

**Characterization of the Molecular Mechanism by which SMN
Regulates mRNA Translation**

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for the MSc degree in Cellular and Molecular Medicine
August, 2017

Cellular and Molecular Medicine
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Abstract

Despite our understanding of the role of the survival motor neuron protein (SMN) in cytoplasmic small ribonucleoprotein (snRNP) assembly, it is unclear how loss of this protein causes motor neuron degeneration in Spinal Muscular Atrophy (SMA). It could be explained by defects in functions that are specific to tissues most affected in SMA. In neurons, SMN localizes to neuronal RNA granules, RNA-containing foci in axons. They regulate many aspects of mRNA fate which include transport along neurites, mRNA stability, and mRNA translation. Most recently, our work provided evidence for SMN's role in mRNA translation. Specifically, we demonstrated that SMN associates with polyribosomes and may repress translation of specific mRNA targets. Our group demonstrated that SMA-causing mutations within the Tudor domain of SMN completely abolished this activity. This indicates the potential significance of this novel SMN function in the SMA pathology. To further investigate SMN's function in regulating translation, our group performed a proteomic screen on polysome-containing sucrose gradient fractions. We identified and validated novel interacting partners for SMN that may act as co-factors to regulate translation. DDX5 (an RNA helicase) is an unexpected novel interacting partner as it is known for its role in micro-RNA processing. Moreover, we observe that FMRP, a recognized protein in translational complexes, is required for the presence of SMN and DDX5 in polysomal fractions. With these latest findings, we updated our model of the molecular mechanism by which SMN regulates translation. This work provides more insights on how SMN regulates translation, a newly uncovered role for SMN in motor neurons. Identification of the molecular targets that are misregulated due to loss of this function may reveal new information on the pathogenesis of SMA.

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List of Abbreviations

- 293Ts: Human embryonic kidney cells 293T
- Anxa2: Annexin a2
- BSA: Bovine serum albumin
- CARM1: co-Activator associated arginine methyltransferase 1
- cDNA: complementary Deoxyribonucleic acid
- Co-IP: co-Immunoprecipitation
- Cpg15: Plasticity-related gene 15
- DDX5: DEAD-Box Helicase
- EWS: Ewing sarcoma breakpoint region 1
- FMRP: Fragile X Mental Retardation Protein
- G-quartets: G-quadruplexes
- GAP43: Growth-associated protein 43
- GST: Glutathione S-transferase
- HuD: Hu antigen D
- IGF2BP1: insulin like growth factor 2 mRNA binding protein 1
- IgG: immunoglobulin G
- KSRP: KH-type splicing regulatory protein
- MEF: Mouse embryonic fibroblast
- miRNA: micro-RNA
- MN-1: Motor neuron-derived cells
- mRNA: messenger Ribonucleic acid
- mRNPs: Messenger ribonucleoproteins

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PVDF: Polyvinylidene fluoride

RBPs: RNA-binding proteins

RPL7: Ribosomal protein L7

RT-PCR: Reverse transcription Polymerase chain reaction

SDS: Sodium Dodecyl Sulfate

shDDX5: short hairpin RNA silencing DDX5

shRNP: Heterogeneous nuclear ribonucleoproteins

SMA: Spinal Muscular Atrophy

SMN: Survival of Motor Neuron Protein 1, telemetric; 2, centromeric

snRNPs: small ribonucleoproteins

STEK: SV40 large T-antigen embryonic knockout

TDRD3: Tudor domain-containing 3

UTR: Untranslated region

WT: Wild Type

Acknowledgements

First, I would like to start by dedicating this work to my late grandfather, who always encouraged me to pursue my goals and education.

I would like to express my gratitude to my supervisor Dr. Jocelyn Côté for his guidance throughout my learning experience, and for giving me the opportunity to join his research laboratory. I will also take this chance to thank my advisory committee members, Dr. Trinkle-Mulcahy and Dr. Kothary, for their input in my research project.

I am also grateful to all of those whom I have had the pleasure to work with during this project. Specifically, I would like to thank each of my lab colleagues for sharing their expertise and knowledge: Dr. Mitch Baldwin, Dr. Alan Morettin, Nasim Haghbandish, Andréanne Didillon, Janik Laframboise, Geneviève Paris and, Amir Haghbandish. I especially appreciate Nasim's encouragement, input, and advice during long and labour-intensive experiments (polysome profiling) and, most importantly, for the inestimable friendship we developed.

Finally, I am grateful to my parents and siblings, who have provided moral and emotional support in my life. I am also grateful to my friends and extended family members who have supported me along the way. Most importantly, I wish to thank my loving and supportive partner, Griff, whose love and guidance are with me in whatever I pursue.

Chapter 1: Introduction

1. Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disorder in humans. SMA, considered the leading cause of infant mortality, has a carrier frequency of 1 in 40, and an incidence of 1 in 6000 (Monani 2005; Ogino et al. 2002; Feldkotter et al. 2002; Sugarman et al. 2011). Werdnig and Hoffman were the first to describe SMA as a neuromuscular disorder. In patients, they observed a loss of anterior horn cells resulting in progressive muscle weakness and death at early age (Werdnig 1891; Hoffmann 1893). Indeed, this disease targets alpha motor neurons in the ventral horn of the spinal cord, and this results in denervation and atrophy of muscles in the limbs and trunk (Melki 1997). The clinical symptoms include denervation of skeletal muscle, muscle weakness, paralysis, and eventual death due to respiratory failure (Lefebvre et al. 1998). Based on the onset and severity of the disease, from severe to mild, the SMA phenotype is classified into types I, II and III (Lefebvre et al. 1997). More recently, a more severe form was identified, type 0 (Gathwala et al. 2014).

SMA is caused by a mutation or deletion in the *SMN1* gene (Wirth 2000). Humans carry an additional copy of the *SMN* gene, *SMN2* (McAndrew et al. 1997). *SMN1* codes for functional SMN, whereas *SMN2* predominantly produces a truncated isoform SMN Δ 7. This is caused by differing nucleotides within exon 7 of the genes; unlike *SMN1*, *SMN2* has a Thymidine instead of a Cytosine. The difference affects the splicing profile, where 90% of the mRNA produced by *SMN2* results in exon 7 skipping (Lorson et al. 1999; Monani et al. 1999). The SMN Δ 7 isoform is less stable and only partially functional (Cho & Dreyfuss 2010; Burnett et al. 2009; Vitte et al. 2007). However, a higher copy number

of the *SMN2* gene is associated with reduced disease severity (Wirth et al. 2006). Since the *SMN2* copy of the gene is present in all patients, it is the most promising therapeutic target. Consequently, SMA therapies that enhance *SMN2* transcription, correct *SMN2* exon 7 skipping, or increase stability of SMN and/or SMN Δ 7 proteins were proposed; some of them are in clinical studies (Seo et al. 2013). Others are approved treatments, such as Spinraza (also known as Nusinersen or ISIS-SMNRx). This treatment is a modified antisense oligonucleotide designed to bind to the *SMN2* pre-mRNA matching an intronic splicing silencer in intro7; this promotes the inclusion of exon 7 (Meijboom et al. 2017).

2. Survival Motor Neuron Protein

SMN is a 38 kDa ubiquitously expressed protein. The gene encoding for the SMN protein contains seven exons, and its amino acid sequence across species shows conservation in higher vertebrates (**Figure 1**) (Singh et al. 2017). Structurally, the exons 2a and 2b encode for a domain that is essential for SMN's interaction with Protein 1 (SIP1, mostly known as Gemin2), self-association, and p53 (Seo et al. 2013; Young et al. 2002; Young et al. 2000). The middle region of SMN, encoded by exon 3 of the *SMN* gene, contains a barrel-like conserved domain, termed the Tudor domain. This domain, composed of beta-sheets that form a hydrophobic pocket, acts as a methyl arginine "sensor" allowing protein-protein interactions (Sattler et al. 2001; Kim et al. 2006; Cote & Richard 2005). It interacts with dimethylated arginine and glycine RGG/RG-rich motifs in proteins, including a large number of RNA-binding proteins (RBPs) (Thandapani et al. 2013; Cote & Richard 2005). Sm proteins, Heterogeneous nuclear ribonucleoproteins (hnRNP) Q and R, insulin like growth factor 2 mRNA binding protein 1 (IGF2B1, also known as IMP1), Hu antigen D (HuD), and KH-type splicing regulatory protein (KSRP) are some of proteins that interact

with SMN via its Tudor domain in a methylation-dependent fashion (Sattler et al. 2001; Mourelatos et al. 2001; Tadesse et al. 2008; Hubers et al. 2011; Fallini et al. 2014). Downstream to the Tudor domain, SMN contains a proline-rich sequence known to interact with Profilins (a family of proteins that control the actin dynamics in the cell) (Giesemann et al. 1999). The C-terminal region contains the most conserved motif of SMN, the YG box. Together with the last sixteen amino acids, the YG box facilitates the self-oligomerization that is critical for SMN's stability and subcellular localization (Lorson et al. 1998; Martin et al. 2012). The C-terminal region also enables SMN's interaction with the dead-box helicase (Gemin3) and the RNA-binding Protein Fragile X Mental Retardation Protein (FMRP) (Piazzon et al. 2008; Charroux et al. 1999).

SMN controls various aspects of RNA metabolism in each cellular compartment. It was first observed in the nucleus and the cytoplasm (Burlet et al. 1998; Pagliardini et al. 2000; Francis et al. 1998). In the nucleus, SMN localizes to foci-like structures called Gemini of Cajal bodies "Gems" (Liu & Dreyfuss 1996). Cajal bodies are rich in transcription factors implicated in the post-transcriptional modification of nuclear RNAs and small ribonucleoproteins (snRNPs), each components of the splicing machinery. SMN is largely known to play an essential role in the formation of these nuclear gems (Burlet et al. 1998). In the cytoplasm, SMN is found as part of a multi-protein 50S complex composed of Gemins2-8 and the protein Unrip. This complex increases the efficiency and specificity of snRNP assembly, SMN's most well-understood function. Interestingly, studies found correlation between a reduced ability to perform assembly of Sm proteins onto snRNA and the severity of SMA. Other studies found splicing defects in SMA conditions that could be caused by a faulty or less efficient formation of the snRNP complexes (Baumer et al. 2009).

