (Q92945) KSRP, rat MARTA1 (Q99PF5), chick ZBP2 (AF461020), xenopus VgRBP71 (AAN77160) and human FBP1 (NP_003893). Identical residues are represented as dots. Residue numbering is shown on the right for each entry, excluding gaps. Degree of conservation is also illustrated using a foreground color scale from blue (100%) to red (0%). Yellow shading identifies methylated arginine residues at RG motifs and red shading identifies methylated arginine residues at R(X) motifs. Methylated residues are labeled above the mouse KSRP sequence. Green boxes denote the presence of P-G-M motifs surrounding methylation sites.

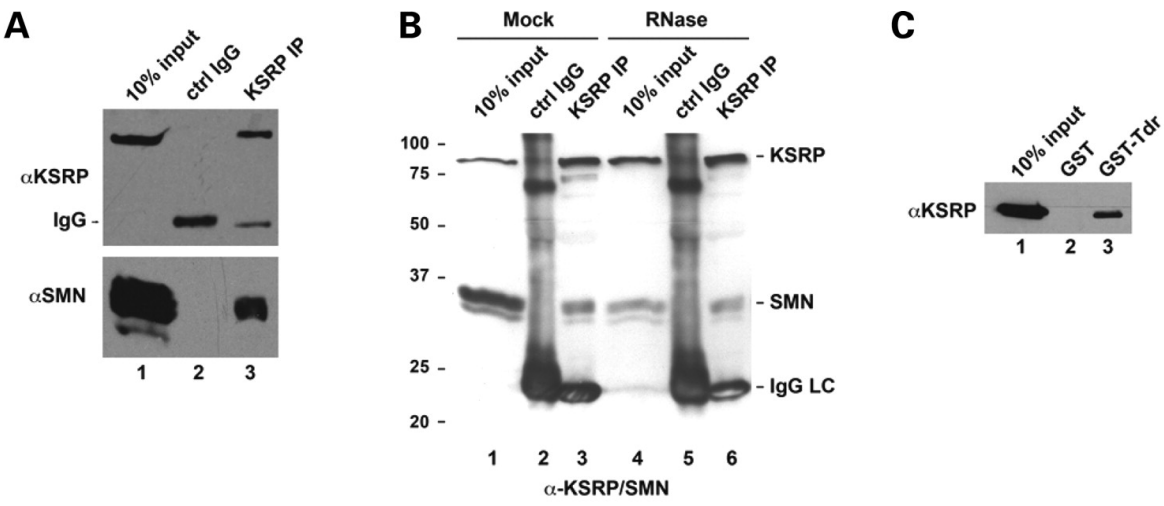


Figure 2. KSRP interacts directly with the Tudor domain of SMN. Endogenous KSRP was immunoprecipitated (IP) from N2a cells and the resulting immunoprecipitated proteins analyzed by western blot using antibodies against KSRP (top) and SMN (lower). Migration of the antibodies immunoglobulin heavy chain (IgG) is indicated (**A**). The same experiment was performed with or without pre-treatment of the cell lysate with RNase A, showing that the interaction between KSRP and SMN is RNA-independent (**B**). Total cell lysate was prepared from N2a cells and was subjected to binding assays using immobilized GST and SMN Tudor domain (GST-Tdr). Retained proteins were then immunoblotted for the presence of KSRP (**C**).

moiety. Total cell extracts from N2a cells were next subjected to GST pull-down experiments with either GST alone or a GST fusion with the Tudor domain of SMN (Fig. 2C; GST-Tdr). Endogenous KSRP bound to the GST-Tdr column, but not to GST alone, suggesting that the interaction was mediated through the SMN Tudor domain (Fig. 2C, lanes 3 and 2, respectively). ^{35}S-labeled KSRP was produced *in vitro* using rabbit reticulocyte lysates and used in GST pull-down experiments. Again, KSRP bound specifically to the Tudor domain of SMN, but not to GST alone (Fig. 2D, lanes 3 and 2, respectively), a strong indication that the interaction is likely direct. Strikingly, binding

was abolished by intragenic Tudor mutations found in human patients with severe Type I SMA (Fig. 2D, E134K, Q136E, I116F and A111G, lanes 4-6 and 10, respectively) (Lefebvre et al. 1998; Sun et al. 2005; Cusco et al. 2004). This was also true when the E134K mutation was tested in the context of the full-length SMN protein (Fig. 2E, lanes 3 and 4), suggesting that the Tudor domain is the main feature contributing to KSRP binding. Taken together, these results show that KSRP can interact directly with the Tudor domain of SMN, and most importantly, the lack of binding to the *Smn* mutations that are associated with severe Type I SMA suggests that this interaction is functionally significant to the etiology of the disease.

We have recently shown that the E134K Tudor mutation abolished interaction with several methylated proteins in cells (Cote and Richard 2005). However, it is not known if the Q136E and I116F mutations also affect binding to arginine methylated proteins. To assess this, HeLa cell extracts were incubated with either GST alone, GST-Tudor or GST-TdrE134K, Q136E and I116F mutants. Following extensive washes, bound proteins were resolved by SDS-PAGE, transferred to PVDF and immunoblotted with a mixture of sDMA-specific antibodies SYM10 and SYM11 (Boisvert et al. 2002; Boisvert et al. 2003). As previously reported (Cote and Richard 2005), the wild-type (WT) Tudor domain bound a number of cellular sDMA-containing proteins from the subset of polypeptides recognized by SYM10/11, which include p80-coilin and Sm proteins B/B´, D1 and D3 (Fig. 2F, lane 3). As was seen for E134K, the Q136E and I116F amino acid substitutions abolished the binding to sDMA-containing interactors (Fig. 2F, lanes 4, 5 and 7, respectively). These mutations also abolished binding to aDMA-containing cellul-

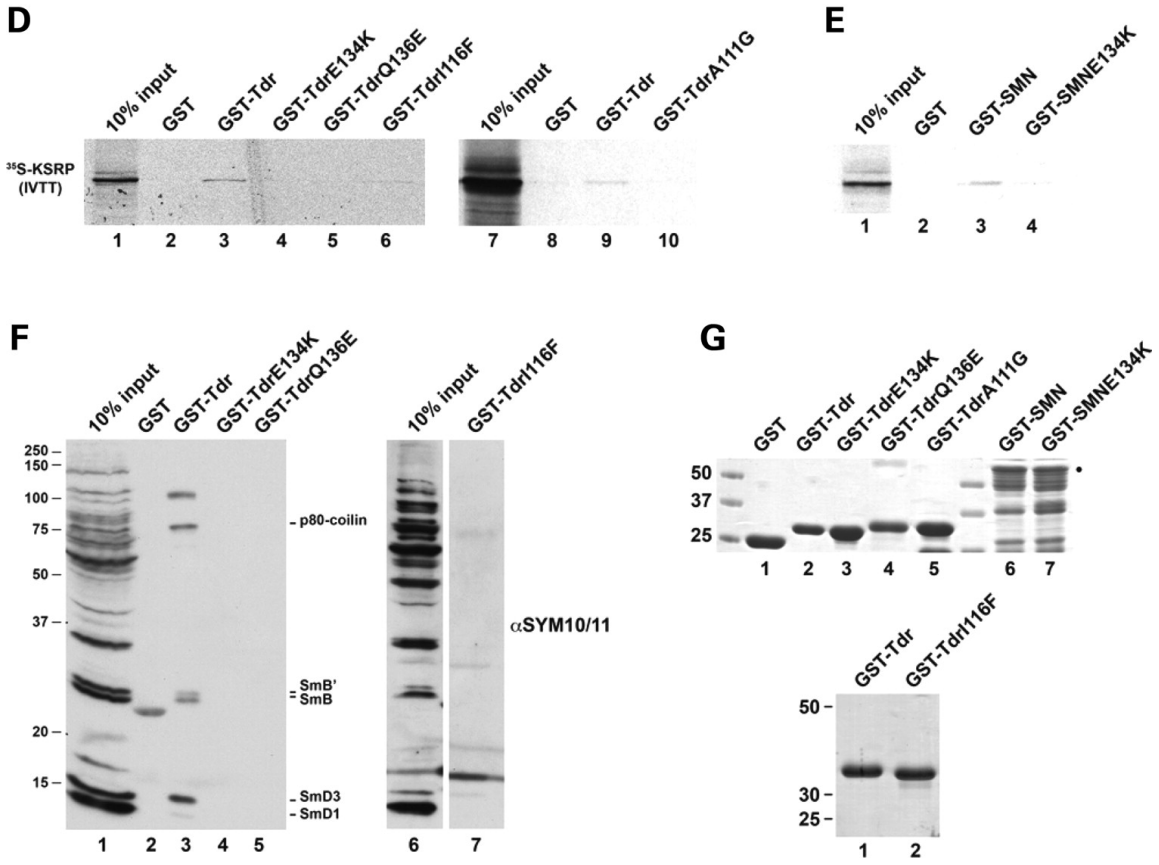


Figure 2 (continued). KSRP interacts directly with the Tudor domain of SMN. ^{35}S-labeled KSRP was produced *in vitro* using transcription/translation coupled (IVTT) rabbit reticulocyte lysates and subjected to binding assays using immobilized GST, SMN Tudor domain (GST-Tdr) or SMA-causing mutant alleles of the Tudor domain (GST-TdrE134K, GST-TdrQ136E, GST-TdrI116F and GST-TdrA111G) (**D**). The E134K mutation also abolishes binding to KSRP when introduced in the context of the full length SMN protein (**E**). GST pull–downs were performed using the same series of immobilized GST-fusion proteins except that HeLa cell lysates were used as a source of proteins. Immunoblotting was with a mixture of sDMA-specific SYM10 and SYM11 antibodies. Lanes 1–5 and 6–7 represent distinct experiments (**F**). GST-fusion proteins used for these experiments were resolved by SDS–PAGE and stained using Coomassie Brilliant Blue. The black dot on the right indicates the migration of the full length GST-SMN fusions (**G**).

ar proteins, as assessed by immunoblotting with antibodies ASYM24 (Boisvert et al. 2003), ASYM25 (Cote et al. 2003), and αH3R17me2a (Cheng et al. 2007) (data not shown).

KSRP and SMN colocalize in neuronal processes foci

Since an interaction between SMN and KSRP has been established, we next wanted to assess if they colocalized in neuronal cells. The intracellular localization of SMN and KSRP in fixed cells is well-established. SMN localizes mostly in the cytoplasm and concentrates in bright nuclear foci corresponding to Cajal bodies in most cell types, as seen in cycling N2a cells (Fig. 3A, panel c). In contrast, KSRP is diffusely distributed throughout the nucleoplasm at steady state, and is excluded from nucleoli (Fig. 3A, panel b). Hence, apart from the obvious overlap in the nucleoplasm diffused staining, no colocalization was observed between KSRP and SMN in undifferentiated N2a cells (Fig. 3A, panel d). N2a cells were induced to differentiate and grow long neuritic processes after addition of dibutyryl-cAMP for 48 h in low-serum conditions. It has been documented that KSRP concentrates in foci that are thought to be sites of *c-src* transcription upon neuronal differentiation (Hall et al. 2004). We find that these foci were often juxtaposed, but never completely overlapping with SMN-containing Cajal bodies in differentiating N2a cells (Fig. 3A, panel h; white arrow). Both KSRP and SMN were also found in granular foci along neuritic extensions (Fig. 3B, panel a-c). Interestingly, in neurites, 23.6 $\pm$ 2.5% of KSRP foci also contained SMN, and 21.4 $\pm$ 3.5% of SMN foci contained KSRP (Fig. 3B, bar graph). This is consistent with the relative amount of SMN that can be co-immunoprecipitated with KSRP, and suggests that these two proteins may interact predominantly in neuronal projections *in vivo*.

