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

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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 CARM1 methyltransferase 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, p21cip1/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 among the leading genetic causes of infant deaths (1). Typical clinical characteristics are symmetrical muscular weakness, ultimately leading to muscle atrophy, paralysis and death from respiratory distress within ~2 years of age (2). 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 (3). SMA is caused by disruption of the Smn1 gene (4) and the level of functional SMN protein in patients correlates with severity of the disease (3,6). 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 (7,8). 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 9).

SMN exists in the cell as part of a stable macromolecular complex consisting of at least eight tightly associated components that include Gemin2–8 and unrip (reviewed in 10). 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 (10–12). Although not much is known about the role of individual Gemin proteins, their presence is required for the integrity of the SMN complex and its function in snRNP assembly (reviewed in 10). After additional cytoplasmic remodeling steps, the mature snRNPs, along with the SMN complex, are imported into the nucleolus, where they transit into discrete nuclear foci termed Cajal bodies for further maturation before ultimately functioning in splicing (reviewed in 13). 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 Gemin (14–16). Besides the stably associated Gemin core, SMN can also interact with a plethora of different binding partners (12,17,18). 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 (13,19,20).

Arginine methylation has been implicated in several cellular processes, including protein subcellular localization, transcription, RNA processing and signal transduction (21). At the molecular level, arginine methylation acts mainly by regulating protein–protein interactions (22). Protein arginine N-methyltransferases (PRMTs), a family now comprised of nine members in higher eukaryotes, are classified as either Type I (PRMT1, 3, 6 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 (21,23). 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 (24–27). 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 (27–30). 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 (26–28,31–34.35 and references therein). Strikingly, single point mutations in the Tudor domain that are sufficient to abrogate interactions with methylated proteins (27) are also associated with severe forms of SMA (36–38), 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 (10,11,39,40), and it was postulated that reduced levels of available spliceosomal snRNPs in SMA might lead to alternative splicing defects selectively more detrimental to motoneurons (41). 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 (42,43–47). Finally, it was recently shown that SMN does not interact with Sm proteins in motoneuron axons (48,49), 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 (50). 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 (51–55). 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 down-regulated in spinal cord tissues from mild SMA model mice, and this correlates with increased p21<sup>cip1/waf1</sup> 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 (56,57). 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 (35). 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 (58), 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 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). 

35S-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) (36–38). 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

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). 35S-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 trans-PMA-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).
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 (27). 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 (31,50). As previously reported (27), 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/B₀, 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 cellular proteins, as assessed by immunoblotting with antibodies ASYM24 (50), ASYM25 (59) and αH3R17me2a (35) (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, 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

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).
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 (60). 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 ± 2.5% of KSRP foci also contained SMN, and 21.4 ± 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.

**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 (59). Specifically, N2a cells were incubated with [methyl-3H]-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 contrast, Sam68 is methylated in cells (59) 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 run 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 35S-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 3H-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 from N2a cells and analyzed by tandem mass spectrometry. Interestingly, in neurites, 23.6 ± 2.5% of KSRP foci also contained SMN, and 21.4 ± 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.

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 (35). 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.

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 (62). 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).

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 (63), β-actin which associates with chick ZBP2 (56) and p21 a known target of KSRP (64). 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).

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.
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 (67).

Since previous studies have shown that KSRP binds directly to the 3′-UTR of p21 (64), 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 (t1/2 = 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 (t1/2 = 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).

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 (68) 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, (65,66). 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 (67).
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 (69). 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

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 (∙ ≤ 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). 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 (t1/2) 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).
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 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 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). 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).

It was recently reported that reducing SMN levels by RNA interference in neuron-like PC12 cells results in differentiation defects (70) 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 24 h 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.

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). 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).
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, 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, p21Sn1P1, 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 mRNA (71), 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 1 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 (72). This study characterized an SMA-causing mutation in the Tudor domain (A111G), which can still support snRNP assembly (73), 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-immunoprecipitated 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 (64). 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 structures 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 (48) 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 (49,71,74). 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 (47). The chick homolog of KSRP, ZBP2, is also involved in β-actin mRNA localization (56), and KSRP was recently identified as a component of β-actin-enriched RNA granules in embryonic rat brains (75). 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 (76). 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 (76), 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 structures 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 (50), 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 (77,78).

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 (35). 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 (35). 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 (35). 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 (59,79,80). 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 corepressor for nuclear receptors, by regulating its interaction with Exportin 1/CRM1 (81). 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 (82). 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 (82). 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 (83) and mammalian systems (84).