Even though splicing is a ubiquitous function, a drastic decrease in SMN levels seems to lead to motor neuron degeneration indicating that SMN has cellular specific functions as well.

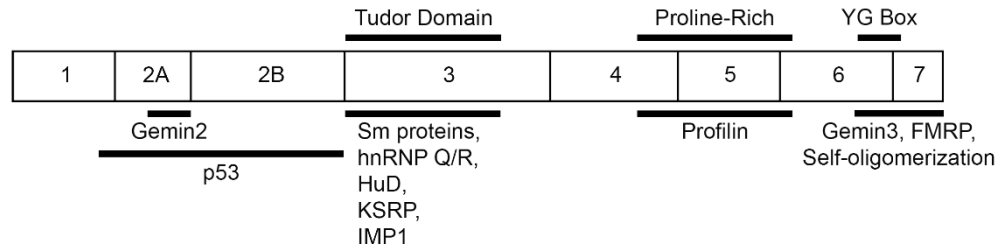


Figure 1: Diagram representation of the SMN protein and its domains. The numbers indicate the exons. The boxes represent the domains encoded by the exons. Below the boxes are the proteins shown to interact with SMN.

3. SMN in Motor Neurons

Even though there is evidence for the involvement of other tissues and cell types in severe cases, SMA is considered a motor-neuron-specific disease (Bowerman et al. 2014; Boyer et al. 2014). During development of the human central nervous system, the concentration of SMN changes from being mainly in the nucleus to a more axonal localization (Giavazzi et al. 2006). Studies observed an accumulation of SMN in the axons and growth cones of neuron-like cells in vitro, where it is found in a complex that does not contain Sm proteins (Fan & Simard 2002). Consistently, reduced SMN levels impairs the maturation of neuromuscular junctions in SMA mice (Kariya et al. 2008). However, our understanding of the cellular and molecular mechanisms through which mutations/deletions in the *SMN1* gene lead to these selective motor neuron loss remains controversial. Therefore, many ongoing studies seek to understand SMN's function in the molecular pathways implicated in neurodegeneration.

3.1. SMN in mRNA Trafficking and Local Translation

In motor neuron cells, growth cone formation and axonal guidance are highly dependent on local protein synthesis at the growth cones (Campbell & Holt 2001; Ming et al. 2002; Zhang et al. 2002; Wu et al. 2005; Verma et al. 2005; Leung et al. 2006; Lin & Holt 2007; Vogelaar et al. 2009). In order for translation to occur locally, silenced mRNAs are transported along axons. These mRNAs are believed to be trafficked in transport messenger ribonucleoproteins (mRNPs), also known as neuronal RNA granules in neurons (Kiebler & Bassell 2006). Neuronal RNA granules contain motor proteins, RNA binding proteins, one or more mRNA(s), and components of the translational machinery (Elvira et al. 2006; Lee 2012). These granules are highly heterogeneous foci-like structures that are assembled at the soma of motor neurons (Villace et al. 2004; Elvira et al. 2006; Jonson et al. 2007; Fritzsche et al. 2013). More recently, studies showed results that suggest SMN could chaperone protein recruitments other than snRNP assembly (Donlin-Asp et al. 2016; Donlin-Asp et al. 2017). Even though more insight is needed, there is strong evidence that SMN could have a crucial role in the fate of neuronal RNA granules (**Figure 2**), which helps us further understand why motor neurons are more sensitive to a decrease in functional SMN levels.

Endogenous SMN was seen to localize to neuronal RNA granules (Zhang et al. 2003). Using live imaging, it was observed that these SMN-containing granules also exhibit bidirectional movement between the cell body and the growth cone in rat spinal motor neurons (Zhang et al. 2003). Additionally, SMN plays a role in the localization of some mRNAs and RNA-binding proteins to axonal growth cones (Rossoll et al. 2003; Akten et

al. 2011; Fallini et al. 2011; Hubers et al. 2011; Rage et al. 2013; Fallini et al. 2014; Saal et al. 2014; Donlin-Asp et al. 2017). For instance, SMN plays a role in the localization of Poly-Adenylated (Poly(A)) mRNAs and a pool of specific transcripts (Fallini et al. 2011). The abnormal localization of β -actin mRNA in SMA motor neurons was the first example uncovered (Rossoll et al. 2003). More transcripts were revealed by a recent RNAseq analysis of axonal mRNAs in cultured SMA motor neurons (Rage et al. 2013; Saal et al. 2014). Other groups have also identified growth-associated protein 43 (GAP43) and plasticity-related gene 15 (cpg15)/neuritin mRNAs as additional transcripts that are mislocalized in SMA conditions (Akten et al. 2011; Fallini et al. 2016). Furthermore, defects in mRNA localization are accompanied by decreased levels of SMN-interacting RNA-binding proteins HuD, KSRP, and IMP1 within axonal neuronal RNA granules (Hubers et al. 2011; Fallini et al. 2011; Fallini et al. 2014). These defects reported upon SMN-deficiency are caused by a dysregulation in either trafficking or assembly. A more recent study revealed that SMN facilitates mRNP assembly of IMP1 protein with β -actin mRNA. They observed smaller IMP1 granules in SMN-deficient cells; Additionally, assembly defects cause decreased association of IMP1 with the cytoskeleton and defective IMP1 localization (Donlin-Asp et al. 2017).

A number of studies have also reported local translation defects in SMA-like conditions (Rathod et al. 2012; Kye et al. 2014). For instance, β -actin mRNA local translation via Laminin signaling is altered in SMN-deficient motor neurons. Because Laminins play an important role in axon growth, its misregulation indicates that the regulatory mechanism for local protein synthesis of actin in axon terminals are disrupted in SMA (Rathod et al. 2012). In another study, neuritin mRNA local translation in axons was observed to be

regulated locally by SMN and its interacting RNA-binding protein HuD (Fallini et al. 2016). More recently, alterations in the function of micro-RNA (miRNA) were reported in SMA mice, suggesting that SMN may also have an indirect role in translation repression through the RNA interference pathway (Haramati et al. 2010). miRNAs are non-coding RNA molecules that target specific mRNA transcripts in order to recruit the RNA-induced silencing complex (RISC). Another group demonstrated that reduced SMN levels triggered differential expression of specific mRNAs via miRNA. For instance, SMN regulates axonal local translation of mTOR mRNA via miR-183 (Kye et al. 2014). Since then, several other miRNAs were reported to be aberrantly expressed in SMA, including miR-9, miR-206, miR-132, and miR-431 (Wang et al. 2014; Catapano et al. 2016; Wertz et al. 2016).

Neurons are highly polarized cells; therefore, deficiency of proper neuronal RNA granule assembly/transport and local translation may underlie SMA pathogenesis. For example, β -actin promotes branching and GAP43 promotes elongating of axons when their respective mRNAs are targeted to axons for localized translation. Overall, SMN has common and cell-specific molecular functions, and in motor neurons it is important in maintaining axonal well-being.

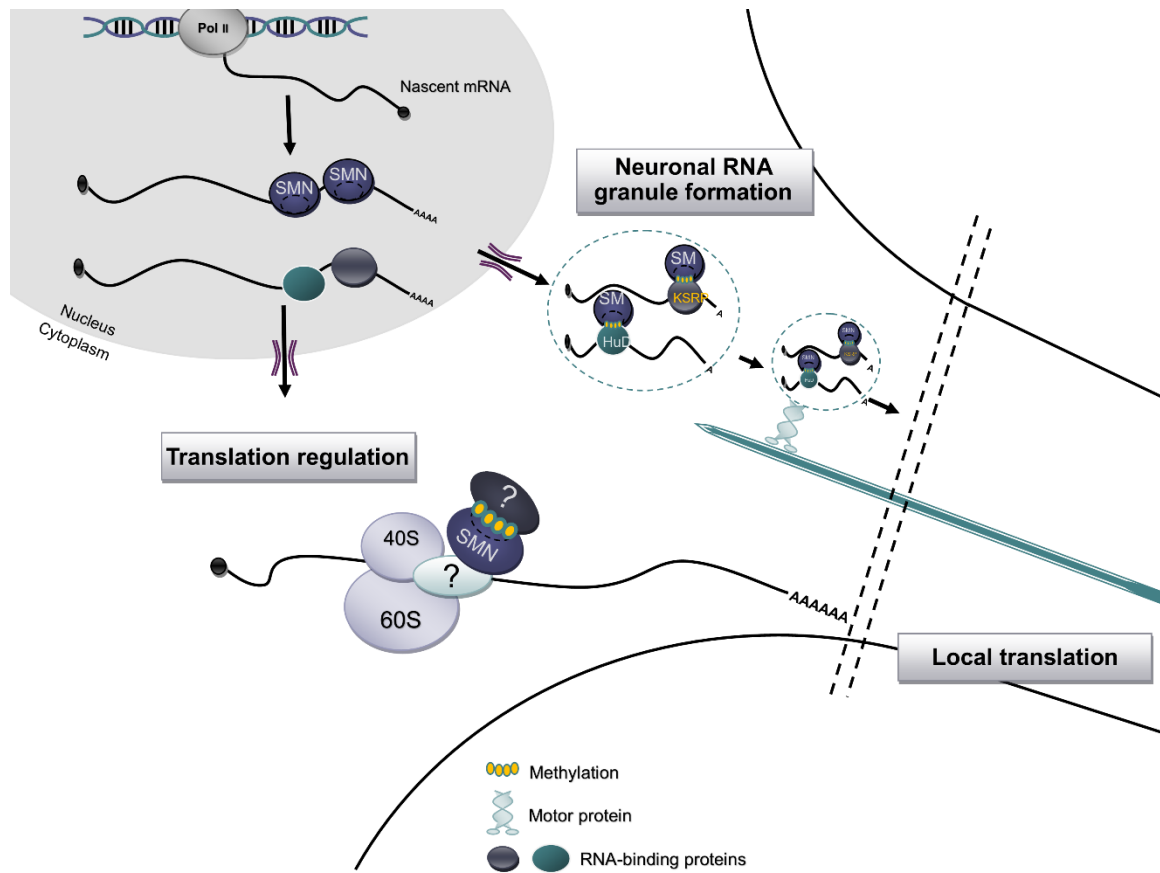


Figure 2: The role of SMN within a neuronal unit. The diagram represents the proposed molecular models by which SMN regulates mRNA fate within a neuronal unit. First, an mRNA is transcribed then transported to the cytoplasm, possibly accompanied by SMN. In the cytoplasm, the mRNA is subject to some sort of mRNA translational regulatory mechanism, where SMN has a role in its regulation. Other mRNA (or mRNAs) could be assembled into neuronal RNA granules. This will allow the transport of the mRNA along neurites. SMN was also associated with this function. Finally, upon arrival to the subcellular compartment, an mRNA is known to be locally translated in response to subcellular cues. Local translation is another function that is regulated by SMN, however the molecular mechanism is yet to be determined.