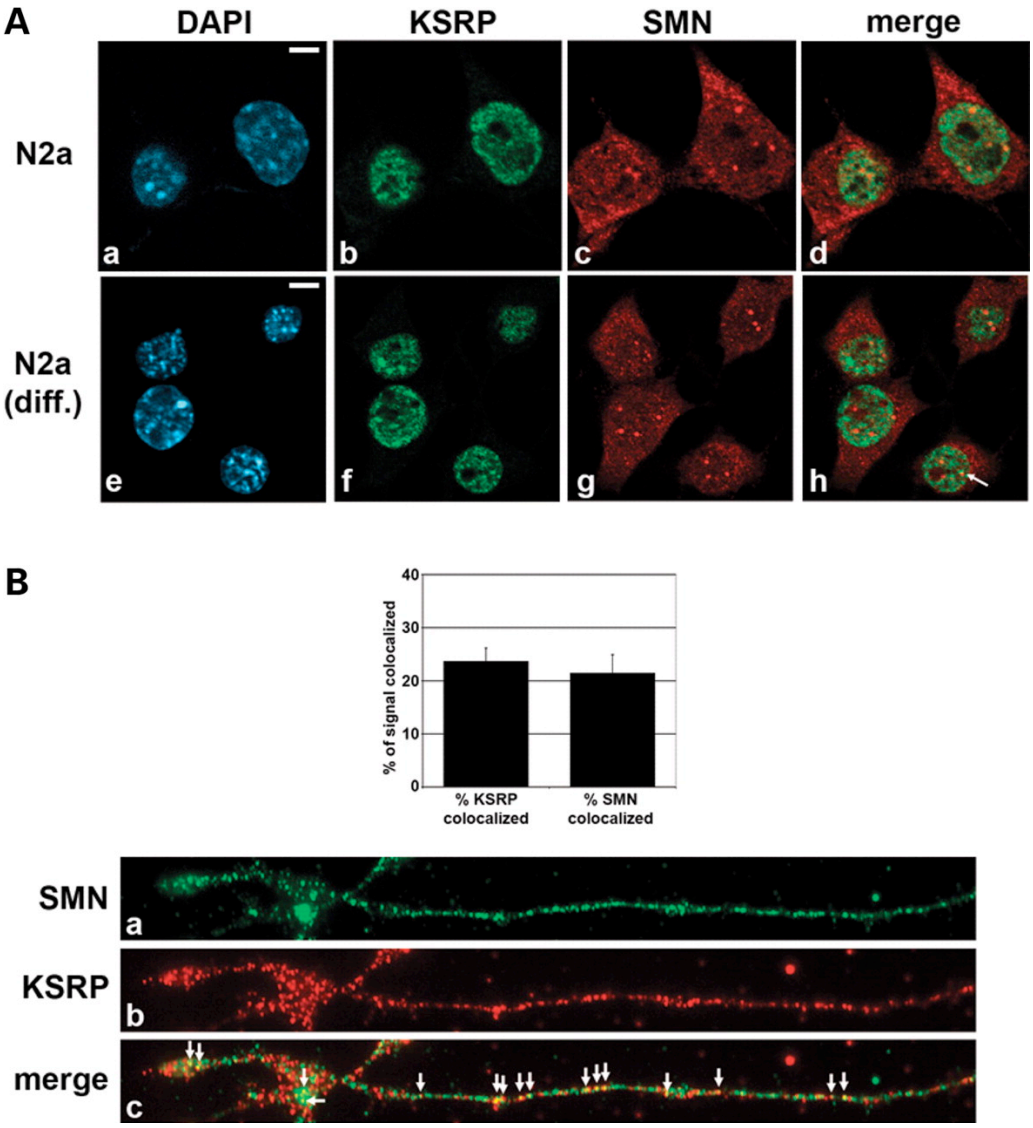


Figure 3. KSRP colocalizes with SMN in neurites of differentiating N2a mouse neuroblastomas. Mock-treated and differentiated N2a cells were labeled for immunofluorescence with KSRP (b and f) or SMN (c and g) antibodies. Cell nuclei were counter-stained with DAPI (a and e). Merged images are shown in (d) and (h) and reveal no colocalization between KSRP and SMN in the cell body. Bars, 10 μm (**A**). Images captured at 63X and focusing on neurites reveal the presence of both SMN (a) and KSRP (b) in bright granular foci along extending neurites. Foci where both proteins colocalized are marked with arrows in the composite panel. 23.6 ± 2.5% of KSRP-positive foci also contained SMN, and 21.4 ± 3.5% of SMN-positive foci contained KSRP (bar graph shows the mean % of signal colocalized ± SEM; n = 20 neurites) (**B**).

KSRP is an arginine methylated protein *in vivo*

To determine whether endogenous KSRP is methylated in cells, an *in vivo* methylation assay was used as previously described (Cote et al. 2003). Specifically, N2a cells were incubated with [methyl-^{3}H]-*L*-methionine in the presence of translation inhibitors, cycloheximide and chloramphenicol. After immunoprecipitation with antibodies against KSRP, SMN or Sam68, proteins were separated by SDS-PAGE and subjected to fluorography. SMN is known not to be methylated and thus serves as a negative control in this assay to ensure that no labeling occurs through translation (Fig. 4A, left panel, lane 1). In

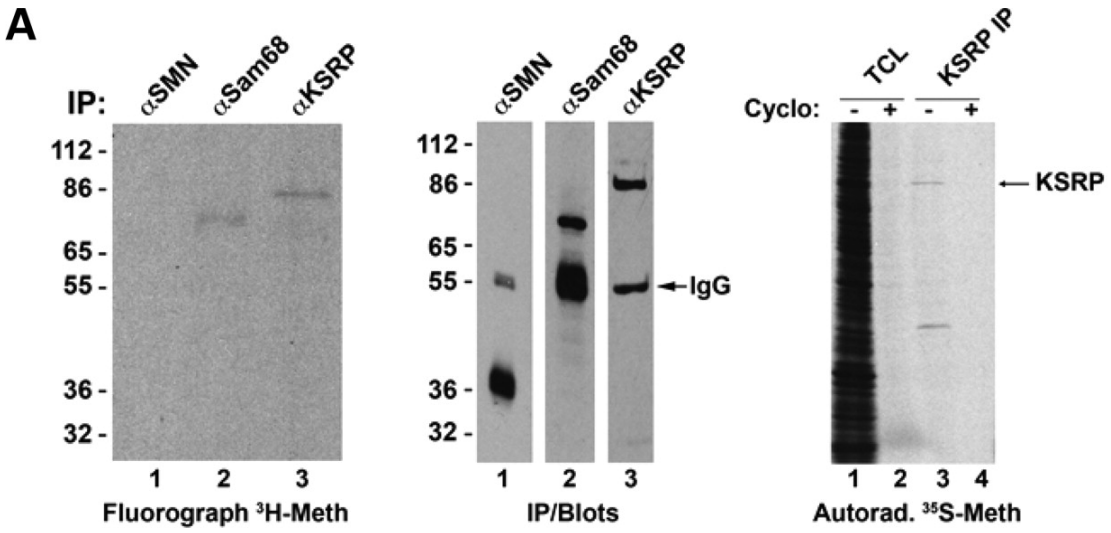


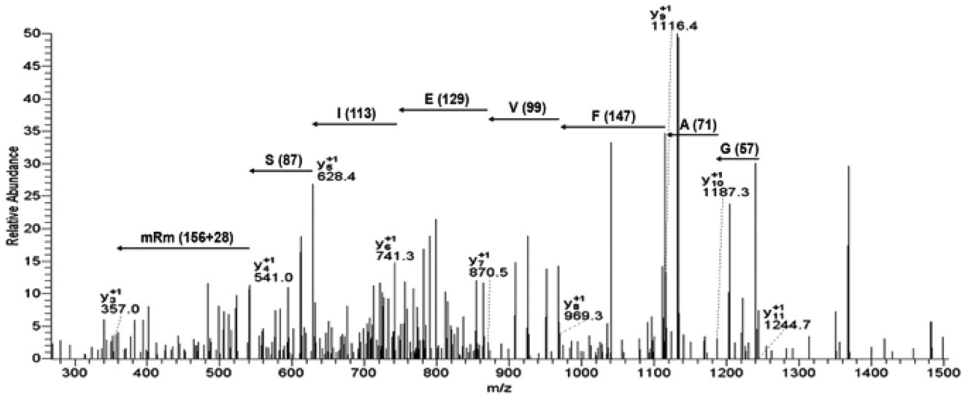
Figure 4. KSRP is arginine methylated *in vivo*. N2a cells were incubated with cycloheximide (Cyclo) and L-[methyl-^{3}H]methionine for 3 h. Indicated endogenous proteins were immunoprecipitated (IP) with respective specific antibodies and *in vivo* methylation was visualized by fluorography (left). An aliquot of the IP was analyzed by western blot (middle). SMN serves as a negative control and Sam68 as a positive control in this assay. The same experiment was done using ^{35}S-methionine (right) to confirm efficient inhibition of translation and that the observed labeling for KSRP is indeed from post-translational methylation (**A**).

contrast, Sam68 is methylated in cells (Cote et al. 2003) and comparable signals were obtained for this protein and KSRP (Fig. 4A, left panel, lanes 2 and 3), indicating that KSRP is methylated *in vivo*. Aliquots of each immunoprecipitation were ran on a separate gel and analyzed by western blot to confirm efficient and equivalent immunoprecipitation (Fig. 4A, middle panel). Finally, the same experiment was performed using ^{35}S-*L*-methionine in the presence or not of translation inhibitors (Fig. 4A, right panel). Labeled KSRP was efficiently immunoprecipitated from mock-treated cells, but no signal was detected when the immunoprecipitation was performed from cells grown in the presence of translation inhibitors (Fig. 4A, right panel, lanes 3 and 4, respectively), confirming that the above ^{3}H-labeling observed for KSRP is indeed from post-translational methylation.

To identify the precise residues modified by arginine methylation, endogenous KSRP was immunoprecipitated from N2a cells and analyzed by tandem mass spectrometry (LC-MS/MS). Specifically, the immunoprecipitate was digested with chymotrypsin and GluC overnight at 30ºC. The resulting peptide mixture was resolved by reverse-phase chromatography and the peptides fragmentation data collected using a LCQ Deca XP (One representative y-ion series spectrum is shown in Fig. 4B). The raw data were processed with Bioworks software version 3.1 and searched against NCBI mouse database. Using this approach, 71.5% of the KSRP protein was sequenced and 14 arginine residues were found to be either mono- or dimethylated (MMA or DMA, respectively; Fig. 4C). Among all nine predicted RG motifs, seven harbored DMA, R40 was not modified and no peptide information could be obtained for R479 (Figs. 1 and 4C). Surprisingly, 7 additional arginine residues were found to be methylated within non-

RG motifs, namely RA, RQ, RD and RI (Figs. 1 and 4C). Most PRMTs characterized to date target RG dipeptides for methylation. However, CARM1 is a PRMT that was found to often methylate non-RG sequences, including RP motifs (Cheng et al. 2007; Lee and Bedford 2002). Hence, considering these findings, it is very likely that more than one enzyme, most probably including CARM1, will be responsible for KSRP methylation *in vivo*.

B



C

Residue	Status	Evidence	Residue	Status	Evidence
$R^{40}GG$	not modified	4 sequences	$R^{385}I$	DMA	2 sequences[2]
$R^{81}A$	DMA	2 sequences	$R^{412}G$	DMA	6 sequences[2]
$R^{83}Q$	MMA	1 sequence	$R^{414}G$	DMA	6 sequences[2]
$R^{163}Q$	MMA	4 sequences[1]	$R^{416}G$	DMA	6 sequences[2]
$R^{215}G$	DMA	2 sequences[2]	$R^{443}GG$	DMA	2 sequences[2]
$R^{217}GG$	MMA	2 sequences	$R^{463}Q$	DMA	2 sequences
$R^{263}A$	MMA	1 sequence[1]	$R^{479}G$	nd	no peptides
$R^{307}D$	MMA	1 sequence			

[1]missing peaks in ion series makes this identification probable, but needs confirmation; [2]MMA was also detected in at least one sequence

Figure 4 (continued). KSRP is arginine methylated *in vivo*. Endogenous KSRP was immunoprecipitated from five 150 mm dishes of N2a cells and subjected to LC-MS/MS mass spectrometry. A representative y-ion series fragmentation spectra is shown. A shift of 28 in mass is observed for R463, indicative of a dimethylated arginine. The b-ion series was left out for clarity (**B**). Sequence coverage by the identified peptides is represented by the shaded areas in the KSRP protein. Results obtained from the mass spectrometry analysis are summarized. MMA is for monomethylated arginine and DMA is for dimethylated arginine (**C**).

Arginine methylation by CARM1 regulates the interaction of KSRP with SMN

Since arginine methylation is known to regulate the interaction between SMN and many of its interactors, we wanted to determine whether the interaction of KSRP with SMN was also regulated by arginine methylation. We recently reported that CARM1 regulated the interaction of SMN with CA150, another protein that harbors RG/PGM motifs (Cheng et al. 2007). To determine if the interaction between KSRP and SMN is CARM1-dependent, KSRP was immunoprecipitated from WT and Carm1$^{-/-}$ mouse embryonic fibroblasts (MEFs). SMN was co-immunoprecipitated with KSRP from WT MEFs, to a similar extent as was observed in N2a cells (Fig. 5A, lane 3). However, SMN was not present at detectable levels when the immunoprecipitation was performed with lysates from the Carm1$^{-/-}$ cells (Fig. 5A, lane 6). This result suggests that KSRP needs to be methylated by CARM1 to interact stably with the Tudor domain of SMN.

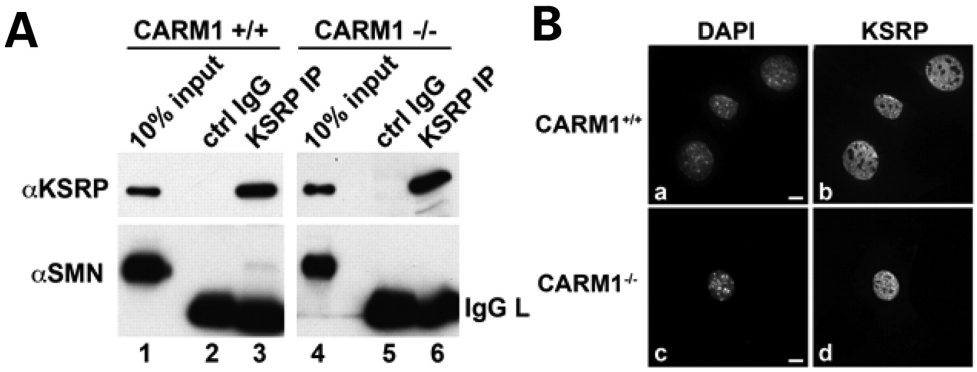


Figure 5. The interaction between KSRP and SMN depends on CARM1 in MEFs. Endogenous KSRP was immunoprecipitated as in Fig. 1A, but using total cell lysates from wild-type (WT) or *Carm1*$^{-/-}$ MEFs as a source of proteins. Presence of KSRP and SMN was detected by immunoblotting as indicated. Migration of the antibodies immunoglobulin light chain is indicated (**A**). The localization of KSRP remains unchanged in *Carm1*$^{-/-}$ MEFs. WT and *Carm1*$^{-/-}$ MEFs were grown on glass coverslips and labeled for immunofluorescence with KSRP antibodies (b and d). Cell nuclei were counter-stained with DAPI (a and c). Bars, 5 μm (**B**).