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 (70). 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 (85). 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 (47,70). 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 (70), 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 p21cip1/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 Smm exon 7 directed to neurons or skeletal muscle, which causes severe motor axonal or myofiber degeneration, respectively (67). 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 (67). 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 (67), 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 86). 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 (64). 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 (66,67,87). 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 Snm2 can compensate for the loss of functional Smn1 during embryonic development (88,89), although the precise contribution of the Snm2 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 motorneurons (90). 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 (45). 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 (91). Finally, a ‘dual dysfunction’ hypothesis was recently put forth (10), 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 (10). 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 SMA. This 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 (10). 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.

In vivo methylation assays

In vivo methylation was conducted essentially as described previously. Briefly, N2a cells were grown for 3 h in methionine-free DMEM (Wisent, St-Bruno, Qc, Canada) containing 10% (v/v) dialyzed fetal calf serum and 10 μCi/ml of l-[methyl-3H]-methionine (85 Ci/mm; 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-[35S]-methionine (175 Ci/mm; 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 and visualized by fluorography.

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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−/− MEFs 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), supplemented with 20% heat-inactivated fetal bovine serum (Wisent, St-Bruno, Qc, Canada). N2a cells were grown for 3 h in methionine-free DMEM (Wisent, St-Bruno, Qc, Canada) containing 10% (v/v) dialyzed fetal calf serum and 10 μCi/ml of l-[methyl-3H]-methionine (85 Ci/mm; 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-[35S]-methionine (175 Ci/mm; 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 and 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 (58). 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 an LCQ Deca XP (ThermoFinnigan, CA, USA) system at 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 (27). GST-TdrQ136E, GST-TdrI116F and GST-TdrA111G were generated using overlap extension mutagenesis PCR (92) with the following mutagenic oligonucleotides (5′-tagagaggaggaatactgtgcc-3′) and (5′-ggagcaatggtacctgggtaaat-3′) for Q136E, (5′-cattgctcattggttaagag-3′) and (5′-cattgaatatcaatggaagact-3′) for I116F, and (5′-attaacccagattctctcta-3′) and (5′-agaagcaatgcccatctctcta-3′) for A111G, while T7 (863-882) and BGHR (1022-1039) were used as flanking primers. myc-SMN-ncDNA (31) was used as template for each mutagenic reaction. All resulting PCR fragments were first substituted back into myc-pcDNA3.1 (Invitrogen) at the EcoR1 site and then the TdrR1fwd (5′-ccggaattcggagtaatcggg-3′) and TdrR1rev (5′-ccggaattcgtttctctctctcata-3′) primers were used to amplify the portion of the cDNA encoding the Tudor domain. This fragment was in turn inserted into pGE-4T2 (Acc. No. U13854; GE Healthcare, Piscataway, NJ, USA) at the EcoR1 site. To generate GST-SMN and GST-SMNQ134K, the WT and mutant cDNA was isolated from the corresponding myc-SMN-pcDNA construct and subcloned into pGE-4T2 using BamHI and XhoI 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. Following induction, cells were spun down, resuspended in 10 ml of 1× PBS in the presence of Complete™ (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 000g. 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-[35S]-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 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 Zeiss Axiovert 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 HR M 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 Bsal sites of the psirNA-hneo vector (InvivoGen, Burlington, ON, Canada), obtained from Rashmi Kohathy (Ottawa Health Research Institute). The oligonucleotide sequence for sh_Smn_519 has been reported elsewhere (93). 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'-gauagcaaguccagauag-3') and J-048766-05 (5'-gcuacaucuccaauagaa-3'), respectively, were ordered from (DHarmaco, 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 (65) 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 (66). Briefly, Smn+/− and Smn−/− littermates were euthanized by intraperitoneal 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 from the vertebral canal, by applying pressure with 1 ml 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 Immunoblotting.

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, USA). cDNAs were amplified using specific forward and reverse primers for CARM1 (5'-agctgtgcagtcctcgtg-3', 5'-agctgtgcagtcctcgtg-3'), GAPDH (5'-accaagtctcatctcattac-3', 5'-tgcagctgtgcagtcctcgtg-3'), S12 (5'-ggagctcgagctctg-3', 5'-cctctagatgtttaaa-3'), GAP43 (5'-acaaagctttggagacactttg-3', 5'-tataattttggactcctcagac-3'), p21 (5'-tatatttggacacttctcagac-3', 5'-tataattttggactcctcagac-3'), β-actin (5'-tgacagactctcagagactt-3', 5'-tgacagactctcagagactt-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 (94). PCR products were electrophoresed on 2% 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 treshold 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 (94). 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 000g 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 RNA was incubated with 10 μg of protein extracts in a decay buffer (modified from 95) 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 ± 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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