4. SMN in Regulating mRNA Translation

After transcription and RNA maturation, the resulting mRNA is used as a template for protein synthesis. As with most cellular processes, mRNA translation is regulated. Many mechanisms have been proposed and investigated to elucidate this function. Translation is regulated at different stages, ranging from initiation (before the first pioneer round of

translation) to stalled elongating ribosomes (Kozak 1992; Kapp & Lorsch 2004; Fabian et al. 2010; Hinnebusch 2014). The regulation of mRNA translation is crucial in maintaining proper neuronal function (reviewed in (Wang et al. 2016)). Its misregulation is believed to lead to known neuronal disorders and can be caused by a deficiency of key proteins.

SMN is now widely known to associate with and regulate the translational machinery. First, SMN was observed to associate with elongating ribosomes and repress the translation of specific mRNA targets. The translation of the coactivator-associated arginine methyltransferase 1 (CARM1) mRNA was the first target identified. More specifically, SMN repressed CARM1 mRNA expression via its mRNA coding sequence (Sanchez et al. 2013). CARM1 is upregulated in SMA mice and in SMA type I patient cells. This has a crucial role in the maintenance of the proliferative state of motor neuron derived cells via posttranslational marks (methylation) on other proteins (Hubers et al. 2011). In addition, SMA-causing mutations within SMN completely abolished translational repression (Sanchez et al. 2013). This is an indication that SMN could have an effect on cellular fate via translational regulation of mRNA targets.

Second, a recent study confirmed the association of SMN with the translational machinery. They demonstrated that SMN, alongside the Tudor domain-containing 3 (TDRD3) protein, regulates translation in human cancer cells by recruiting the RNA-binding protein Aven to elongating ribosomes (Thandapani et al. 2015). They also determined the presence of G-quadruplexes (G-quartets) RNA secondary structures within mRNA coding sequence (reviewed in (Song et al. 2016)). In addition, they found that an RNA helicase (DHX36) is recruited to this regulatory complex and is believed to help destabilize the G-quartet structures (allowing the translational machinery to go through). Finally, they predicted,

based on a bioinformatic analysis, that CARM1 mRNA contains G-rich structures within its coding sequence and that they could potentially form G-quadruplexes.

Overall, these studies suggest that SMN regulates mRNA translation at the elongation stage. One current model of translation regulation at this stage is stalled elongating ribosomes. It was first proposed by the Darnell group when they observed that the RNA-binding protein FMRP can reversibly stall ribosomes during elongation on target mRNAs (Stefani et al. 2004; Darnell et al. 2011). However, the molecular events and the factors that constitute the complex involved in this regulatory mechanism are still unclear. It also remains to be seen what other mRNA targets are involved in the regulatory mechanism mediated by SMN.

5. Rationale, Hypothesis and Objectives

5.1. Rationale

Recent literature, including key contributions from our lab, support a critical role played by SMN in regulating translation. However, the molecular mechanism(s) by which it mediates its effect on translation remains unclear. Previous work from our group has provided evidence that SMN associates with polyribosomes and represses the translation of specific mRNA targets (Sanchez et al. 2013). The same study showed that SMN's Tudor domain is crucial for its regulatory translation activity but is not required for SMN's association with the translational machinery. Therefore, we favor a model where SMN's interaction with polyribosomes would be mediated through a domain distinct from the Tudor domain, and might either be direct or involving one or more additional interacting partners. In turn, the requirement of SMN's Tudor domain for function implies that it may

interact with a co-factor through this domain in order to mediate its impact on translation (**Figure 3**).

To look for other interacting proteins that may regulate translation alongside SMN, our group previously performed, as an unbiased approach, a proteomic screen by pulling down GFP-SMN and its interacting proteins from polysome-containing sucrose gradient fractions. The RNA helicase DEAD-Box Helicase 5 (DDX5) was one of the putative proteins revealed by the mass spectrometry experiment. DDX5 is a member of the microprocessor complex involved in miRNA maturation in the nucleus (Salzman et al. 2007; Dardenne et al. 2014). More recently, a study showed evidence of DDX5 association with polysomes (Berg et al. 2015). The same study demonstrated that the loss of another RNA-binding protein (JAKMIP1) in vivo disrupts the polyribosome association profile of DDX5, overall making DDX5 an ideal candidate to study. Next, we performed a literature analysis to further investigate other potential interacting proteins. We mainly looked for RNA-binding proteins that interact with SMN and are known translation regulators. The RNA-binding protein FMRP is an established protein in the translational mechanism (Darnell et al. 2011; Mazroui et al. 2002). FMRP is also known to interact with SMN (Piazzon et al. 2008), however not through its Tudor domain. But FMRP would still potentially fit the profile to play a role as a co-factor for SMN in its translational regulatory function. Thus, we have investigated FMRP as one of SMN's cofactors in addition to DDX5. This work provides more insights on the molecular mechanism by which SMN regulates translation. Identification of the molecular targets that are misregulated due to the loss of this function may reveal new information on the pathogenesis of SMA.

5.2. Hypothesis

I propose that FMRP and DDX5 functionally interact with SMN to regulate mRNA translation.

5.3. Objectives

- Characterize the biochemical and functional interaction between DDX5 and SMN.
- Investigate if and through which mechanism FMRP may act as a cofactor for SMN in regulating mRNA translation.

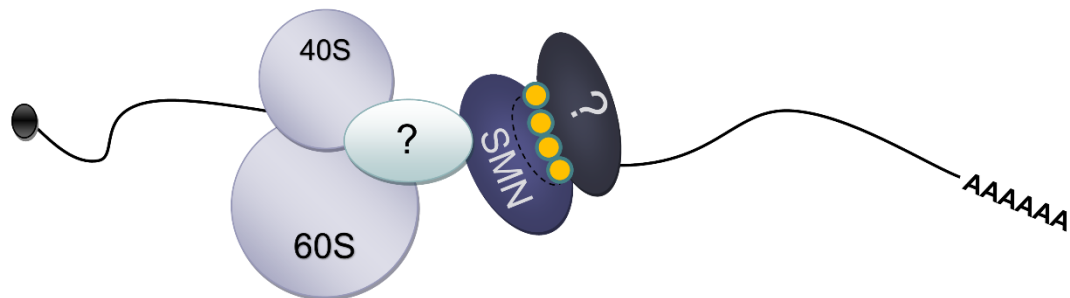


Figure 3: Proposed molecular mechanism by which SMN could be regulating mRNA translation within polyribosomes. SMN regulates translation through its Tudor domain, with which it could be interacting with a co-factor to accomplish this function. SMN is found within elongating ribosomes independently of its Tudor domain, therefore this co-localization with polyribosomes is most likely mediated through another interacting protein. The circle of dotted lines within the representation of SMN represents the Tudor domain. The four yellow circles represent a potential methylation of the interacting protein.

Chapter 2: Material and methods

1. Cell culture, transfection, and generation of transient knockdown cell lines

Cell Maintenance. MN-1, 293T, MEF, and STEK cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS; ThermoFisher), and 2% penicillin/streptomycin (Multicell; Wisent). They were cultured at 37 °C with 5% CO₂. For protein extraction, RNA isolation, and co-Immunoprecipitation, cells were passaged at 80-90% confluency.

Transfection of Plasmid DNA. The transient transfections for the luciferase were done using Lipofectamin 2000 (Invitrogen). 293T cells were seeded a day prior to transfection at a density of approximately 80%, as the transfection will affect cell survival. These cells were transfected using the Lipofectamine 2000 (Invitrogen), according to the manufacturer protocol. Plasmid DNA was diluted in OptiMEM and complexed with the Lipo 2000 in 1:1 ratio (mg cDNA: ml Lipo 2000). The mixture was left at room temperature for a minimum of 20 minutes. The complexes were added to the cells incubating in 1 ml OptiMEM. Media was replaced 3 hours later. For the luciferase assay, the cells were transfected for 24 hours prior to use.

To generate a transient knockdown of DDX5 in 293T cells, we did a transient transfection. 293T cells were seeded a day prior to transfection at a density of approximately 80%. These cells were transfected using the Lipofectamine 2000 (Invitrogen), according to the manufacturer protocol. The vector pGIPZ containing a target sequence shRNA (GE Dharmacon) targeting the DDX5 gene was used for the transient knockdown. pGIPZ empty vector was also transiently transfected, as a control, using the same conditions. The cells

were incubated with the complex plasmid DNA and Lipo 2000 for 48 hours to allow sufficient knockdown.