Arginine methylation by CARM1 regulates KSRP intracellular localization in neuronal cells

Arginine methylation is known to regulate the intracellular localization of many RNA-binding proteins. Since CARM1 can regulate the interaction between KSRP and SMN, we sought to determine if the intracellular localization of KSRP was also affected in the absence of this enzyme. WT and Carm1$^{-/-}$ MEFs were fixed, permeabilized and subjected to indirect immunofluorescence using antibodies against KSRP (Fig. 5B). KSRP was found to be diffusely distributed throughout the nucleoplasm and excluded from nucleoli in WT MEFs (Fig. 5B, panel b), and this localization was not affected in Carm1$^{-/-}$ cells (Fig. 5B, panel d). In order to assess this in a neuronal setting, we used MN-1 cells, a motor neuron-neuroblastoma hybrid cell line that has been used previously for functional studies involving motor neuron proteins (Salazar-Grueso et al. 1991). CARM1 expression was efficiently knocked-down in MN-1 cells using transiently transfected RNA duplexes, as determined by RT-PCR (Fig. 6A). Control and transfected MN-1 cells were processed for indirect immunofluorescence with KSRP antibodies as described above (Fig. 6B). Strikingly, KSRP gradually accumulated in the cytoplasm with decreasing levels of CARM1 (Fig. 6B, panel e and f). Moreover, we observed that reduced CARM1 levels in these cells induced neurite outgrowth (Fig. 6C, panel a-c and d-f).

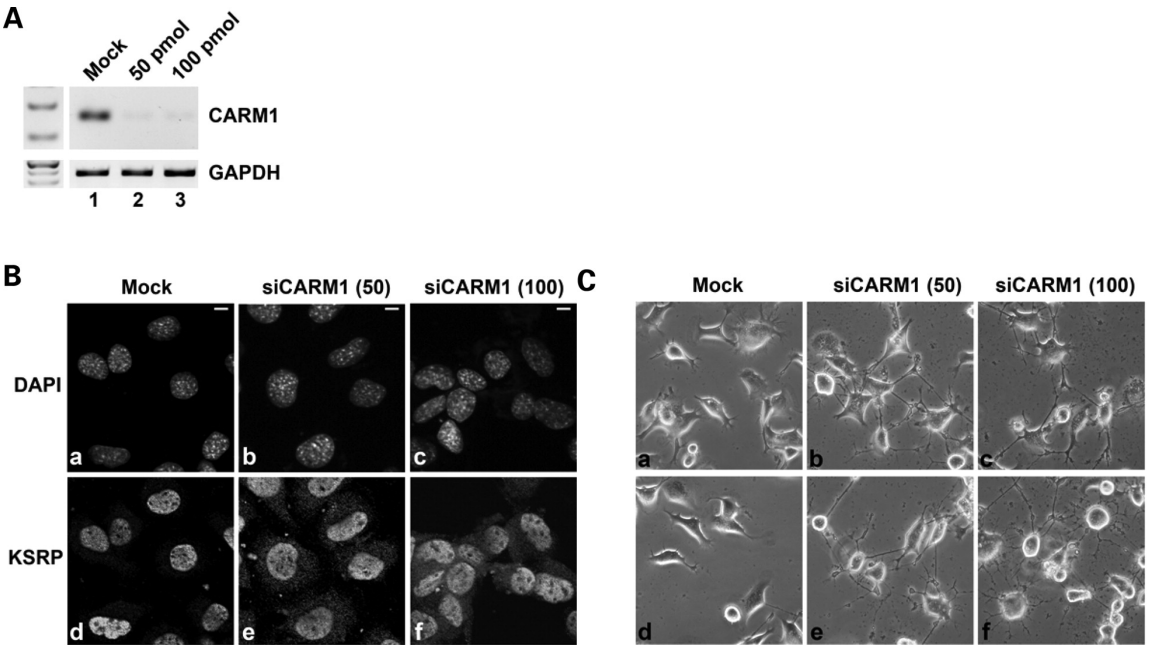


Figure 6. Arginine methylation by CARM1 regulates KSRP intracellular localization in MN-1 mouse motoneuron-like cells. CARM1 expression was knocked-down in MN-1 cells using RNA interference. Semi-quantitative RT–PCR was performed to assess the extent of knockdown obtained after 48 h with 50 and 100 pmols of siRNA duplex targeting CARM1 mRNAs. GAPDH serves as control for cDNA input (**A**). Mock- and siRNA-transfected MN-1 cells were labeled for immunofluorescence with KSRP antibodies, revealing gradual increase in cytoplasmic staining (d–f). Cell nuclei were counter-stained with DAPI (a–c). Bars, 10 μm (**B**). Reducing CARM1 levels by RNA interference in MN-1 cells induces neurite outgrowth. Phase contrast images for two independent experiments are shown in (a–c) and (d–f) (**C**).

A KSRP mRNA target is stabilized in SMA tissues

KSRP is involved in many aspects of RNA metabolism (see section *Introduction*), but its role in recruitment of specific mRNAs for degradation by the exosome has recently received considerable attention. Given that KSRP interacts and colocalizes with SMN in neurites of neuronal cells, we wanted to assess if the function of KSRP in mRNA stability would be affected in SMA neuronal tissues. To do this, we focused on transcripts that have previously been shown to be targets for KSRP or its homologues, such as GAP43,

whose 3′-UTR is bound by FBP1 (Irwin et al. 1997). β-actin which associates with chick ZBP2 (Gu et al. 2002) and p21 a known target of KSRP (Briata et al. 2005). To first confirm that mouse KSRP indeed interacts with GAP43 and β-actin mRNAs, KSRP was immunoprecipitated from N2a cells and the associated RNA extracted and amplified by RT-PCR with primers specific for these two transcripts. As expected, both GAP43 and β-actin mRNAs were present in the KSRP immunoprecipitate (Fig. 7A, lanes 2 and 5), but not in the control IgG immunoprecipitation (Fig. 7A, lanes 3 and 6).

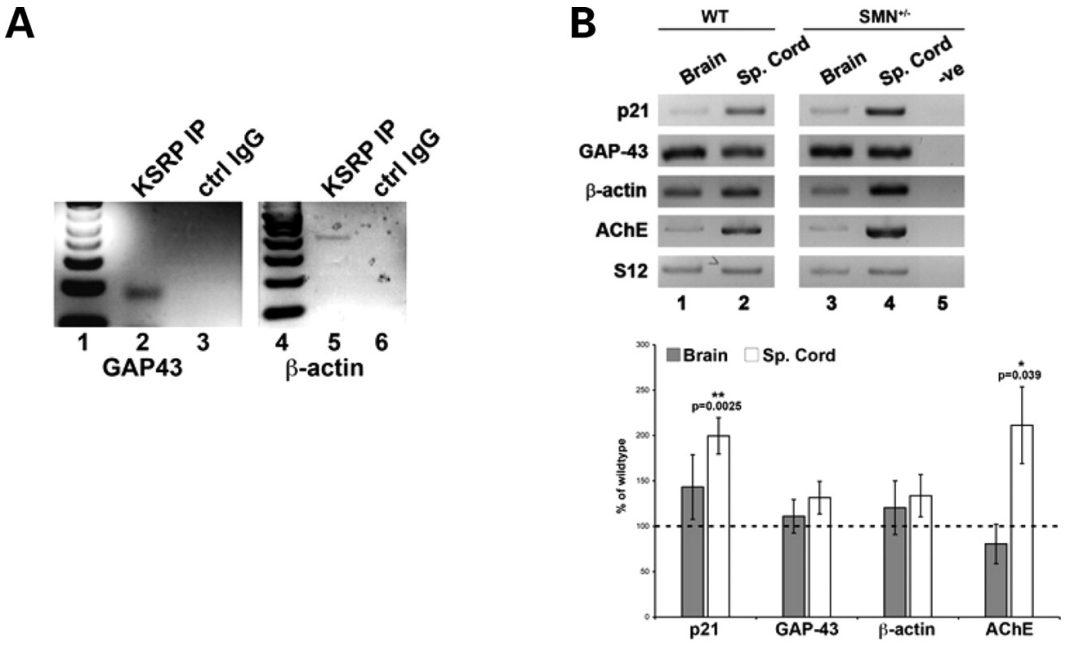


Figure 7. A KSRP mRNA target is stabilized in SMA tissues. KSRP interacts with GAP43 and β-actin mRNAs. Endogenous KSRP was immunoprecipitated from N2a cell extracts and the RNA isolated from the immunoprecipitate was analyzed by RT–PCR with primers specific for GAP43 and β-actin mRNAs (**A**). Increased p21 and AChE mRNA levels are observed in spinal cord of *Smn*$^{+/-}$ mice, as determined using semi-quantitative RT–PCR. In contrast, no differences are observed for GAP43 and β-actin mRNA. '–ve' (lane 5) indicates a no RT control. mRNA levels ± SEM ($n \geq 3$) were normalized to corresponding S12 level and plotted in the bar graph as percent of WT levels (dotted line). Statistical significance is indicated for p21 and AChE (**B**).

Total RNA was prepared from WT and *Smn* heterozygote mouse brain and spinal cord tissues. Mice heterozygous for Smn show up to 50% motor neuron attrition by 6 months of age and are used as a model for mild SMA in humans (Jablonka et al. 2000; Balabanian et al. 2007). Using semi-quantitative RT-PCR, steady-state mRNA levels for GAP43 and β-actin were found to be equivalent in brain and spinal cord of WT and $Smn^{+/-}$ mice (Fig. 7B, compare lanes 1-2 with lanes 3-4 of respective panels and see bar graph below). In contrast, p21 mRNA levels were significantly ($P = 0.0025$) increased in the spinal cord of $Smn^{+/-}$ mice (Fig. 7B, compare lanes 2 and 4 and see bar graph below). This result is in agreement with previous studies reporting increased levels of p21 mRNA and protein in both mouse and human SMA spinal cord samples (Olaso et al. 2006).

Since previous studies have shown that KSRP binds directly to the 3′-UTR of p21 (Briata et al. 2005), we next examined whether the increased level of p21 mRNA was due to an increased transcript stability. To assess this, an *in vitro* stability assay was used, where total RNA from WT mouse brain was incubated for specific time intervals in the presence of protein extracts from WT and $Smn^{+/-}$ mice spinal cords. RNA was then recuperated from each time point and semi-quantitative RT-PCR performed with primers specific for p21 and S12 mRNAs. As shown in Fig. 7C, p21 mRNA levels decay relatively quickly ($t_{1/2} = 11.4$ min) and decrease to ~20% of time 0 values within 30 min, as opposed to S12 mRNA levels, which stay constant throughout the incubation period (Fig. 7C, lanes 1-6 and solid line in graph). Strikingly, p21 mRNAs decay with a slower rate ($t_{1/2} = 27.9$ min) when incubated in $Smn^{+/-}$ protein extracts (Fig. 7C, lanes 7-12 and dotted line in graph). As expected, since their level was the same in WT and $Smn^{+/-}$ spinal cord,

we did not observe significant differences in the mRNA stability of GAP43 and β-actin mRNAs (data not shown).

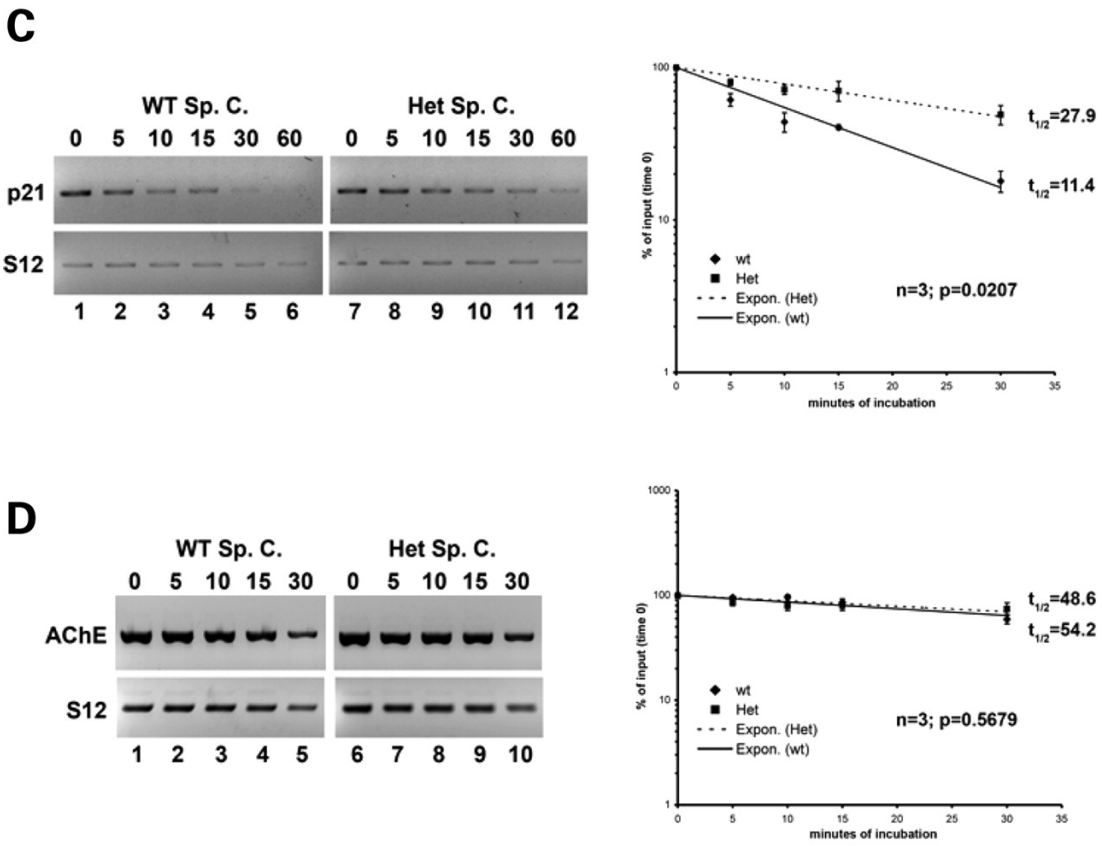


Figure 7 (continued). A KSRP mRNA target is stabilized in SMA tissues. *In vitro* mRNA stability assays were performed with protein extracts from WT or *Smn*$^{+/-}$ spinal cord tissues (Het). Representative ethidium bromide-stained gels displaying p21 and S12 RT–PCR products following 0, 5, 10, 15, 30 and 60 min of incubation with protein extracts. Mean values ± SEM (n = 3) were normalized to S12 levels and plotted as percent of input at time 0 versus time of incubation in minutes. Increased stability is observed for p21 mRNA in *Smn*$^{+/-}$ spinal cord extracts, as revealed by the longer half-life ($t_{1/2}$) calculated from the regression (**C**). The same assays were performed for AChE mRNA and revealed no significant differences in stability between WT and SMA spinal cord tissues (**D**).

To demonstrate the specificity of our assay, we wished to use an mRNA that was known to be regulated at the level of its mRNA stability, but was not a target of KSRP.

The acetylcholinesterase (AChE) mRNA was chosen for this purpose. It was shown by several groups to be regulated post-transcriptionally (Deschenes-Furry et al. 2005a) and we have confirmed that it is not bound by KSRP in our KSRP immunoprecipitation/RT-PCR experiments (data not shown). Even though a significant (P = 0.039) increase was detected for AChE mRNA in $Smn^{+/-}$ spinal cord, as compared with WT tissues (Fig. 7B, compare lanes 2 and 4 and see bar graph below), no significant difference in rate of decay was observed in the *in vitro* mRNA stability assay (Fig. 7D). Taken together, these results therefore suggest that the difference in p21 levels observed between WT and mild SMA spinal cord is due to increased mRNA half-life, likely as a result of an intrinsic difference in the abundance and/or function of a specific factor(s) in SMA tissues. Since this effect is specific to p21 and is not seen for AChE, it is consistent with the possibility that KSRP is one such factor and might be misregulated in the absence of normal levels of SMN.

KSRP is down-regulated in the presence of low levels of SMN

Based on these observations, we next sought to get some insights into the mechanism by which SMN could influence KSRP function. Previous reports have shown that reduced levels of SMN can sometimes cause a reduction in the amount of its interacting partners (Helmken et al. 2003). In order to determine if this was the case for KSRP, we used RNA interference to knockdown either SMN or KSRP in N2a neuronal cells (Fig. 8A). Efficient knockdown was obtained using either transient transfection of siRNA duplexes (KSRP k/d; Fig. 8A, lane 2) or stable expression of shRNA hairpins from a plasmid vector (SMN k/d; Fig. 8A, lane 3). Strikingly, KSRP protein levels were reduced upon

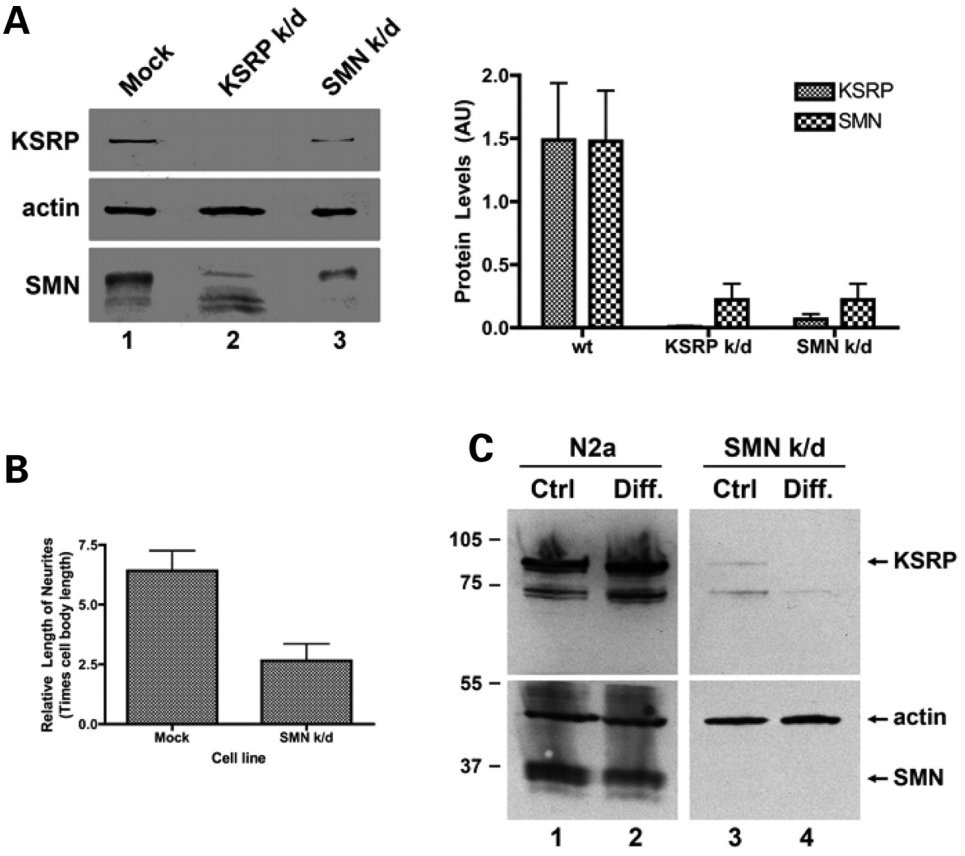


Figure 8. KSRP is misregulated in the absence of SMN. KSRP and SMN protein levels are co-regulated. RNA interference was used to knockdown the expression of KSRP or SMN as indicated. Immunoblots show that KSRP levels are reduced when SMN is knocked-down and vice versa. Protein levels were normalized to actin levels. Linear detection of the immunoblot signal was obtained using fluorescence-coupled secondary antibodies and an Odyssey Infrared Imaging System. The mean values ± SEM (n = 3) are plotted in the bar graph on the right (**A**). Reducing SMN expression by RNA interference results in differentiation defects in N2a cells treated with dibutyryl-cAMP and low serum for 48 h. Shown is a bar graph where the relative length of neurite extensions (expressed as times cell body length) was plotted for N2a (Mock) or N2a SMN knockdown (SMN k/d). Mean values ± SEM from 100 cells were used to generate the graph (**B**). KSRP is down-regulated following induction of differentiation in N2a cells where SMN expression was knocked-down. Protein samples were obtained from N2a cells and SMN knocked-down N2a cells, mock-treated (Ctrl) or treated with dibutyryl-cAMP and low serum for 24 h (Diff.). Immunoblotting for KSRP, SMN and actin is shown (**C**).

SMN knockdown and the reciprocal observation was also true (Fig. 8A, compare lanes 2 and 3 for the respective panels and see bar graph). Linear detection of immunoblot signals was obtained using fluorescence-coupled secondary antibodies and an Odyssey Infrared Imaging System. Equal loading was confirmed by probing the same membrane with antibodies against F-actin (Fig. 8A, middle panel).

It was recently reported that reducing SMN levels by RNA interference in neuron-like PC12 cells results in differentiation defects (Bowerman et al. 2007) and we have made similar observations for our N2a SMN knockdown cell line (Fig. 8B, H. Tadesse and J. Côté, unpublished data). Hence, we next wanted to determine if these defects could be correlated with KSRP levels. Normal and stable SMN knockdown N2a cells were induced to differentiate as above using dibutyryl-cAMP. Total proteins were obtained from mock-treated and differentiating cell lines and used for immunoblotting (Fig. 8C). As seen above, KSRP protein levels were greatly reduced in N2a lines stably engineered to produce reduced levels of SMN, as compared with parental N2a cells (Fig. 8C, compare lanes 3 and 1, respectively). Following 24h of treatment with dibutyryl-cAMP and low serum concentrations, KSRP protein levels were found to be further decreased in the SMN knockdown lines, while KSRP levels remained unaffected in the parental N2a cells (Fig. 8C, compare lanes 4 and 2, respectively). These results suggest that in the absence of SMN, induction of the normal neuronal differentiation program may be defective, at least in part, due to aberrant down-regulation of KSRP protein levels.

Finally, we wished to determine if this down-regulation of KSRP levels could also

be observed in SMA mice neuronal tissues. Total RNA was first prepared from WT and *Smn*$^{+/-}$ mouse brain and spinal cord tissues. KSRP transcript levels were assessed by semi-quantitative RT-PCR and were found to be significantly higher in brain versus spinal cord tissues (Fig. 8D, lane 1 and 3, respectively), but this profile remained unchanged in mild SMA mouse tissues (Fig. 8D, lanes 2 and 4). To determine whether that was also the case at the protein level, total brain and spinal cord protein extracts were prepared from WT and *Smn*$^{+/-}$ mice, and equal amounts of protein were subjected to immunoblotting for KSRP and Sam68 (Fig. 8E). In accordance with its mRNA levels,

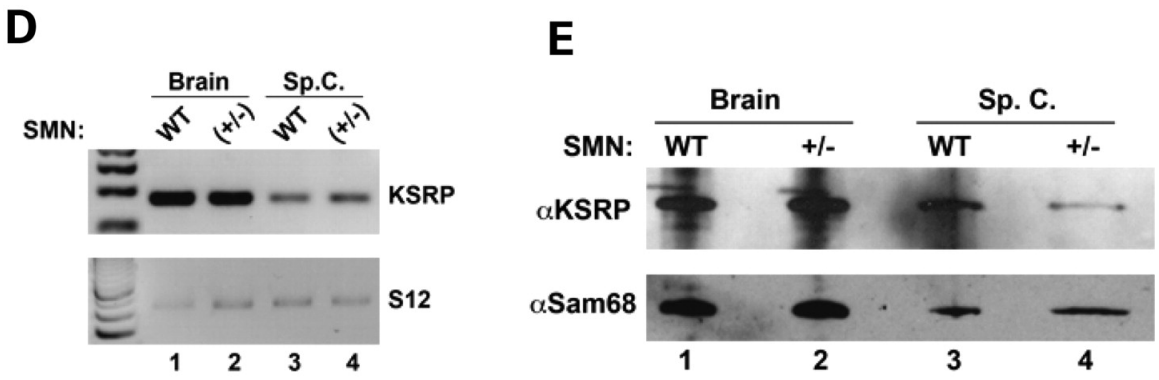


Figure 8 (continued). KSRP is misregulated in the absence of SMN. Total RNA was prepared from brain and spinal cord of WT and *Smn*$^{+/-}$ mice and KSRP mRNA level was determined by semi-quantitative RT–PCR (**D**). Total protein extracts were prepared from brain and spinal cord of WT and *Smn*$^{+/-}$ mice. KSRP protein level was determined by immunoblotting and normalized to Sam68 levels (**E**).

KSRP protein levels were ~2-fold higher in WT brain, as compared with spinal cord (Fig. 8E, lanes 1 and 3, respectively). However, KSRP protein levels were lower in the spinal cord of mild SMA mice, while remaining constant in brain (Fig. 8E, compare lanes 3 and 4 with lanes 1 and 2, respectively). Sam68 protein levels, which were used to confirm

equal loading, were also slightly higher in brain tissues, but remained unchanged in *Smn*$^{+/-}$ tissues (Fig. 8E, bottom panel). Taken together, these observations suggest that KSRP is abnormally down-regulated, specifically at the protein level, in the presence of reduced levels of SMN. Moreover, this effect is seen only in spinal cord tissues, suggesting that down-regulation of KSRP may contribute to the tissue-specificity observed in SMA.

DISCUSSION

We report here that KSRP is an arginine methylated protein *in vivo* and interacts with the Tudor domain of *Smn*, the causative gene for SMA. These two proteins also colocalized in granule-like foci of neuritic processes in differentiating N2a cells. Strikingly, this interaction is abrogated by Tudor domain mutations found in human patients affected with severe Type I SMA, a strong indication of its functional significance to the etiology of the disease. We also report for the first time that Q136E and I116F Tudor mutations, behave similarly to the previously characterized E134K mutation, and cause loss of Tudor interactions with several cellular methylated proteins. Moreover, we show that the CARM1 methyltransferase is required for the SMN/KSRP interaction, as well as for normal localization of KSRP in neuronal cells. Finally, KSRP is misregulated in the absence of SMN, and this correlated with increased mRNA stability of its mRNA target, $p21^{cip1/waf1}$, in spinal cord of mild SMA model mice.

KSRP is a novel SMN interacting protein

SMN is thought to exist in most cells as part of a stable multi-protein complex known as the 'SMN complex'. Besides its stable interaction with the core components of the SMN complex, SMN also potentially interacts with a surprisingly large number of proteins, localized in different sub-cellular compartments and involved in varied cellular processes. We report here a novel interaction between SMN and KSRP, a protein more abundantly expressed in neuronal cells and, that is potentially implicated in several aspects of RNA metabolism. Endogenous SMN was co-immunoprecipitated with KSRP from neuronal N2a cells (Fig. 2A). However, the relative abundance of KSRP and SMN

in the total cell lysate as compared with the immunoprecipitate suggests that the interaction is not stoichiometric and that only a portion of the total SMN pool is likely in complex with KSRP. This is consistent with the notion that the SMN complex interacts with a plethora of so-called 'substrates' at any one time and consequently suggests KSRP is not a core component of the SMN complex. It is worth noting that we were not able to observe the reciprocal co-immunoprecipitation, by using antibodies directed against SMN to perform the immunoprecipitation and then blotting for the presence of KSRP (data not shown). This could be explained by the relative abundance of the KSRP/SMN complexes, or alternatively could be due to epitope inaccessibility in the context of the interaction. Because SMN also interacts with hnRNP R, which like KSRP (Fig. 7A), is associated with the β-actin mRNP (Rossoll et al. 2002), it was important to determine if the interaction between SMN and KSRP was direct. Our experiments have indeed provided several evidence to support a direct, and RNA-independent interaction: (i) RNase treatment does not prevent the interaction, (ii) the Tudor domain is sufficient to mediate the interaction even though it does not harbor an RNA binding activity and (iii) the interaction can be recapitulated with KSRP produced by *in vitro* translation, using rabbit reticulocyte lysates that have been commercially pre-treated with RNases to eliminate endogenous mRNAs.