2. Protein extraction, SDS-PAGE and Western blot

Cells from 10 cm plates were washed with 1X PBS three times, then lysed with the lysis buffer, radioimmunoprecipitation (RIPA) containing 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM ethylene tetraacetic diamine acid (EDTA), 1% nonyl phenoxyethoxyethanol 40 (NP-40), 0.5% sodium deoxycholate (NaDOC), 0.1% sodium dodecyl sulfate, and 1 complete, EDT-free protease inhibitor Cocktail (Roche). The extract was mixed then incubated on ice for 15 minutes to insure proper lysis. Next, the extract was centrifuged at 16100 rcf for 15 minutes. The supernatant was then collected for SDS-PAGE. The protein quantification was done using the Bradford method (BioRad) according to manufacturer protocol. The standard curve for the Bradford assay was done using Bovine serum albumin (BSA). To quantify the protein concentration, the optical density (OD) was measured using a spectrophotometer with a wavelength of 595 nm. The concentrations of proteins were obtained by extrapolating the OD value to the standard curve. Equal concentration of protein contained in the numerous extracts were then mixed with equal amount of 5X Laemmli buffer containing 25% glycerol, 125 mM Tris HCl pH 6.8, 4% SDS, 700 mM β -mercaptoethanol, and 0.1% bromophenol blue. The extracts were heated at 95 °C for 8 minutes, then loaded onto a 10% gel for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were then transferred onto an Immobilon-P polyvinylidene difluoride membrane (PVDF, from Millipore: 0.45 μ m pores). The membrane was blocked for 1 hour in 5% non-fat milk in 1X PBS with 0.05% Tween 20 (PBS-T). The primary and

secondary antibodies were mixed in 5% non-fat milk in 1X PBS. The membranes were incubated with the primary antibodies for either one hour at room temperature, or overnight at 4 °C depending on the antibody. Primary antibodies include: SMN (1:3000; BD Biosciences), Tubulin (1:1000; Sigma), Anxa2 (1:4000; Proteintech), CARM1 (1:10000; Bethyl), DDX5 (1:10000; Santa Cruz Biotechnology), FMRP (1:2; Hybridoma), RPL7 (1:20000; Novus Biologicals), and EWS (1:10000; Bethyl). After the incubation period, the membrane was washed 3 times for 5 minutes each with PBS-T. Next, the membranes were incubated with the secondary horseradish peroxidase (HRP) conjugated antibodies (goat anti-mouse or mouse anti-rabbit; Jackson ImmunoResearch) for one hour at room temperature. The membranes were again washed 3 times for 5 minutes each with PBS-T before exposure to film (Clonex) in the presence of the chemiluminescent HRP substrate (Millipore).

3. RNA isolation, Reverse transcription and Polymerase Chain reactions (PCR)

RNA was isolated from 10 cm plates of either MEF or STEK cells using Trizol reagent (Invitrogen) according to the manufacturer protocol. The RNA was suspended in 30 µl DEPC treated water, then quantified using a spectrophotometer with a wavelength of 260 nm. DEPC treated water was used as a negative control.

For the reverse transcription (RT), the first step consisted of incubating 1 µg of RNA with 1 µM oligo dT for 10 minutes at 65 °C. Then the reverse transcription was done using 0.5 mM deoxyribonucleotide (dNTPs; Invitrogen), 20 U RNasin (Promega), and 10 U AMV

reverse transcriptase enzyme (Promega) in AMV reverse transcriptase buffer. The samples were then incubated at 42 °C for 1 hour.

Polymerase chain reactions (PCR) were done using 20 ng of the respective cDNA, 2 X GoTaq green master mix (Promega) and 400 nM of the respective forward and reverse primers. The primers used were:

CARM1 (F): 5'- AGCTGTGCAGTACTTCCAGTTCTA -3'

CARM1 (R): 5'- ATTGTTACTCTTCACCAGGACCTC -3'

Anxa2 (F): 5'- AAGGGAGGCTCTCAGCGATAC -3'

Anxa2 (R): 5'- TCCACTCCTTTGGTCTTGACTG -3'

GAPDH (F): 5'- TGCTGAGTATGTCGTGGAGTCT -3'

GAPDH (R): 5'- ATCATACTTGGCAGGTTTCTCC -3'

The PCR incubation temperatures and times for each the respective primers were:

CARM1 and Anxa2:

1. 98 °C for 600 seconds
2. 98 °C for 30 seconds
3. 57 °C for 30 seconds
4. 72 °C for 30 seconds
5. Steps 2-4 were cycled 31 times
6. 72 °C for 600 seconds

GAPDH:

1. 95 °C for 300 seconds
2. 98 °C for 30 seconds
3. 57 °C for 30 seconds

4. 72 °C for 30 seconds
5. Steps 2-4 were cycled 20 times
6. 72 °C for 600 seconds

4. Co-immunoprecipitation

For Co-immunoprecipitation, cells at 50-60% confluency were plated 24 hours in advance. The cells were rinsed 3 times with 3 mL 1X PBS. Cells were pelleted by centrifugation at 6 000 rcf for 5 minutes at 4 °C. The cells were lysed in 1 ml lysis buffer containing 20 mM Tris pH 8, 137 mM NaCl, 10% Glycerol, 1% NP-40, and a protease inhibitor cocktail. Next, the cells were sonicated twice for 4 seconds, with 10 seconds rest on ice, at 20% duty cycle (Branson Sonifier 450). To pre-clear the cell lysate, 25 µL of A/G agarose bead slurry (50% beads 50% 1X PBS; Protein A/G PLUS-Agarose; Santa Cruz Biotechnology) was added to each sample, then tumbled for 30 minutes. Cell lysates were centrifuged briefly to discard the beads. The final concentration of lysate was standardized between all conditions in a given experiment. The extracts were incubated with 2 µg of the respective primary antibodies on the rotating wheel for 1 hour at 4 °C. Next, 25 µL of A/G agarose bead slurry (50% beads 50% 1X PBS; Protein A/G PLUS-Agarose; Santa Cruz Biotechnology) was added to each of the samples, then incubated for 1 hour on the rotating wheel at 4 °C. The immunoprecipitates (the beads) were washed 6 times with 1 ml cold lysis buffer. Upon the final wash, 30 µL distilled water and 10 µL 5X Laemmli was added to the beads. The samples were resolved by a 10% SDS-PAGE, then transferred onto a PVDF membrane as previously described.

5. Sucrose density gradient fractionation and polysome profiling

Either brain mouse tissues or cultured cells were used for this experiment. The mouse brains were harvested then flash-frozen in liquid nitrogen before making a powder to use for lysis. Cultured cells were first plated in 10 cm plates and incubated for 48 hours to obtain a confluency at 80-90 %. The cells' media was changed 24 hours before collection of the cells. For each condition 4 to 6 plates were used. For the lysis preparation, the cells were washed 3 times with ice cold 1X PBS, lysed with lysis buffer containing 20 mM Tris-HCl pH 7.4, 150 mM sodium chloride (NaCl), 1.25 mM Magnesium chloride (MgCl₂), 1% NP40, 1mM Dithiothreitol (DTT), 8 U/mL RNasin (Promega), and 1 cOmplete, EDT-free protease inhibitor Cocktail (Roche). The extracts were lysed using a syringe 1CC and a 28G needle. The lysate was then incubated on ice for 15 minutes, then was centrifuged for 20 minutes at 16 000 rcf, at 4 °C. The supernatant was quantified using a spectrophotometer with a wavelength of 260 nm. The sucrose gradients (15-50% (w/w)) were prepared in 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 3 mM MgCl₂, 8 U/mL RNasin (Promega), and 1 cOmplete, EDT-free protease inhibitor Cocktail (Roche) using the Hoefer Scientific gradient maker. 20 OD of each sample was loaded on the sucrose gradient before ultracentrifugation at 37 0000 rpm for 2 hours and 30 minutes using a SW41Ti swinging rotor. After centrifugation, the polysome profile was monitored using a 254 nm wavelength with ISCO UV-6 UV detector. Using a fraction collector, 1 mL fractions were collected using the fraction collector (Brandel).

The proteins from each fraction were isolated using a methanol/chloroform protocol. 150 µL of each fraction was mixed with 600 µL methanol, then 150 µL of chloroform was added to the mixture. Next, a 450 µL of distilled water was added before mixing well. The

samples were then centrifuged with the speed of 16 000 rcf for 5 minutes at 4 °C. The top aqueous layer was discarded, and 650 µL of methanol was added to the bottom layer then inverted three times. The samples were next centrifuged with the speed of 16 000 rcf for 5 minutes at 4 °C. The pellet containing the proteins was dried then diluted in distilled water and 5X Laemmli. The samples were run on a 10% SDS-PAGE gel and transferred onto a PVDF membrane as previously described.

6. Luciferase assay

Plasmids used for this experiment is pGL4.14 backbone. The 3'UTR of CAMR1 was subcloned upstream of the Firefly, and a cmv promoter was also added upstream of it. A Renilla plasmid was also used to control for transfection efficiency.

Transfected cells were washed 3 times with 1X PBS and harvested and lysed in 400 µL 1X passive lysis buffer (Dual-Luciferase Reporter Assay Kit; Promega). Extracts were centrifuged at 16 000 rcf for 5 minutes to pellet cellular debris, and the supernatants were transferred to a new tube. 5 µL of each sample was pipetted to into 5 mL polystyrene cuvettes, then 50 µL of LAR II solution (Dual-Luciferase Reporter Assay Kit; Promega) was added to each sample. The Firefly luciferase activity was measured for 10 seconds (as relative luciferase units) using the Monolight 3010 Luminometer (Applied Luminescence Laboratory). Next, 50 µL of Stop & Glo Reagent was added to each sample to measure the Renilla luciferase activity.