Most importantly, the interaction with KSRP was completely abrogated by naturally-occurring Tudor mutations found in human patients with severe Type I SMA (see Fig. 2D and E). This strongly suggests that the interaction with KSRP is functionally significant for the pathophysiology of the disease. A recent study has, for the first time,

uncoupled SMN function in snRNP assembly from its potential contribution to motor axons function and/or differentiation (Carrel et al. 2006). This study characterized an SMA-causing mutation in the Tudor domain (A111G), which can still support snRNP assembly (Shpargel and Matera 2005), but fails to rescue motor axon defects in SMN-deficient zebrafish. The authors speculated that this mutation might prevent the interaction of SMN with one or more arginine methylated proteins in motoneuron axons, and we now report that one such protein that fails to interact with the A111G patient allele is KSRP. Hence, in human SMA patients with missense mutations in the Tudor domain, loss of interaction with KSRP may contribute to the etiology of the disease (see below). However, it remains crucial to determine the behavior of such SMA-causing Tudor mutations and KSRP in severe SMA models where the human *Smn2* gene is present. Intriguingly, the amount of SMN co-immunoprecipated with KSRP was less when the experiment was performed in non-neuronal cells (compare Fig. 5A with Fig. 2A), which suggests the existence of tissue-specific mechanism(s) in place to regulate the KSRP/SMN interaction. Such mechanisms could potentially include post-translational modifications of KSRP, like for example arginine methylation (see below) and/or phosphorylation. Indeed, it was recently reported that KSRP becomes phosphorylated by p38 MAP kinase during skeletal muscle differentiation, and this is proposed to regulate its interaction with the 3′-UTR of specific mRNAs involved in the myogenic differentiation program (Briata et al. 2005). Whether similar events take place during neuronal differentiation remains to be determined.

The fact that the association between KSRP and SMN seems more prevalent in

neuronal cells is also consistent with the intracellular colocalization of these two proteins, which, at least in N2a cells, is restricted to a subset of granule-like foci in projecting neurites (Fig. 3). The ~23% of colocalization observed for KSRP and SMN is consistent with the efficiency of co-immunoprecipitation (Fig. 2A and B). Zhang and collaborators (Zhang et al. 2006) reported that SMN and Gemin2-3 colocalized in 40-45% of foci in cultured hippocampal neurons, while the random coincidence of SMN and synaptophysin signals was at ~15%. Hence, this suggests that the colocalization we observed for SMN and KSRP is above the threshold of random coincidence. The localization of SMN in neuritic foci has been reported previously, although the nature, precise composition and function of these foci is not fully understood (Sharma et al. 2005; Rossoll et al. 2002; Zhang et al. 2003). One of the best characterized examples is the colocalization of SMN with hnRNP R in axons, where it is thought to act in concert with this protein to mediate β-actin mRNA localization to neuronal growth cones (Rossoll et al. 2003). The chick homolog of KSRP, ZBP2, is also involved in β-actin mRNA localization (Gu et al. 2002), and KSRP was recently identified as a component of β-actin-enriched RNA granules in embryonic rat brains (Elvira et al. 2006). Interestingly, it was proposed that heterogeneity in the protein and RNA composition of mRNP transport granules may contribute to differential translation, as that involved in neuronal plasticity (Vanderklish and Edelman 2005). In support of their hypothesis, the authors of that study performed co-labeling experiments in axonal processes and showed that various RNA-binding proteins exhibit largely non-overlapping distributions in granules (Vanderklish and Edelman 2005), somewhat reminiscent of the profile we observed here for KSRP and SMN. However, the functional significance of SMN and KSRP colocalization in a subset of these stuctures in

neurites will require further studies.

Arginine methylation regulates the interaction of KSRP with the tudor domain of SMN

KSRP was among the ~200 proteins identified in our previous proteomic identification of arginine methylated protein complexes from HeLa cells (Boisvert et al. 2003), although that study did not differentiate bona fide methylated proteins from co-purifying contaminants. We have now confirmed using tandem mass spectrometry that KSRP does harbor at least 14 methylated arginines that are either mono- or dimethylated. However, it came as somewhat of a surprise that in addition to the predicted RG dipeptides, seven arginine residues were methylated within RA, RQ, RD and RI motifs. As mentioned above, CARM1 is the most likely candidate for methylation at these sites, although the implication of PRMT6 can not be ruled out since this enzyme was able to methylate both RG motifs as well as a peptide derived from HIV Tat, harboring RA, RQ and RR motifs (Boulanger et al. 2005; Frankel et al. 2002).

We recently reported that CA150, a protein that is thought to provide a molecular bridge between transcription and splicing machineries, interacted with the Tudor domain of SMN in a CARM1-dependent fashion (Cheng et al. 2007). This interaction was mediated through the N-terminal portion of CA150, which harbors several RG and PGM motifs, and it is partly based on the presence of several of these motifs in KSRP (see Fig. 1) that we predicted that this protein might also be a novel SMN interacting protein. This suggests that presence of RG/PGM motifs in a protein might constitute a strong predictor

for the identification of novel SMN interactors. In addition, it can be inferred that these interactions will likely be regulated by arginine methylation. For example, CA150 was found to be methylated by both PRMT5 and CARM1 in cells, although only CARM1 regulated its interaction with the Tudor domain of SMN (Cheng et al. 2007). Interestingly, the exact opposite observation can be made for another well-known SMN interactor, SmB; i.e. it can be methylated by both PRMT5 and CARM1, but its interaction with the Tudor domain of SMN is only regulated by PRMT5 (Cheng et al. 2007). We have shown here that the co-immunoprecipitation of SMN with KSRP was dependent on the presence of CARM1, which, following on the results recently obtained with CA150, support the notion that CARM1 may regulate a number of SMN interactions. Nevertheless, as mentioned above, our experiments can not rule out that PRMT5, and/or another PRMT, could also methylate KSRP and regulate its interaction with SMN.

Arginine methylation affects intracellular localization of KSRP: a link with neuronal differentiation?

Arginine methylation is known to influence the intracellular localization of a number of RNA-binding proteins (Cote et al. 2003; Smith et al. 2004; Nichols et al. 2000). Hence, since CARM1 regulated the interaction of KSRP with SMN, we looked for an effect of this methyltransferase on KSRP intracellular localization. Even though no effect was observed in embryonic fibroblasts derived from *Carm1* knockout mice, a gradual accumulation of KSRP in the cytoplasm was observed upon siRNA-induced knockdown of CARM1 expression in motor neuron-like MN-1 cells (see Figs. 5B and 6A and B).

The mechanism by which arginine methylation can regulate intracellular localization remains unclear in most cases. Nevertheless, it was recently proposed that arginine methylation could regulate the nucleocytoplasmic localization of RIP140, a ligand-dependent co-repressor for nuclear receptors, by regulating its interaction with Exportin 1/CRM1 (Mostaqul Huq et al. 2006). It is not known whether KSRP normally shuttles between the nucleus and cytoplasm even though its steady-state localization is clearly nuclear. In any case, this mechanism would not provide an explanation for the fact that this effect of CARM1 on localization is seen in neuronal MN-1 cells, but not in fibroblasts.

Interestingly, CARM1 was recently implicated in the regulation of neuronal differentiation: namely, it was proposed that its activity is required for the maintenance of neuronal cells in a proliferative state (Fujiwara et al. 2006). In this study, the authors provided evidence suggesting that this effect was mediated, at least in part, through regulation of HuD RNA binding properties by CARM1 methylation during NGF-stimulated PC12 cells differentiation (Fujiwara et al. 2006). Our results using MN-1 cells are consistent with these observations, since knocking-down of CARM1 expression in these motor neuron-like cells efficiently induces their entry and progress through the neuronal differentiation program (Fig. 6C, data not shown). Hence, as discussed above, it is tempting to speculate that post-translational modification of KSRP, including arginine methylation (this study), or phosphorylation, might also contribute to these regulatory pathways through regulation of KSRP interactions and intracellular localization. Moreover, in support of this hypothesis, arginine methylation and phosphorylation have

been shown to participate in coupled pathways to regulate the nuclear-cytoplasmic shuttling of certain RNA-binding proteins involved in mRNA handling, in both yeast (Yun and Fu 2000) and mammalian systems (Hsu et al. 2005).

KSRP is misregulated in the absence of SMN

We have found using an RNA interference approach that reducing the expression of either KSRP or SMN in neuronal cells, resulted in the reciprocal down-regulation of the other protein (see Fig. 8A). Consistent with this, down-regulation of KSRP was also observed in spinal cord tissues of mild SMA model mice (Fig. 8E). However, KSRP mRNA levels were equal between WT and *Smn*$^{+/-}$ spinal cord tissues (Fig. 8D), suggesting the implication of post-transcriptional mechanisms. These could include either regulation at the level of translation or alternatively, at the level of protein stability/turnover. In any case, it will be important to carefully assess the levels of KSRP mRNA and protein in severe SMA model mice and most importantly, in SMA patients. SMN has been implicated in the assembly of different RNP complexes, and it is thought to act as a type of molecular chaperone for components of these complexes. Hence, it is conceivable that SMN may similarly be required for the proper assembly and stability of an RNP complex involving KSRP. This could explain the down-regulation of KSRP when SMN levels are reduced, but implication of a similar mechanism for the reciprocal effect seems more difficult to reconcile. Indeed, the KSRP/SMN complex likely represents only a small fraction of all molecular complexes in which SMN is taking part, and in this context, loss of KSRP should not influence the stability of these other complexes. Roles for SMN or KSRP in translational regulation have not been reported

before, although their presence in neuronal RNA granules suggests that this could be a possibility worthy of further investigation.

In contrast to the effects observed by knocking-down CARM1 expression, reducing SMN levels in N2a cells resulted in differentiation defects, including shorter neurite length following treatment with dibutyryl-cAMP and low serum (Fig. 8B, H. Tadesse and J. Côté, unpublished data). A recent study reported a detailed characterization of similar defects in neuritogenesis when SMN expression was knocked-down in PC12 cells (Bowerman et al. 2007). Interestingly, we observed that down-regulation of KSRP was even more pronounced in N2a SMN knockdown cells after they were grown for 24 h under differentiation conditions (Fig. 8C). Again, these observations suggest that the KSRP/SMN interaction might constitute a key regulatory node in neuronal differentiation signaling pathways.

Aberrant mRNA stability as a novel molecular defect in SMA?

Our assessment of KSRP mRNA target levels revealed no difference for GAP-43 and β-actin mRNAs between WT and mild SMA tissues (Fig. 7B). β-actin mRNAs are transported to the tip of growing axons, where they are locally translated and play key roles in axonal growth and guidance cues (Ming 2006). This observation is consistent with previous reports showing that SMN-deficient neurons exhibit reduced accumulation of β-actin at growth cones, but without affecting overall expression levels (Rossoll et al. 2003; Bowerman et al. 2007). This defect was observed in cultured motoneurons derived from severe SMA model mice (hSMN2;$SMN^{-/-}$), but was also recapitulated in hypomorphic

SMN knockdown cell line models, suggesting that the same should be observed in the mild SMA model mice that we have used in our experiments. Aberrant accumulation of GAP-43 protein was also observed in PC12 cells where SMN had been knocked-down (Bowerman et al. 2007), although the fate of the GAP-43 mRNA was not addressed in that study. Taken together, these results suggest that KSRP could be involved in the transport of these two mRNAs rather than in controlling their stability, although further experiments will be required to demonstrate this directly.

In contrast, we observed increased levels of $p21^{cip1/waf1}$, a known target of KSRP, in spinal cord tissues from $SMN^{+/-}$ mice (see Fig. 7B). Up-regulation of p21 mRNA has been observed previously in mice with homozygous deletion of murine *Smn* exon 7 directed to neurons or skeletal muscle, which causes severe motor axonal or myofiber degeneration, respectively (Olaso et al. 2006). Although some molecular defects observed with these mice were not seen in human SMA patient samples, the up-regulation of p21 was among defects that were consistently observed for both (Olaso et al. 2006). Importantly, we report here for the first time, that this up-regulation is the result of an increased mRNA half-life, which correlates with misregulation of KSRP in the absence of SMN in mild SMA spinal cord (Fig. 7C). Based on the observation of Olaso and colleagues (Olaso et al. 2006), we predict that this should also be the case for human SMA tissues, although this will have to be determined experimentally with spinal cord tissues obtained from severe SMA mice models and human patients. p21 is a negative regulator of the cell cycle and is a key player in terminal differentiation, such as neurite outgrowth and myoblast fusion (as reviewed in (Boulaire et al. 2000). Several

studies have reported that p21 mRNA is up-regulated during neuronal differentiation, and this is known to be mediated, at least in part through post-transcriptional mechanisms, involving AU-rich elements in its 3′-UTR. KSRP can bind these AU-rich elements and regulate p21 mRNA stability during myoblast differentiation (Briata et al. 2005). Thus, it is tempting to speculate that KSRP could play a similar role, along with SMN, during motoneuron differentiation. Nevertheless, the relevance of aberrant p21 stabilization to the etiology of SMA remains to be determined, and in addition to p21, a number of transcripts have been shown to be up-regulated in human SMA patients and various mouse SMA models (Balabanian et al. 2007; Olaso et al. 2006; Anderson et al. 2004). Thus, it is conceivable that some of these mRNAs may also be aberrantly regulated at the level of mRNA stability and contribute to the pathophysiology of SMA.

SMA has not been known as a developmental disorder to this point, largely because *Smn2* can compensate for the loss of functional *Smn1* during embryonic development (Hsieh-Li et al. 2000; Monani et al. 2000), although the precise contribution of the Smn2 gene products remain poorly understood. Intriguingly, it was recently proposed that a cell-specific dysregulation of SMN2 expression could result in a pathological gain of function in SMA motoneurons (Soler-Botija et al. 2005). Moreover, the pattern of expression of SMN in the human germinative neuroepithelium led to the suggestion that it may play a role in neuronal migration and/or differentiation (Giavazzi et al. 2006). An earlier study comparing neuronal death in control and SMA fetuses and neonates led the authors to hypothesize that type I SMA could involve differential, age-dependent responses leading to cell death and motoneuron degeneration during development (Soler-

Botija et al. 2002). Finally, a 'dual dysfunction' hypothesis was recently put forth (Pellizzoni 2007), which suggests that two mechanistically and temporally distinct defects may be responsible for motoneuron degeneration in SMA. According to this view, severe SMA and early developmental defects would mainly be the result of reduced snRNP biogenesis, while milder forms of SMA may, in contrast, develop because of impaired axonal mRNP metabolism (Pellizzoni 2007). Considering that we have used mild SMA model mice tissues in this study, our findings are consistent with KSRP being involved in the etiology of milder forms of human SMA. However, since KSRP no longer interacts with naturally-occurring Tudor mutations found in severe Type I patients, we can not rule out its contribution to severe SMA, and further studies will be required to clarify this issue. Major challenges for the future will lie in the identification of the precise molecular mechanism underlying the pathogenesis of SMA, in the midst of a constantly growing list of functional SMN interactions.

MATERIALS AND METHODS

Cell culture

N2a neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with sodium pyruvate, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 10% fetal calf serum (Wisent, St-Bruno, QC, Canada). Neurite outgrowth was promoted by changing the cells to DMEM medium plus 0.25% serum, and the addition of 5 mM dibutyryl-cAMP (Sigma, St. Louis, MO, USA) for 24-48 h. WT and *Carm1*$^{-/-}$ mouse embryonic fibroblasts were a kind gift of Dr. Mark T. Bedford and were maintained in DMEM medium supplemented with sodium pyruvate, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 10% fetal calf serum (Wisent, St-Bruno, QC, Canada). MN-1 cells were a kind gift of Matthew Butchbach and Arthur Burghes (Ohio State University) and were maintained in DMEM (#11960; Invitrogen, Burlington, ON, Canada), supplemented with 20% heat-inactivated fetal bovine serum (Wisent, St-Bruno, QC, Canada), 2× penicillin-streptomycin (#15140; Invitrogen, Burlington, ON, Canada) and 4 mM glutamine (#25030; Invitrogen, Burlington, ON, Canada). Differentiation of MN-1 was induced by the addition of *trans*-retinol (#R7632; Sigma, resuspended in DMSO to 50 mM) to a final concentration of 50 μM for 24-72 h. All cells were grown at 37 °C and 5% CO_2 in a humidified incubation chamber.

Antibodies

Murine hybridomas producing a mouse monoclonal antibody to KSRP (Ab5) were kindly provided by Dr. Douglas Black (UCLA) and described elsewhere (Hall et al. 2004).

Monoclonal antibodies were purified over a Protein A Sepharose column (Sigma, Saint Louis, MO) following manufacturer recommendations and used at a dilution of 1:1000. The anti-SMN mAb (BD Transduction Laboratories, Palo Alto, CA, USA) and anti-β-actin mAb (Sigma, Saint Louis, MO) were used at a dilution of 1:1500. Anti-Sam68 (Upstate Biotechnology, Upstate, NY) was used at 1:2000. SYM10 and SYM11 antibodies were described elsewhere (Boisvert et al. 2002; Boisvert et al. 2003) and were used at 1:750 and 1:1000, respectively. Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (MP Biomedicals, Solon, OH, USA) were used at a dilution of 1:1000.

***In vivo* methylation Assays**

In vivo methylation was conducted essentially as described previously. Briefly, N2a cells were grown for 3 hrs in methionine-free DMEM (Wisent, St-Bruno, QC, Canada) containing 10% (v/v) dialyzed fetal calf serum and 10 μCi/ml of L-[methyl-^{3}H]-methionine (85 Ci/mmol; Perkin, Elmer, Waltham, MA, USA) in the presence of translation inhibitors (100 μg/ml cycloheximide and 40 μg/ml chloramphenicol). In parallel, labeling was performed with *L*-[^{35}S]-methionine (175 Ci/mmol; Perkin Elmer, Waltham, MA, USA) under the same conditions to control for efficient inhibition of translation. Cells were harvested in RIPA buffer and used for immunoprecipitation (as described below). Following SDS-PAGE, gels were stained in Coomassie Brilliant Blue R-250 for 20-30 min, destained in a 10% methanol (v/v), 5% acetic acid (v/v) destain solution to visualize protein bands, and then soaked in EN^{3}HANCE (PerkinElmer Life Sciences) according to the manufacturer's instructions. Gels were dried *in vacuo*, and

radioactivity was visualized by fluorography.

Immunoprecipitation and immunoblotting

For immunoprecipitations, cells were lysed in RIPA buffer [1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, Complete (Roche, Laval, QC, Canada) protease inhibitors] and incubated on ice with the primary antibody for 1 h with occasional gentle agitation. Then 20 µl of a 50% protein A-Sepharose 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. RNase treatment was performed at 37°C for 1 h with a final concentration of 1 mg/ml of RNase A, as described previously (Chen et al. 1997). Incubation of the cell lysate at 37°C without RNase was taken as mock treatment. Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes for analysis by immunoblotting with the respective antibody. For immunoblotting, cells were lysed in RIPA buffer [1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, Complete (Roche) protease inhibitors]. Cellular debris were removed by centrifugation and the supernatants transferred to clean tubes. Protein concentration was measured spectrophotometrically using the DC Protein Assay reagent (Bio-Rad) and loaded onto a 10% SDS- PAGE. Proteins were transferred to nitrocellulose, blocked with 4% dry milk in 1× TBST and probed with the appropriate primary antibody for 1 h at room temperature (RT) or overnight at 4°C. This was followed by probing with secondary antibody under the same conditions. Where indicated, fluorescence-coupled secondary antibodies were used and the corresponding immunoblots analyzed using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NB, USA), following

manufacturer recommended procedures.

Mass spectrometry

Mass spectrometry was performed as a service by Rulin Zhang (WEMB Biochem Inc., Toronto, ON, Canada). Specifically, KSRP was immunoprecipitated as described above from five 150 mm plates of N2a cells grown to ~90% confluency, the beads were washed extensively with RIPA buffer and 5× with PBS. The immunoprecipitate was then digested with chymotrypsin and GluC overnight at 30°C in 20 mM tris buffer, pH 8.1. After digestion, the sample was cleaned up with C18 ZipTip. The resulting peptide mixture was resolved by reverse-phase chromatography and the peptides fragmentation data collected using a LCQ Deca XP (ThermoFinnigan, San Jose, CA, USA) system with a 90 min gradient of 0.1% acetic acid (buffer A) and 100% acetonitrile (Buffer B). The raw data was processed with Bioworks software version 3.1 and searched against NCBI mouse database.

GST pull-down assays

GST-Tdr and GST-TdrE134K were described elsewhere (Cote and Richard 2005). GST-TdrQ136E, GST-TdrI116F and GST-TdrA111G were generated using overlap extension mutagenesis PCR (Ho et al. 1989) with the following mutagenic oligonucleotides (5'-tagagaggaggaaaatctgtccg-3') and (5'-cggacagattttcctcctctcta-3') for Q136E, (5'-cattgcttcatttgattttaagag-3') and (5'-ctcttaaaatcaaatgaagcaatg-3') for I116F, and (5'-atttacccaggtaccattgcttc-3') and (5'-gaagcaatggtacctgggtaaat-3') for A111G, while T7 (863-882) and BGHrev (1022-1039) were used as flanking primers. myc-SMN-pcDNA

(Boisvert et al. 2002) was used as template for each mutagenic reaction. All resulting PCR fragments were first substituted back into myc-pcDNA$_{3.1}$ (Invitrogen) at the *Eco*R1 site and then the TdrR1fwd (5'-ccggaattcgcagtggaaagttggggacaaa-3') and TdrR1rev (5'-ccggaattcttaattagctacttcacagattgg-3') primers, were used to amplify the portion of the cDNA encoding the Tudor domain. This fragment was in turn inserted into pGEX-4T2 (Acc. No. U13854; GE Healthcare, Piscataway, NJ, USA) at the EcoR1 site. To generate GST-SMN and GST-SMNE134K, the WT and mutant cDNA was isolated from the corresponding myc-SMN-pcDNA construct and subcloned into pGEX-4T2 using *Bam*H1 and *Xho*I sites. All DNA constructs were confirmed by automated DNA sequencing. GST-fusion proteins were overexpressed in *Escherichia coli* codon plus DE-3 (RP) competent cells (Invitrogen, Burlington, ON, Canada) by induction with a final concentration of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Following induction, cells were spun down, resuspended in 10 ml of 1× PBS in the presence of Complete^{TM} (Roche, Laval, QC, Canada) protease inhibitor cocktail and subsequently broken down by sonication (5 pulses of 15 s). Cell debris were discarded through centrifugation for 20 min at 18,000*g*. GST fusion proteins were then purified using glutathione-agarose beads (Sigma, St-Louis, MO, USA). For GST pull-down experiments, fusion proteins were kept on glutathione-agarose as a 50% slurry in 1× PBS. Cell lysates were prepared using lysis buffer (1% Triton X-100, 20 mM Tris pH 7.4 and 150 mM NaCl) or RIPA buffer, as indicated in Figure legends. Twenty microliters of the respective GST-fusion slurry was added and the mixture incubated at 4°C for 30 min with constant end-over-end mixing. The beads were washed twice with lysis buffer and once with 1× PBS. Protein samples were resolved by SDS-PAGE and transferred to

nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) for immunoblotting. Alternatively, a full-length KSRP cDNA (a generous gift from Doug Black) was inserted in the pET-28c vector (Novagen, San Diego, CA, USA). KSRP was then produced from this plasmid using *in vitro* coupled transcription/translation lysates (T7 coupled TnT kits; Promega, Madison, WI, USA) following manufacturer protocols, in the presence of *L*-[^{35}S]-methionine (175 Ci/mmol; Perkin Elmer, Waltham, MA, USA). This programmed lysate was then used as described above for GST pull-down experiments, except that following SDS-PAGE, gels were dried and revealed by autoradiography.

Immunofluorescence and phase contrast microscopy

When prepared for indirect immunofluorescence microscopy, N2a cells and MEFs were cultured directly onto glass coverslips, while MN-1 cells were plated on coverslips that had been pre-coated overnight with poly-D-lysine (#P7280; Sigma, St-Louis, MO, USA) at 100 µg/ml and mouse laminin (#L2020; Sigma, St-Louis, MO, USA) at 2 mg/ml. Cells were fixed with 4% paraformaldehyde in 1× PBS for 15 min at RT and permeabilized with 0.5% Triton X-100 in PBS for 5 min at RT. The cells were incubated with primary antibodies (1:200 for anti-SMN and 1:1 for KSRP hybridoma supernatant) in PBS at RT for 1 h. The cells were washed with 0.1% Triton X-100 in PBS and incubated with the appropriate secondary antibodies (1:600) in PBS, in the dark, at RT for 1 h. Goat anti-mouse coupled to Alexa 488 and goat anti-rabbit coupled to Alexa 594 (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. The cells were washed again with 0.1% Triton X-100 in PBS, counterstained with DAPI, mounted onto glass

slides, and visualized with a Z.1 AxioImager upright microscope (Carl Zeiss Canada). Images were captured through either EC PLAN NEOFLUAR 40X/0.75 M27 or PLAN APO 63X/1.4 OIL M27 objectives and an AXIOCAM HRM R 2.0 CCD camera. For Figures 3A and 6B, pseudo-confocal images were obtained using an APOTOME module. Phase contrast images were obtained on an inverted Axiovert 40CFL microscope (Carl Zeiss Canada). For quantification of colocalization, foci were counted for 20 neurites chosen randomly, and the ratio of foci where both fluorescence signals coincided was expressed as percentage ± SEM for each fluorophore. Colocalization in the *z*-plane was confirmed by acquiring *z*-stacks using the APOTOME module for each neurite used for quantification.

RNA interference

For the establishment of stable N2a cell lines with reduced SMN expression, cells were transfected with sh_Smn_519 which was ligated in the *Bbs*I sites of the psiRNA-hHneo vector (InvivoGen, Burlington, ON, Canada), obtained from Rashmi Kothary (Ottawa Health Research Institute). The oligonucleotide sequence for sh_Smn_519 has been reported elsewhere (Shafey et al. 2005). Lipofectamine transfected cells were selected with 1 mg/ml G418 (Invitrogen, Burlington, ON, Canada) and obtained colonies were cloned using standard procedures. Clones were routinely maintained in normal 10% fetal calf serum media containing 0.6 mg/ml G418. For transient knockdown of KSRP and CARM1, ON-TARGETplus RNA duplexes J-054914-05 (5'-gaauacggaucucgaguuguu-3') and J-048766-05 (5'-gcuacaugcucuucaaugauu-3'), respectively, were ordered from (Dharmacon, Lafayette, CO, USA). Transfection was then performed with 50 or 100

pmol of each RNA duplexes using lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) according to manufacturer's instructions.

Animal tissues

The *Smn*$^{+/-}$ mouse line (Jablonka et al. 2000) was initially obtained from Dr. Michael Sendtner (University of Würzburg, Germany) and was maintained on a C57Bl/6 background at the University of Ottawa Animal Care and Veterinary Services Facility, in accordance with the Institutional Animal Care and Use Committee guidelines. Spinal cords were dissected from 3-6 month-old mice, essentially as described previously (Balabanian et al. 2007). Briefly, *Smn*$^{+/+}$ and *Smn*$^{+/-}$ littermates were euthanized by intraperitonal sodium pentobarbital injection and the cranium was separated from the spine above the first cervical region using a scalpel. The vertebral column was partially liberated through parallel longitudinal incisions on each side and transected at the most distal end. A blunt 21-gauge needle attached to a 10 ml syringe was then used to eject the spinal cord from the vertebral canal, by applying pressure with 1× PBS. In parallel, total brain was pulled out from the severed heads with tweezers, cutting through the cranium bone with dissection scissors. Tissues were washed thoroughly in 1× PBS, flash frozen and grinded to powder in liquid nitrogen and homogenized using a Model PRO 200 homogenizer (PRO Scientific Inc., Monroe, CT, USA). Homogenization was either directly in TriZol for RNA extraction, or in 1× PBS supplemented with a cocktail of protease inhibitors for subsequent protein extraction as described for Immunoblottting.