Chapter 3: Results

1. DDX5 is a novel SMN interacting partner

1.1. SMN interacts with DDX5

In a previous study, using ultracentrifugation through sucrose density gradients, SMN was observed to co-fractionate with elongating ribosomes (polyribosomes), and using a generic Luciferase reporter the same study showed that SMN represses the translation of specific mRNA targets, such as CARM1. In addition, they demonstrated that a naturally occurring point mutation in the Tudor domain, E134, completely abolishes the repressive activity of SMN. Moreover, SMN's Tudor domain is known as a methylated arginine sensor. Altogether, SMN most likely needs to physically interact, via its Tudor domain, with an arginine-methylated co-factor(s). To test the hypothesis that SMN associates with co-factors to regulate translation, using an exploratory approach, our group previously performed a proteomic screen on SMN-interacting proteins within polyribosomes (by pulling down GFP-SMN and its interacting proteins from polyribosome containing sucrose gradient fractions). Several putative proteins were revealed by the proteomic screen (appendix I, table 1). DDX5 is one of the putative interacting proteins identified (appendix I, table 1). DDX5 is mostly known in the literature for playing a role in miRNA processing. However, a study showed evidence of DDX5 association with polyribosomes (Berg et al. 2015). The same study demonstrated that the loss of another RNA-binding protein (JAKMIP1) *in vivo* disrupts the polyribosome association profile of DDX5. Overall making DDX5 an ideal candidate to study. To further study this novel potential SMN interacting protein, a previous student started the biochemical characterization of SMN's interaction with DDX5. Co-immunoprecipitation experiments were used to confirm the

interaction between DDX5 and SMN, which is consistent with the proteomic screen. The same experiment was performed using RNase treatment and the interaction remained present. This suggests that the interaction is independent of RNA (appendix I, figure 1, a). Using GST pull downs, the previous student next determined that the interaction is mediated thorough SMN's Tudor domain (appendix I, figure 1, b). Interestingly, the interaction is abolished using the purified GST-tagged Tudor domain of SMN harboring the severe type I SMA mutation (E134K). In order to further demonstrate this interaction, we looked at the endogenous interaction using DDX5 immunoprecipitation. The DDX5 and SMN endogenous interaction was examined in motor neuron-like cells using co-immunoprecipitation experiments (co-IP). DDX5 was immunoprecipitated from wild type (WT) motor neuron-derived cells (MN-1) and the resulting immunoprecipitate was resolved by SDS-PAGE, transferred on a PVDF membrane, and probed for the presence of endogenous SMN using Western blotting (Figure 4). We observe a signal for SMN in the IP lane compared to the IgG control lane. This demonstrates and confirms the endogenous interaction between DDX5 and SMN. These observations are consistent with the previous results discussed above.

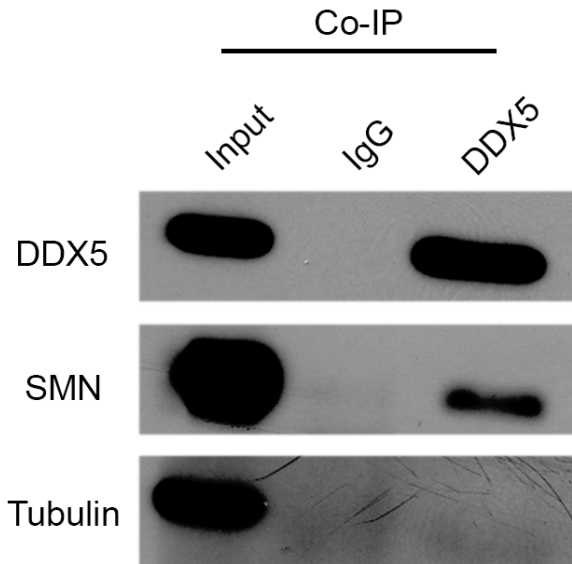


Figure 4: SMN and DDX5 interact in motor neuron-like cell extracts. An immunoprecipitation of endogenous DDX5 was done using wild type (WT) motor neuron-derived cells (MN-1) lysates, then analysed by western blotting for the presence of SMN, DDX5, and α -Tubulin as a negative control. As a negative control for the IP, purified non-immunogenic mouse immunoglobulins G (IgGs) was used. An input of the lysate was run alongside the IgG and IP lanes. The experiment was repeated three times.

2. DDX5 as SMN's novel co-factor in regulating translation

2.1. DDX5 modulates *Anxa2* protein expression

To further explore whether SMN functionally interact with DDX5 to regulate mRNA translation, we proceeded to test if DDX5 has an effect on translation. Our previous studies showed that the global translation of CARM1 is reduced in SMN deficient cells. It was also determined that the regulation could be dependent on the mRNA coding sequence (Sanchez et al. 2013). Our previous results indicated that SMN and DDX5 interact. Subsequently, they would both have an effect on the same mRNA target(s). We could therefore hypothesise that DDX5 could modulate mRNA translation of the same targets. A previous student has already looked into whether DDX5 has an effect on CARM1 mRNA

translation. In DDX5 deficient cells, it was observed that CARM1 protein levels were up-regulated where its mRNA levels remained unchanged (Appendix I, figure 2). These results suggest that DDX5 might have the same regulatory outcome on the same mRNA targets as SMN. In our previous studies only CARM1 was investigated. We therefore used a candidate approach to uncover another mRNA target, we looked at Annexin a2 (Anxa2). First, we used Anxa2 since previous preliminary results showed evidence that SMN could have a stimulating effect on its mRNA expression (Appendix I, figure 3). Indeed, we used sucrose gradient sedimentation with SMA mouse brain tissues and with control mice. Anxa2 mRNA levels were examined by reverse-transcription (RT)-PCR using isolated mRNA from each of the gradient fractions (Appendix I, figure 3). We observed a shift towards the lighter fractions in SMA-like condition compared to control, meaning a reduction in translational levels. Second, Anxa2 has been shown to associate with SMN and this complex is responsible for Anxa2's localization to neurites (Rage et al. 2013; Rihan et al. 2017). These suggest that DDX5 could also have an effect on Anxa2 protein levels in addition to CARM1. Anxa2 protein levels were assessed using western blot analysis. We used lysates from MN-1 cells expressing a short hairpin RNA silencing DDX5 (shDDX5) protein expression, and MN-1 cells expressing control plasmid (pGipz). The lysates were resolved by SDS-PAGE, transferred on a PVDF membrane, and probed for the presence of endogenous Anxa2 using Western blotting (Figure 5). We observed a decrease in Anxa2 protein levels in cells with reduced DDX5 expression.

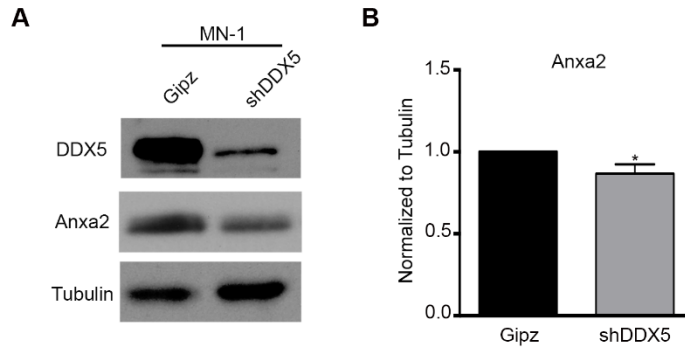


Figure 5: Anxa2 was down-regulated in DDX5 deficient MN-1 cells. Annexin 2 (Anxa2) protein levels were down-regulated in motor neuron-derived cells (MN-1) expressing a short hairpin RNA silencing DDX5 (shDDX5) protein expression compared to MN-1 cells expressing control plasmid (pGipz). (A) Western blot analyses were done to analyze Anxa2 protein levels in MN-1 shDDX5 cells compared to the control cell MN-1 Gipz. Lysates were run on an SDS-PAGE gel, transferred on a PVDF membrane then probed for the presence of endogenous Anxa2 and DDX5 using Western blotting. α -Tubulin was used as a loading control. (B) The bar graph represents quantification of Anxa2 protein levels for three replicates. The quantification was normalized to Tubulin, then to control. n=3; statistical significance is indicated with an asterisk; p=0.0161; t-test

2.2. DDX5 does not regulates CARM1 protein expression through 3'UTR

Before this study, DDX5 is known to be involved in nuclear maturation of miRNAs that silence targets by interacting with the 3' UTR of an mRNA target. Since we hypothesize that DDX5 translationally regulates CARM1 mRNA expression, we proceeded to eliminate the potential regulatory mechanism via miRNA at CARM1's 3'UTR. Indeed, to exclude the possibility that DDX5 affects CARM1 mRNA translation through miRNA processing at its 3'UTR, we employed a luciferase reporter assay (as has been previously established in our laboratory (Sanchez et al. 2013)). Firefly Luciferase plasmids harboring CARM1 3'UTR were transfected into human embryonic kidney cells 293T (293T) expressing a short hairpin RNA silencing DDX5 (shDDX5) alongside 293Ts expressing control plasmid (pGipz). We also transfected a plasmid harboring Renilla to control for transfection efficiency. 293T cells were used for their ease of transfection and to maintain

the transient DDX5 knockdown (Figure 6A). The Luciferase activity was quantified in both conditions expressing the Firefly reporter in DDX5 knockdown cells and control cells. The Firefly activity was normalized to Renilla and then to control (Figure 6B). We observed no significant change. **Even though a positive control will be needed,** this assay shows evidence that upon modulating DDX5 expression, the changes in CARM1 protein levels were potentially not a result of 3'UTR miRNA processing by DDX5. Thus, it is most likely that DDX5 affects CARM1 through translational regulation.