Semi-quantitative RT-PCR

Total RNA was extracted from N2A cells and WT and *Smn*$^{+/-}$ mice tissues using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to manufacturer's instructions. RNA concentration was measured with a spectrophotometer, and RNA quality was determined by agarose gel electrophoresis. First strand cDNA synthesis was performed using 5 μg total RNA and the AMV Reverse Transcriptase (Promega, Madison, WI, USA) with an oligo-dT (18) primer. PCR were performed in a 25 μl reaction mixture containing 0.5 μl of cDNA using *Taq* DNA polymerase (Qiagen, Mississauga, ON, Canada). cDNAs were amplified using specific forward and reverse primers for CARM1 (5'-agctgtgcagtacttccagttcta-3'; 5'-attgttactcttcaccaggacctc-3'), GAPDH (5'-accacagtccat gccatcac-3'; 5'-tccaccaccctgttgctgta-3'), S12 (5'-ggaaggcatagctgctgg-3'; 5'-cctcgatgacat ccttgg-3'), GAP43 (5'-aacattgtttcttggtgttatg-3'; 5'-tattaatattttggactcctcagaacg-3'), p21 (5'-tcactctgtgtgtcttaatta-3'; 5'-aggactgttcctccggtatagg-3'), β-actin (5'-tgacagactacctcatgaa gatcc-3'; 5'-agtaatctccttctgcatcctgtc-3'), and AChE (5'-atagcaagcaggagcgctgct-3'; 5'-ggt ggctgtaacagtttattgg-3'). PCR cycling parameters consisted of an initial activation step at 95°C for 3 min followed by denaturation at 94°C for 1 min; annealing was for 30 s at a temperature determined empirically for each primer pair using gradient PCR; and extension at 72 °C for 1 min followed by a 7 min elongation step at 72°C. Cycle number was also determined empirically for each primer pair in order to stay within the linear range of amplification as described previously (Deschenes-Furry et al. 2005b). PCR products were electrophoresed on 2.0% agarose gels and visualized by ethidium bromide staining. Results were captured on a Kodak Gel Logic 200 and quantified using the accompanying Kodak 1D analysis software. Obtained values for each mRNA were

normalized to corresponding S12 values, averaged from at least three independent experiments (with tissue preparations from different animals) and then expressed as percent of WT ± SEM in the bar graph. One-way analysis of variance was performed to evaluate the difference in p21 mRNA levels in WT and *Smn*$^{+/-}$ brain and spinal cord tissues. Unpaired *t*-tests were performed to determine whether the differences seen within tissues were significant, with a threshold set at $P < 0.05$. For immunoprecipitation/RT-PCR experiments, cell extracts (equalized for total protein concentration) were incubated overnight at 4°C with beads and affinity-purified anti-KSRP or mouse IgG control antibody. The beads were washed four times with IPP-50 buffer (25 mM Tris·HCl; 50 mM KCl; 2 mM MgCl2; 1 mM DTT; 0.01% Triton X-100). RNA extraction and cDNA synthesis was then performed as above.

***In vitro* mRNA stability assay**

Total RNA was prepared from mouse brain using TriZol reagent (Invitrogen, Burlington, ON, Canada) according to manufacturer's instructions. Cytoplasmic protein fractions were prepared from WT and *Smn*$^{+/-}$ total brain and spinal cord tissues essentially as described previously (Deschenes-Furry et al. 2005b). Briefly, tissues were homogenized in MOPS buffer (10 mM MOPS•NaOH, pH 7.2, 200 mM NaCl, 2.5 mM magnesium acetate) with 100 μM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, and 1 Complete Mini, EDTA-free Protease Inhibitor Cocktail tablet (Roche Applied Science, Laval, QC, Canada). Homogenates were centrifuged (10 000*g* for 15 min at 4°C), and the resulting supernatants were stored at -80°C until further use. After optimization of protein and RNA concentrations, 2.5 μg of total brain RNA was incubated with 10 μg of protein

extracts in a decay buffer (modified from Chen et al. 2001) containing 100 mM KCl, 2 mM magnesium acetate, 10 mM Tris•Cl pH 7.6, 2 mM dithiothreitol, 10 mM creatine phosphate, 2 U/µl creatine phosphokinase, 1 mM ATP, 0.4 mM GTP, 0.1 mM spermine, 2 mM dithiothreitol, and 0.15 U/µl RNAguard (GE Healthcare Life Sciences, Piscataway, NJ, USA) in a final volume of 25 µl and incubated at 37°C. At intervals of 0, 5, 10, 15, 30 and 60 min, aliquots were removed, and the reaction stopped by adding 100 µl of stop buffer containing 400 mM NaCl, 25 mM Tris•Cl pH 7.6 and 0.1% SDS. RNAs were then extracted using TriZol reagent and precipitated with ethanol in the presence of glycogen (20 µg) as a carrier. p21 and AChE mRNAs were then detected by semi-quantitative RT-PCR as described above. Products were quantified, normalized to S12 and averaged (n = 3) as above, and then plotted as percent of time 0 $\pm$ SEM versus time. An exponential regression was obtained for each set of data and used to derive half-lives (time at which mRNA levels would reach 50% of input levels). Paired t-tests were performed to determine the significance with a threshold value set at $P < 0.05$.

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Conflict of Interest statement. None declared.

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3. General Discussion

3.1 Summary

We have shown here for the first time, that KSRP is an arginine methylated protein. We identified a novel interaction between KSRP and the Survival of Motor Neuron (SMN) protein, and more specifically, its Tudor domain (Tadesse et al. 2008). We also demonstrated that SMN and KSRP colocalize in granule-like foci of axonal processes of differentiating neuroblastoma N2a cells. We also showed that this interaction between KSRP and the SMN Tudor domain, was abrogated by Tudor domain mutations found in severe Type I SMA: E134K, I116F, and Q136E. Methylation by protein arginine methyltransferase CARM1 is required for this interaction, as well as the normal localization of KSRP in neuronal cells. KSRP protein expression was also decreased in the absence of SMN, both in our SMN knockdown cell line and in SMA tissues. This decrease in KSRP, an mRNA decay-promoting factor, resulted in the stabilization of its mRNA target $p21^{Cip1/WAF1}$, in SMA tissues (Tadesse et al. 2008). These results, discussed in detail in the manuscript above, provide key functional roles for SMN and its arginine methylated interactor that we identified here, KSRP, in SMA. We highlight below, some major findings since our work, that could provide further critical insights into the functional roles of KSRP and SMN in the pathophysiology of SMA.

3.2 HuD, an arginine methylation-dependent SMN interacting partner

Following our work with KSRP described above, our lab sought to identify other similar candidates that may exist in a complex with SMN, and may be involved in neuronal pathways related to SMA. In this vein, HuD, another RNA binding neuronal-specific protein, was identified as a potential target to pursue. HuD, similarly to KSRP, regulates mRNA stability through specific interactions with ARE-containing 3'UTRs. Mammalian *Drosophila* homologs of embryonic lethal abnormal vision (ELAV), Hu proteins, are involved in nearly every aspect of post-transcriptional regulation (Hinman and Lou 2008). HuD is a neuronal-specific family member that promotes differentiation and neuronal function of many types of neurons, including motor neurons (Akamatsu et al. 2005; Deschenes-Furry et al. 2006). Fujiwara et al. had just demonstrated that methyl-

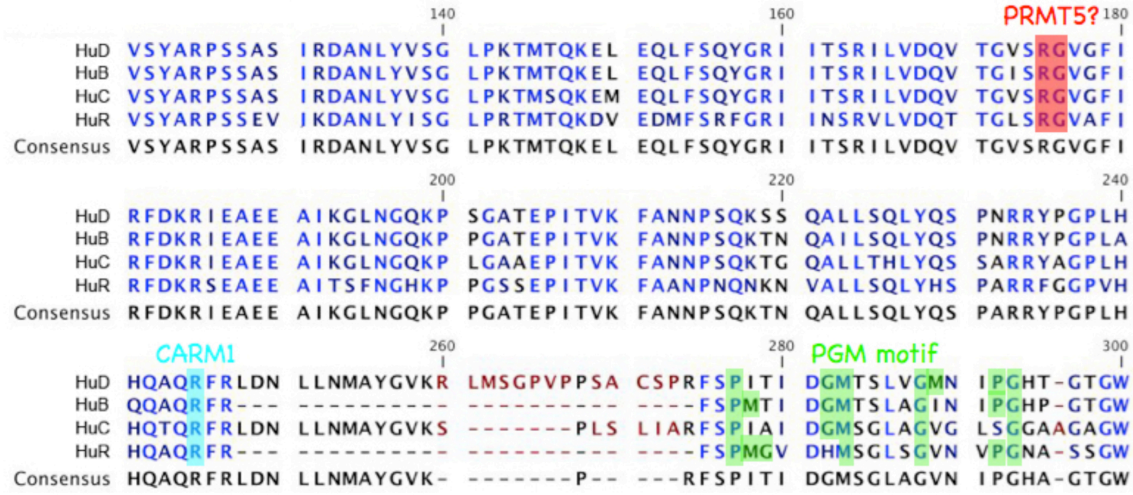

Figure 1. Arginine Methylation of the Hu Family of Proteins. Arginine Methyl Transferase CARM1 (PRMT4) produces asymmetrically dimethylated arginine residues (*blue shading*) flanked by P-G-M motifs (*green shading*) like those found in the Hu family of proteins [Hu family degree of conservation is illustrated using a foreground color scale from blue (100%) to red (0%)]. Similar to KSRP (see Chapter 2 – Figure 1), HuD also contains P-G-M, and potential PRMT5 methylated RG motifs (*red shading*).

ation of HuD by CARM1 maintained neuronal PC12 cells in proliferative state by promoting p21 mRNA decay, through negative regulation of HuD RNA binding properties (Fujiwara et al. 2006). Moreover, in our own work in motor neuron-like (MN-1) cells, knocking-down CARM1 induced neuronal differentiation (see Chapter 2. Manuscript - Figure 6C). p21 is a negative cell cycle regulator, essential to terminal differentiation (Boulaire et al. 2000). Therefore, upon CARM1 decrease, and thus decrease in its methylation of HuD, HuD can further bind and stabilize p21 mRNA, increasing its levels. This led us to speculate whether CARM1 may regulate p21 levels in SMA, through a similar pathway to that of KSRP (**Figure 2**).

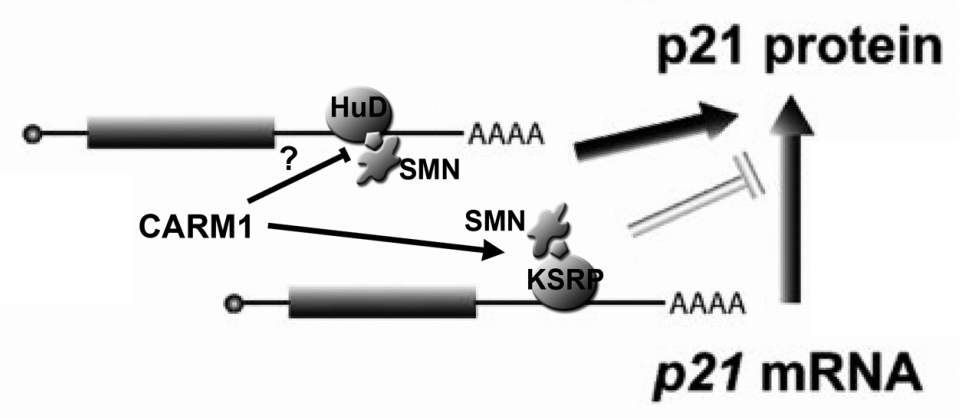

Figure 2. Role of CARM1 methylation in p21 regulation. Putative model mechanisms by which p21 mRNA could be upregulated in SMA. Methylation of KSRP by CARM1 leads to its interaction with SMN. However, in the absence of SMN, destabilizing ARE-binding protein KSRP is misregulated, as is, its regulation of p21 mRNA levels through the mRNA decay pathway; resulting in the increase of p21 mRNA and protein levels (Tadesse et al. 2008). CARM1 methylation of HuD negatively regulates its direct interaction with p21 mRNA (Fujiwara et al. 2006), with what involvement from SMN?

Members of our lab have now demonstrated HuD as being another arginine methylated interactor of SMN (**Figure 1**) (Hubers et al. 2011). This interaction has since been

confirmed by other groups, that have also shown HuD as a factor responsible for the axonal localization of poly(A) mRNA in primary motor neurons (Fallini et al. 2011), and in a complex with SMN that interacts with candidate plasticity-related gene 15 (cpg15)/neuritin mRNA, in spinal motor axons (Akten et al. 2011). KSRP and HuD are methylated by CARM1 on similar proline glycine and methionine (PGM) motifs (**Figure 1**), seemingly enhancing their binding to the Tudor domain (Tadesse et al. 2008; Hubers et al. 2011). This interaction with HuD was also lost upon Type I SMA mutations on the Tudor domain (Hubers et al. 2011). HuD and KSRP are thus analogous in many facets, and known regulators of mRNA stability, with similar mRNA targets, like $p21^{Cip1/WAF1}$. As for KSRP, SMN interaction with HuD was found to be essential for the latter's recruitment into these dense neuronal RNA granules, along with its mRNA targets. HuD overexpression led to p21 mRNA upregulation and strongly induced neuronal differentiation of MN-1 cells, even rescuing the SMA-like motor neuron defects normally seen in the MN-1 *Smn* hypomorph line (Hubers et al. 2011).

Due to the effects of HuD on MN-1 differentiation noted above, we also sought to characterize KSRP upon N2a differentiation (see **Appendix I**). So far, we have observed the levels of KSRP to be highest at peak differentiation times for the N2a cells (~24 hours). This also correlated with the methylation profile of symmetrically dimethylated arginine (sDMA) proteins and the arginine methyltransferase, PRMT7. Unlike SMN, we found KSRP and arginine methylation to be dynamic during N2a differentiation. It would be of interest to see whether CARM1, which we see as differentially expressed during the differentiation of MN-1 cells, is also affected during the differentiation of N2a cells.

3.3 KSRP Protein: Functional Updates

To date, the multi-functional KSRP protein has been found to be implicated in various biological functions including, most recently, in the regulation of viral translation, through an interaction with the internal ribosomal entry site (IRES) of the Enterovirus 71 (as reviewed in (Gherzi et al. 2010).

KSRP's main known function still remains as an ARE-BP, involved in regulating mRNA stability via recruitment of the decay machinery, and leading to mRNA decay. In the past, serum withdrawal at early phases of myoblast differentiation was shown to induce activation of the p38 MAPK pathway, and phosphorylation of KSRP at Threonine 692 (Briata et al. 2005). KSRP's ability to interact with ARE-containing myogenic transcripts, including p21 and myogenin is impaired by this phosphorylation (Briata et al. 2005). Briata et al. have now brilliantly demonstrated that a dynamic switch between separate KSRP functions is also determined by AKT/PI3K signaling (Briata et al. 2012). Two distinct KSRP functions: promoting mRNA decay and its newly identified function - favoring miRNA maturation - are in fact regulated by AKT/PI3K, in opposite ways (Briata et al. 2012).

Indeed, KSRP has been shown to bind the terminal loop (TL) of miRNA precursors, and interact with both Drosha and Dicer, as a co-regulator of maturation of a group of miRNAs (Trabucchi et al. 2009; Ruggiero et al. 2009). Primary miRNAs (pri-miRNAs) are first processed to these precursor miRNAs (pre-miRNAs), and later, to mature miRNAs. This miRNA maturation mechanism is comprised of two controlled endonucleolytic cleavages carried out by multiprotein complexes, that include the two

RNAse III enzymes Drosha and Dicer, in that order (Filipowicz et al. 2008). A role for KSRP as a co-activator in the regulation of pri-let-7a maturation into pre-let-7a, (Trabucchi et al. 2009) - antagonizing that of co-repressors hnRNPA1 (Michlewski and Caceres 2010) and Lin-28 (Briata et al. 2011) - has been proposed. In light of these works now demonstrating KSRP's involvement in microRNA precursor processing, it is worth examining whether any novel data on microRNAs involved in motor neuron degenerative diseases, and even more specifically SMA, could in fact be related back to KSRP's recently recognized function.

In 2010, Haramati et al. demonstrated *in vivo* that miRNA activity was essential for the long-term survival of postmitotic spinal motor neurons (Haramati et al. 2010). Mice that couldn't process miRNAs in their spinal motor neurons showed highly SMA-like features: "including sclerosis of the spinal cord ventral horns, aberrant end plate architecture, and myofiber atrophy with signs of denervation (Haramati et al. 2010)." These spinal motor neurons with defects in miRNA processing also showed a specific upregulation of the neurofilament heavy subunit (NEFH), a protein previously implicated in motor neuron degeneration (Haramati et al. 2010). NEFH is a target of miR-9, an miRNA they found to be specifically downregulated in their $SMN1^{mut}$ models (Haramati et al. 2010). miR-9 is an essential regulator of motor neuron specification and columnar formation (Otaegi et al. 2011). Yet another study has now revealed selective loss of LMC (Lateral Motor Column) and PGC (Preganglionic Column) neurons upon dicer disruption (Chen and Wichterle 2012). This suggests a more complex role of dicer function in the specification and eventual survival of motor neurons; and not just simply through regulation of miR-9 microRNA (Chen and Wichterle 2012). A strong possibility that

such a crucial role of the miRNA processing pathway in motor neuron survival and specification, could also well encompass KSRP's new function in miRNA processing.

In other work showing KSRP's involvement in microRNAs biogenesis, Trabucchi et al. noted that upon KSRP decrease, expression of the microRNA miR196a, was also reduced by more than 1.5-fold (Trabucchi et al. 2009). miR196a is responsible for the silencing of CUGBP, Elav-like familin member 2 (CELF2). CELF2 acts and stabilizes AR (Andogen Receptor) mRNA. Expansion of the polyglutamine tract (polyQ) of the androgen receptor (AR-polyQ) is what causes Spinal and Bulbar Muscular Atrophy (SBMA), whose hallmarks include: "proximal muscular atrophy, weakness, contraction fasciculation and bulbar involvement (Miyazaki et al. 2012)." Miyazaki et al. showed that viral delivery of miR-196a improved the SBMA phenotype, by silencing CELF2 and thus AR mRNA. SBMA is a neurodegenerative disorder at times misdiagnosed as ALS, but with causes distant from that of SMA, that has been referred to in the past as X-linked SMA (Miyazaki et al. 2012). Nonetheless, these works highlight the importance of the miRNA processing pathway, including KSRP, in neuromuscular disorders involving the degeneration of motor neurons.

KSRP is a protein that has multiple functions and multiple sites of activity, from the nucleus to the synapse, and interacting with different proteins and divergent RNA sequences. These add to the challenge of defining KSRP-containing neuronal RNP complexes, and identifying RNA targets within these. Therefore, although its myogenic role in muscle cell proliferation and differentiation is well-defined; more work is surely needed to also further uncover its functions in the neuronal system.

3.4 SMN, axonal function updates

Ever since studies showing the presence of SMN in the dendritic ends and axonal branch points of cultured primary neurons (Jablonka et al. 2000), and a predominant axonal localization during sprouting and axonogenesis of human spinal cord (Giavazzi et al. 2006), the idea of an axon-specific SMN function has been widely accepted. SMN associates with a vast array of proteins, including apoptotic regulators, growth factors, and viral / cellular transcription factors (as reviewed in Fallini et al. 2011). Axonal localization however, enables its association with two distinctive families of proteins: (i) *RBPs*, that regulate the stability, transport, and local translation of mRNAs in neurons, and (ii) *cytoskeletal dynamics associated proteins.*

Although no novel RBP, such as KSRP and those already mentioned above and in **Table I** below (Coady and Lorson 2011) have been identified since as being associated with SMN, functional axonal data for the existing ones has been forthcoming. In earlier works, SMN knockdown in zebrafish had shown promising specific motor neuron pathfinding and presynaptic neuromuscular junction (NMJ) defects (Carrel et al. 2006). Although β-actin mRNA and protein was considerably lower in motor neurons cultured from SMA mouse models versus wildtype mice (Glinka et al. 2010), regrettably, most of the zebrafish defects did not carry over to mouse models, whose majority of NMJs seem to have a normal, though delayed, development (**Figure 3**) (Murray et al. 2010; also reviewed in Liu-Yesucevitz et al. 2011; Fallini et al. 2011; Coady and Lorson 2011). For the time being, axonal outgrowth and synaptogenesis defects stemming from SMN's axonal function, as it relates to RBPs, is being deemed as an improbable primary developmental defect in SMA *in vivo*; slightly shifting the focus to *cytoskeletal dynamic*

TABLE 1 | Previously Identified Survival Motor Neuron (SMN) Interacting Proteins Are Shown

SMN Interacting Factor	Function	Direct/Indirect	Motifs
RNPs/RNA metabolism			
hnRNP-Q	RNA metabolism	Direct	RRM, RGG
hnRNP-R	RNA metabolism	Direct	RRM, RGG
hnRNP-U	RNA metabolism	Direct	SAP, SPRY, RGG
FMRP	RNA metabolism	Direct	KH
KSRP/FBP2/ZBP2/MARTA1	RNA metabolism	Direct	KH, RGG
U1A	Pre-mRNA splicing	ND	RRM
Galectin 1 and 3/LGALS1	Pre-mRNA splicing	Indirect	GLECT
RNA helicase A	Transcription	Direct	DSRM, DEXD, HELIC, RGG
RNA polymerase II	Transcription	Direct	–
Rpp20	tRNA/rRNA metabolism	Direct	–
Nucleolin	rRNA metabolism	Indirect	RRM, RGG box
ISG20	ssRNA degradation	ND	EXOIII
TIAR	Stress granules		RRM
TDP43	Pre-mRNA splicing/transcription	ND	RRM
NFAR1/2	RNA metabolism	Direct	DZF, DSRM

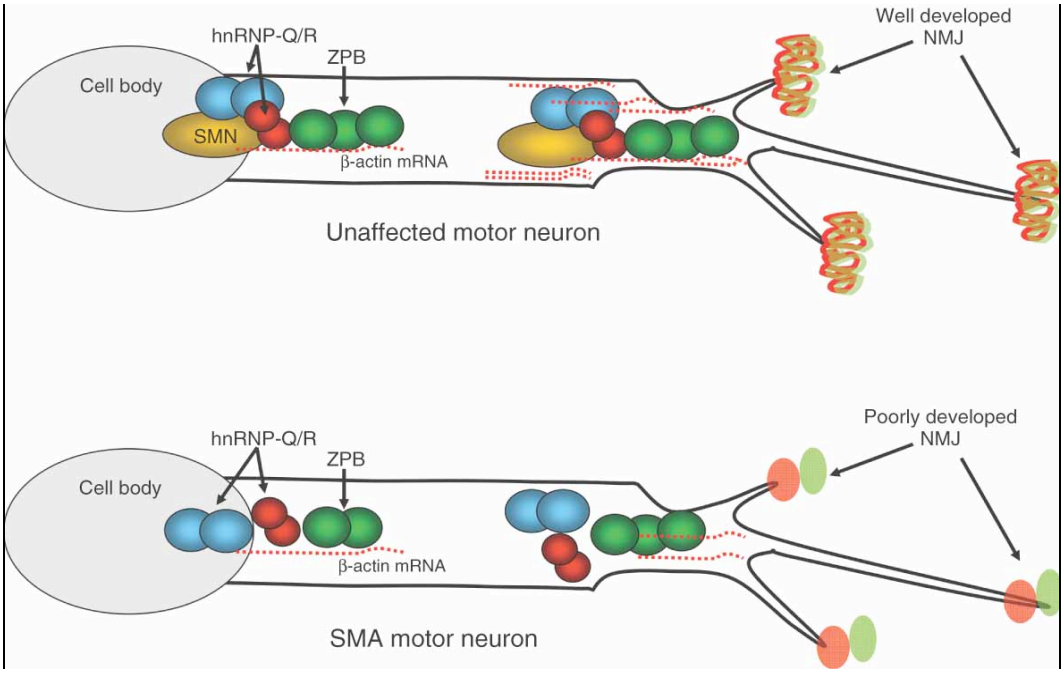

Figure 3. Proposed role for SMN in a novel neuronal RNP complex. Axonal SMN-RNP complexes are composed of axonal pools of SMN that are uncomplexed with Sm

proteins and the entire Gemin complex. SMN-RNP forms that contain hnRNP-Q/R (*red/blue*), ZBP (*green*) and β-actin mRNA have been identified (as reviewed in (Coady and Lorson 2011). In unaffected motor neurons (top panel), these complexes are actively transported to the distal ends of the axon, resulting in the accumulation of β-actin mRNA, and well-developed NMJs. Whereas in SMA (bottom panel), low SMN levels result in reduced levels of β-actin mRNA accumulation and poorly developed NMJs (modified from (Coady and Lorson 2011).

associated proteins. Further evidence of this is the much hailed recent identification and characterization of SMA modifying gene, Plastin 3/T-Plastin (PLS3), which participates in actin dynamics, and stabilizes filamentous actin (Oprea et al. 2008). Transient overexpression of PLS3 was enough to rescue axon length and outgrowth defects associated with SMN down-regulation in embryonic SMA mice motor neurons and zebrafish (Oprea et al. 2008). A Rho-kinase (ROCK) inhibitor has also been shown to correct NMJ defects and extend survival (Bowerman et al. 2009, 2010), in further show of the importance of actin dynamics, in SMA; proving that SMN axonal function associated with *RBPs* and/or *cytoskeletal dynamics associated proteins* is vital to motor neurons.

3.5 Conclusion

We identified an arginine methylated RNA binding protein, KSRP, which interacts with SMN in a methyl-dependent fashion. We also demonstrated the misregulation of this protein in SMA conditions, in the absence of low functional levels of SMN. We have proven that SMN indeed has a functional role, along with its interactors, in RNP complexes; and that the presence of arginine methylation is a key factor. These results above support our stated hypothesis and fulfill the objectives outlined for this research project. We hope this data adds to further the understanding into SMA pathophysiology.

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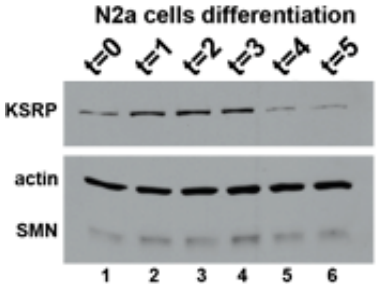
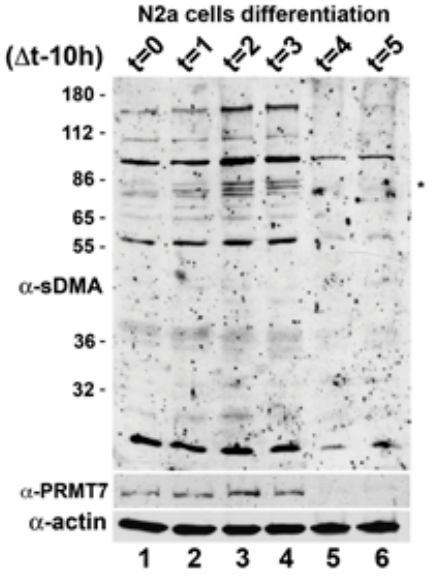
APPENDIX I

(a)



(b)



(a) KSRP, SMN and (b) symmetrically dimethylated arginine proteins (sDMA), PRMT 7, levels upon N2a differentiation. KSRP and arginine methylation are dynamic during N2a differentiation (t=10h).