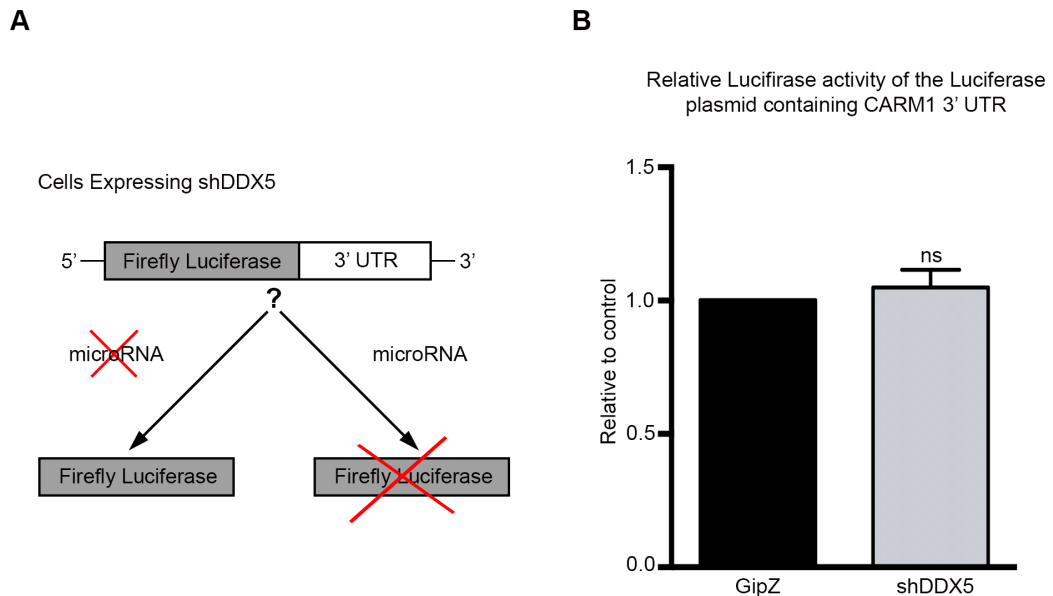


Figure 6: DDX5 does not regulate CARM1 protein expression through its 3'UTR. (A) The Luciferase assay was done to look at the potential effect of microRNA on CARM1 3'UTR. Firefly Luciferase reporters containing CARM1 3' UTR were transiently transfected for 24 h into the Human embryonic kidney cells 293T (293T) expressing a short hairpin RNA silencing DDX5 (shDDX5) protein expression and 293T expressing control plasmid (pGipz). Then, the Luciferase activity was quantified. (B) The bar graph represent the Firefly Luciferase activity normalized to Renilla Luciferase to control for transfection efficiency, then normalized to control (n=3; ns: not significant; p=0.2571; t-test).

2.3. SMN is required for DDX5's association with polysomes

SMN is found within elongating ribosomes and it is likely that DDX5 would co-fractionate with SMN. Therefore, we next proceeded to look if DDX5 associates with the translational machinery and co-fractionates with elongating ribosomes, and if SMN is required for its recruitment to these fractions. To test this, and to support that DDX5 most likely does not regulate CARM1 protein expression through 3'UTR, we used sucrose gradient sedimentation with SMA mouse brain tissues and with control mice. SMA mice harbor a defective copy of SMN protein, commonly known as delta 7 mouse. This mouse model harbors an SMN2 copy combined with a copy of $\Delta 7$ SMN cDNA transgene. Cytoplasmic extracts from SMA mouse brain tissues and control mice were used for gradient sedimentation at 105 000g. This sedimentation speed was used to remove the SMN 50S complex from the heavy fractions; the complex is involved in snRNP biogenesis. Total proteins were extracted from each fraction, resolved by SDS-PAGE, and subjected to immunoblotting with the indicated antibodies. We observed the sedimentation of DDX5 within the elongating ribosomes (Figure 7). We also observed a reduction of DDX5 levels within elongating ribosomes of SMA brain tissues in comparison to wild type, and an increase of DDX5 protein levels in the 80S cut-off.

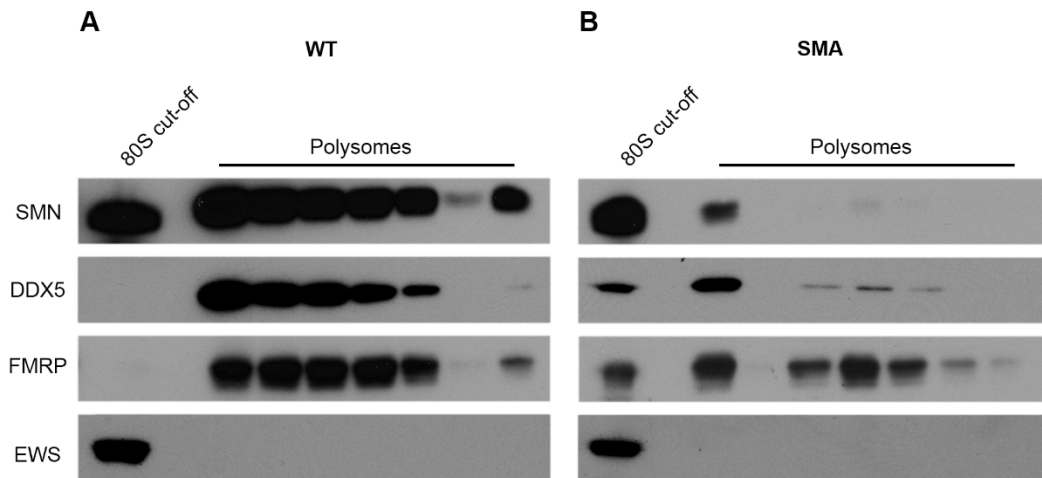


Figure 7: SMN is required for DDX5 localization to polysome fractions. Mouse brain tissues cytoplasmic lysates, from either wild type mice (WT) or mice with SMA type 1 phenotype, were subjected to sucrose gradient sedimentation at 105 000g. Polyribosomes were depleted of the 50S SMN core complex, there was an 80S cut-off at the top of the gradient. The components include ribosomal subunits, monosomes (80S) and, polyribosomes. Total proteins were extracted from each fractions, resolved by SDS-PAGE, and subjected to immunoblotting with the indicated antibodies. EWS antibody was used as a control for light fractions and, FMRP was used as a control for the polysomal fractions. The experiment was repeated three times.

3. FMRP as SMN's novel co-factor to regulate translation

3.1. FMRP modulates *Anxa2* and *CARM1* mRNA translation

Our previous results demonstrated that SMN's Tudor domain is required for its translational activity but not its association with polyribosomes. We also determined that DDX5 could be interacting with SMN's Tudor domain. It remains therefore to uncover an additional cofactor that facilitates SMN's interaction with polyribosomes. We used a candidate approach to find a potential protein for this role. FMRP is a known translation regulator of elongating ribosomes, and is known to interact with SMN. We therefore proposed that FMRP is as an additional cofactor to regulate mRNA translation alongside SMN. Hence, we looked at the protein and mRNA levels of CARM1 and Anxa2 in the

absence of FMRP. Anxa2 and CARM1 protein levels were assessed using western blot analysis. We used lysates from immortalized *Fmr1* knockout mouse embryonic fibroblast cells, SV40 large T-antigen embryonic knockout (STEK) (Mazroui et al. 2002) and wild type MEF (mouse embryonic fibroblast) cells as control. The lysates were resolved by SDS-PAGE, transferred on a PVDF membrane, and probed for the presence of endogenous CARM1 and Anxa2 using Western blotting (Figure 8). We observed an increase in CARM1 protein levels in STEKs and a reduction of Anxa2 protein levels. We have previously seen this pattern in our DDX5 and SMN knockdown cells. To confirm that the effect is not a result of upstream regulation, we looked at the mRNA levels of both targets. Total RNAs were isolated from STEK and control MEF cells. CARM1 and Anxa2 mRNA levels were examined using reverse-transcription (RT)-PCR, then ran on an agarose gel. We observed no significant change in their mRNA levels, suggesting that the effects were translational.

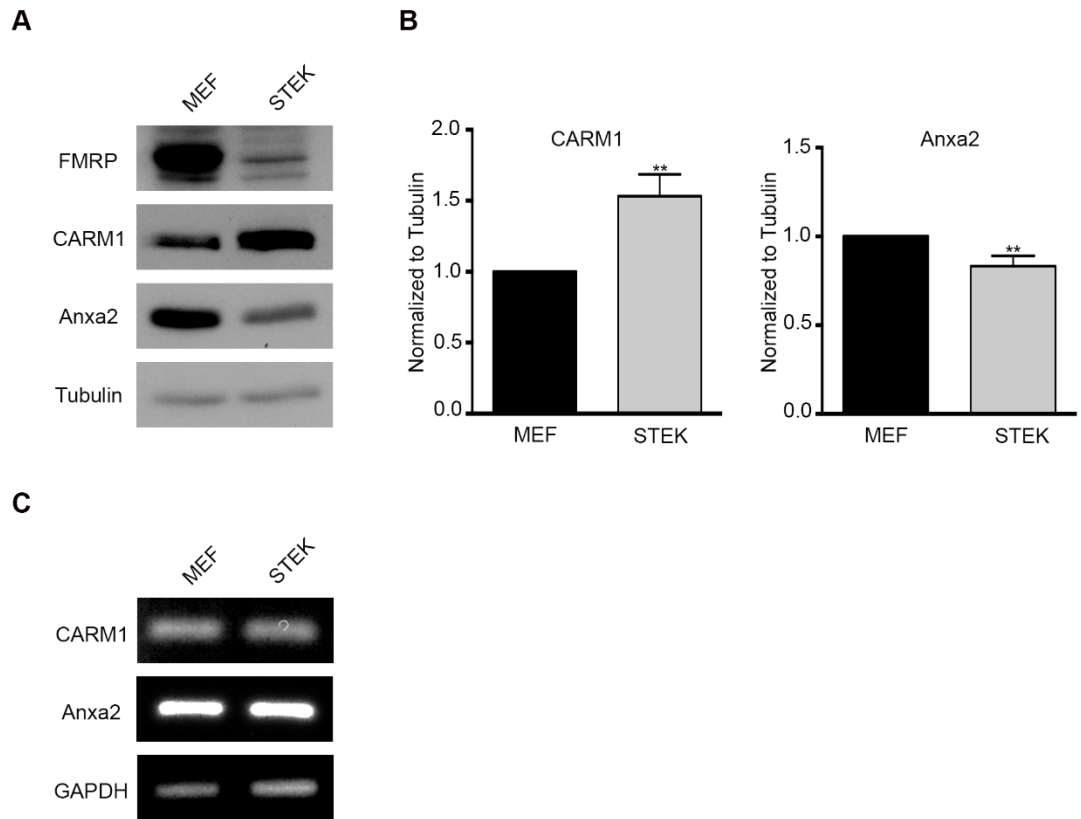


Figure 8: FMRP regulates CARM1 and Anxa2 mRNA translation. CARM1 protein levels were up-regulated in SV40 large T-antigen embryonic knockout cells (STEK) compared to mouse embryonic fibroblasts (MEF). Anxa2 protein levels were down-regulated. Their mRNA levels did not change. (A) Western blot analyses were done to analyze CARM1 and Anxa2 protein levels in the STEKs compared to the control cell line MEF. Lysates from either MEF or STEK cells were run on an SDS-PAGE gel, transferred on a PVDF membrane, then probed for the presence of endogenous CARM1 and Anxa2 using Western blotting. α -Tubulin was used as a loading control. (B) The bar graphs represent CARM1 and Anxa2 protein level quantification for three replicates. The quantification was normalized to Tubulin, then to control. CARM1 expression is represented on the left graph (n=3; statistical significance is indicated with asterisks; p=0.0038; t-test) and Anxa2 on the right (n=3; statistical significance is indicated with asterisks; p=0.0075; t-test). (C) Total RNAs were isolated from MEF and STEK cells. CARM1 and Anxa2 mRNA levels were examined using reverse-transcription (RT)-PCR, then run on an agarose gel.

3.2. . *FMRP enhances SMN and DDX5 localization to polysomal fractions*

Next, we examined if FMRP is required for the localization of SMN and DDX5 to the polyribosomal fractions. SMN co-fractionates with elongating ribosomes, and we demonstrated in previous experiments that DDX5 co-fractionates with SMN within the polysomal fractions and this is dependent on the presence of SMN. We observed that FMRP could have a potential effect on CARM1 and Anxa2 mRNA translation as well. However, it remains unclear how SMN interacts with elongating ribosomes. We therefore suggest that FMRP is a potential co-factor required in the SMN translational regulatory mechanism. To test this, we used sucrose gradient sedimentation with FMRP knockout cells and MEF cells as control. Cytoplasmic extracts from these cell lines were used for polysome profiling and sucrose gradient fractionation. Total proteins were extracted from each fraction, resolved by SDS-PAGE, and subjected to immunoblotting with the indicated antibodies (Figure 9). FMRP is already known to sediment within elongating ribosomes. We observed a significant reduction of SMN and DDX5 protein levels within polysomes in the absence of FMRP. Thus, suggesting that FMRP is required for the recruitment of both SMN and DDX5 to the elongating ribosomes, and FMRP could be one of SMN's co-factor in regulating translation.

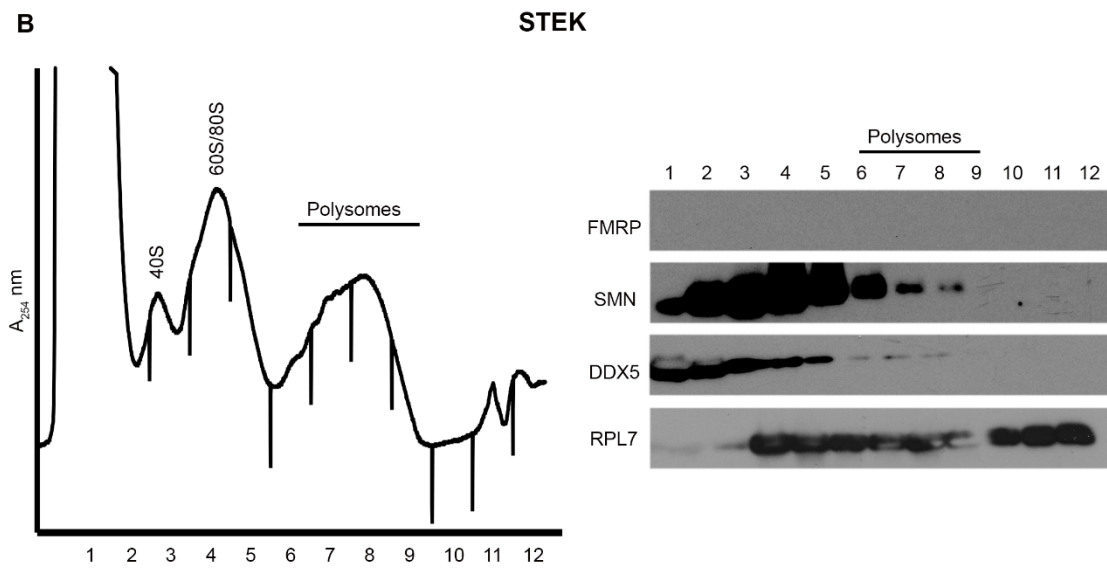
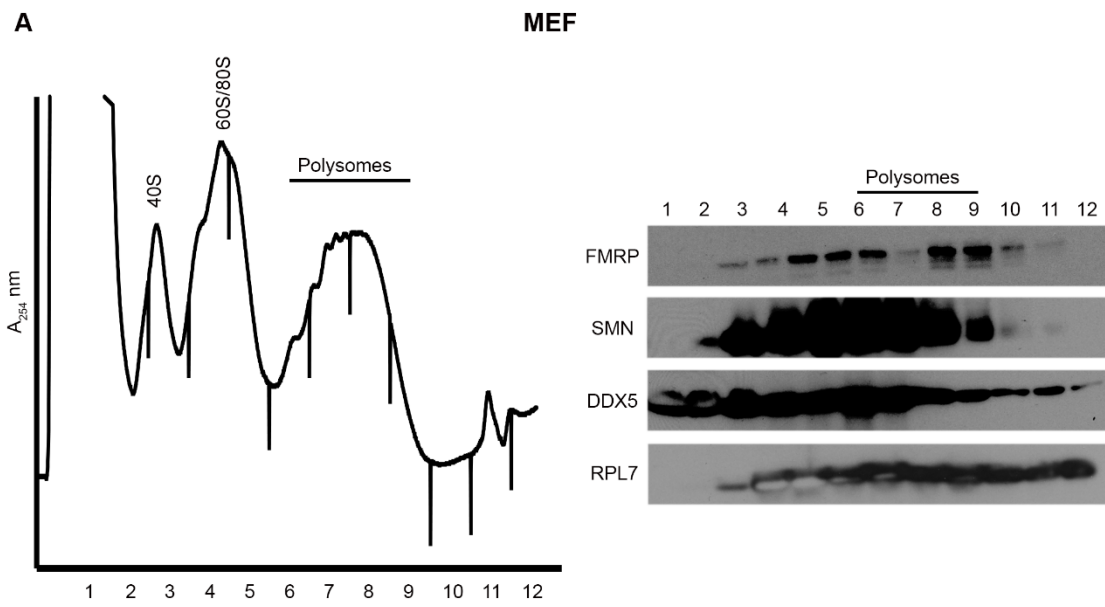


Figure 9: FMRP enhances SMN and DDX5 localization to polysomal fractions. A sucrose density gradient fractionation and polysome profiling was done using cytoplasmic lysates from STEK and MEF cells as control. The left panels represent the polysome profile with all its components: mRNA ribonucleoprotein particles (mRNPs), small ribosomal units (40S), large ribosomal subunits (60S), monosomes (80S), and polyribosomes. Total proteins were extracted from each fraction, resolved by SDS-PAGE, and subjected to immunoblotting with the indicated antibodies, as seen on the right panels. EWS antibody was used as a control for light fractions and RPL7 was used as a control for the polysomal fractions. The fractions from 6 to 9 represent the polysomal fractions. **(A)** Enrichment of DDX5 and SMN protein levels were observed within the polysomal fractions of MEFs. **(B)** A leftward shift of DDX5 and SMN was observed in the STEKs. The experiment was repeated three times.

Chapter 4: Discussion

1. Summary

Spinal muscular atrophy is characterized by selective degeneration of motor neurons caused by insufficient production of the ubiquitous SMN protein. SMN is widely known to regulate many aspects of RNA metabolism. Several studies show its involvement in neuronal RNA granule fate, including assembly, transport along neurites, and translation regulation. Interestingly, our group demonstrated that an SMA-causing mutation (E134K) within SMN completely abolished the capacity of SMN to regulate translation of CARM1 mRNA, which indicates the significance of this function. Our new finding provided evidence that SMN works with additional factors to regulate translation, and supports the hypothesis that SMN is potentially required for the differential regulation of a subset of mRNAs. First, we uncovered that SMN interacts with a novel interacting protein, DDX5. We also reported in this study a novel function for DDX5 in translation regulation, which is previously known for its role in miRNA processing. We next demonstrated that FMRP is required for SMN's interaction with elongating ribosomes, and could work together with SMN to recruit DDX5. Overall, we propose that SMN, DDX5, and FMRP work together to regulate mRNA translation.

2. SMN is potentially required for differential regulation of some mRNA expressions

In previous studies SMN depletion did not seem to have an effect on global translation (Sanchez et al. 2013; Thandapani et al. 2015); This strongly suggests that SMN regulates

only a subset of mRNAs. The repression of the CARM1 mRNA expression was the first target observed, and this regulation is dependent on its coding sequence. Interestingly, our group showed that the CARM1 expression needed to be down-regulated to allow cell cycle exit and induction of the full differentiation program. Subsequent studies suggested that repressing CARM1 mRNA translation could be part of a negative feedback loop regulating other mechanisms in motor neurons (Hubers et al. 2011). Furthermore, in this study I show that FMRP is required for SMN's association with elongating ribosomes. FMRP is a well-known factor in regulating mRNA translation. Using ribosomal foot-printing technique, FMRP was determined to regulate mRNA translation by stalling elongating ribosomes (Darnell et al. 2011). In SMA conditions, some proteins are either down-regulated or up-regulated. Studies show that down-regulation of Plastin3 in SMA might involve a translational mechanism (Hao et al. 2012). Overall, this suggests that SMN could be required for differential regulation of some mRNA translation, which may depend on cellular cues.

It was therefore crucial to identify additional mRNA targets that may be regulated by SMN. Using a candidate approach, we investigated Anxa2 as it is known to associate with SMN along neuronal axons (Rage et al. 2013). We also had preliminary results that indicated that SMN could have an activating role on Anxa2. Interestingly, Anxa2 mRNA contains G-rich structures upstream from its 3'UTR which have been identified as a potential cis-acting element in regulating mRNA translation within elongating ribosomes (Rihan et al. 2017). Even though the Anxa2 protein function is not well understood in motor neurons, it plays a key role in many processes, including membrane organization, ion channel conductance, and F-actin cytoskeleton linkage to the plasma membrane (reviewed in (Bharadwaj et al.

2013)). However, fluorescent in-situ hybridization (FISH) labeling of Anxa2 mRNA was used to demonstrate its co-localization with SMN along neuronal axons (Rage et al. 2013). I therefore hypothesized that a deficiency in the potential proteins that work alongside SMN to regulate translation would change the expression of Anxa2 mRNAs. In this work, discussed in more details in the following sections, we argue that DDX5 and FMRP work together with SMN to regulate mRNA translation. We show that DDX5-deficient and FMRP-deficient cells exhibit a down-regulation of Anxa2 protein expressions. However, we did not take in consideration protein stability/turnover during our study. I therefore propose in future studies to use mRNA isolated from sucrose gradient fractions to look at CARM1 and Anxa2 mRNA shift in control and knockdown cells, and potentially a pulse chase experiment to look at the half-life of their respective proteins. On the other side, preliminary results revealed that Anxa2 could be an additional mRNA targeted by SMN for translational regulation. This suggests that SMN could have opposite effects on Anxa2 and CARM1 mRNA regulation. Indeed, we suggest that SMN could be stimulating Anxa2 mRNA expression in regular conditions. Further study is required to explore this possibility and to understand Anxa2's cellular functions in neurons. Subsequently, we will be able to identify a new regulatory pathway involving Anxa2, allowing us to better understand how a down-regulation of this protein could potentially lead to an SMA phenotype.

Overall, we suggest that SMN could have either a repressive or stimulating effect depending on specific mRNA targets. We propose that this regulatory effect may be dependent on which set of co-factor(s) is recruited. For instance, a study demonstrated that SMN, alongside TDRD3, regulates translation by recruiting the RNA-binding protein Aven to elongating ribosomes (Thandapani et al. 2015), which in turn recruits an RNA

helicase that seem to stimulate the translation of specific mRNA targets. The specific cis-regulatory elements present on a given mRNA could also serve as a tag to whether it will be either repressed or stimulated.

3. DDX5 interacts with the Tudor domain of SMN

Although there is growing evidence that SMN could be involved in translation regulation of a subset of mRNAs, the precise molecular mechanism remains unclear. Using ultracentrifugation through sucrose density gradients, our group has previously demonstrated that SMN associates with polyribosomes and represses CARM1 mRNA expression. In addition, an SMA-causing Tudor domain mutation completely abrogates the repressive activity of SMN, while deletions in either the N-terminal or C-terminal domains of the protein did not have any effect. SMN's Tudor domain is responsible for protein-protein interactions, which has prompted speculation that SMN might be regulating translation in association with another factor. We therefore proceeded to purify and identify the specific components associated with SMN in polyribosomes. To identify these components, a previous lab member performed a proteomic screen by pulling down GFP-SMN and its interacting proteins from polysome-containing sucrose gradient fractions. Several interesting putative proteins were revealed. In this study, we report a new interaction between SMN and DDX5. First, we confirmed the endogenous interaction of SMN with DDX5 in motor neurons using co-immunoprecipitation experiments. Next, another student in our group characterized the biochemical interaction of DDX5 with SMN. Interestingly, functional SMN's Tudor domain was seen to be necessary for this interaction, strongly suggesting that DDX5 could be one of the proteins that interact with

SMN via the Tudor domain to regulate translation. This also suggested a previously undiscovered function for DDX5 in the translational machinery.

4. DDX5 could have a novel function in the Cellular Translation Machinery

After the biochemical characterization of the novel interaction of SMN with DDX5, we proceeded to further explore DDX5's involvement within the translational machinery. Ultracentrifugation through sucrose density gradients from mouse brain tissue revealed that DDX5 was present, alongside SMN, in polyribosome-containing fractions. This result suggests that DDX5 is associated with the translational machinery. In support of this hypothesis, a study demonstrated that the loss of another RNA-binding protein (JAKMIP1) *in vivo* disrupts the polyribosome association profile of DDX5 (Berg et al. 2015). I tested whether or not SMN is important for DDX5's association with polyribosomes and found that DDX5 levels were reduced in polysomal fractions following reduced levels of SMN in the same fractions. Interestingly, we observed that the sedimentation profile of DDX5 moved towards lighter fractions under these conditions.

These results prompted us to hypothesize that DDX5 regulates the expression of CARM1 mRNA in a similar fashion as SMN. In DDX5-deficient cells, a student previously observed that CARM1 protein levels were up-regulated whereas its mRNA levels remained unchanged. I observed reduced Anxa2 protein levels in DDX5-deficient cells. Our analysis clearly indicated that DDX5 somehow contributes to the differential regulation of Anxa2 and CARM1 expressions. However, it remains important to look at the mRNA levels as well to speculate that the regulation is the translational level. On the other side, DDX5 is known to be involved in the nuclear maturation of miRNAs which silence mRNA

expression via 3'UTR association. For instance, a study performed an miRNA profiling using basal breast cancer cells revealed that DDX5 affect the maturation of a subset of miRNAs (Wang et al. 2012). They demonstrated that of these miRNAs, forty of them are downregulated in DDX5 knockdown cells, including the miRNA miR-15a. Another study showed that this miRNA, miR-15a, regulates the posttranscriptional expression of CARM1, by targeting its 3'UTR (Liu et al. 2014). Nevertheless, these are two independent studies with different conditions. Since we demonstrated that DDX5 repressed CARM1 protein expression in our cells, we proceeded to eliminate the potential regulatory mechanism via miRNA at CARM1's 3'UTR. We employed a luciferase reporter assay (as has been previously established in our laboratory). The DDX5 expression was modulated in Cells expressing a Firefly Luciferase plasmid harboring CARM1 3'UTR. This assay revealed that the repression might not involve 3'UTRs, thus, it is most likely that the coding sequence of CARM1 is required to allow regulation by DDX5. However, this experiment has its limitations, and a positive control will be needed to confirm these speculations.

Altogether these results demonstrate and support a potential novel function for DDX5 in regulating translation. Further study will also be required to gain insight into DDX5's possible function in neurons. Even though DDX5 was not directly linked to neurodegeneration, it is shown to be implicated in tau splicing and in association with a novel regulator of neuronal translation (JAKMIP1) (Kar et al. 2011; Berg et al. 2015).

5. FMRP enhances SMN's association with the translational machinery

An SMA-causing mutation completely abolished the repression activity of SMN, while the same mutant remained associated to polyribosomes. In addition, evidence demonstrating

that SMN is capable of binding directly with RNA remains elusive (Lorson & Androphy 1998). We therefore hypothesize that SMN interacts with an additional cofactor to regulate translation, and this interaction is mediated through a domain that is not the Tudor domain. We used a candidate approach to find a potential protein for this role. FMRP is a known translation regulator of elongating ribosomes, and is known to interact with SMN independently of the Tudor domain. FMRP associates through coding sequences with some of its mRNA targets, leading to translational repression via reversibly stalling elongating ribosomes (Stefani et al. 2004; Darnell et al. 2011). In addition, DDX5 is a member of the FMRP-kinesin transport RNP granule, and co-immunoprecipitates with FMRP in vitro and in vivo (Kanai et al. 2004). The binding of DDX5 to FMRP depends on single-stranded RNA, suggesting that the interaction is within an RNA-protein complex (most likely within an mRNP complex) (Berg et al. 2015). We suggested that FMRP could be an additional co-factor to regulate mRNA translation with SMN. Using ultracentrifugation through sucrose density gradients, we demonstrated that FMRP enhances the localization of SMN and DDX5 to the polyribosomal fractions. In addition, we demonstrated an increase in CARM1 protein levels in STEKs and a reduction of Anxa2 protein levels in FMRP-deficient cells. Nevertheless, these changes in protein levels could also be caused by protein degradation/turnover. I therefore propose in future studies to use mRNA isolated from sucrose gradient fractions to look at CARM1 and Anxa2 mRNA shift in control and knockdown cells, and to support further these experiments to do a pulse chase experiment to look at the half-life of their respective proteins.

Overall, we propose that SMN, DDX5, and FMRP work together to regulate translation of a subset of mRNAs. However, evidence showing the direct interaction of these factors

within elongating ribosomes is not investigated yet. To support this hypothesis, I propose to use polyribosomal sucrose gradient fraction for immunoprecipitation experiments using either of the above proteins as a pull-down protein.

6. Conclusion

Together, our observations suggested that SMN, with its interacting proteins FMRP and DDX5, could be playing an essential role in differential regulation of mRNA translation. We therefore propose a revised molecular model in which SMN could be regulating mRNA translation with FMRP and DDX5 (**Figure 10**). Translation regulation is crucial for proper mRNA transport and local translation in subcellular compartments, such as axonal growth cones in neurons, and its aberrance could lead to degenerative disorders such as SMA.

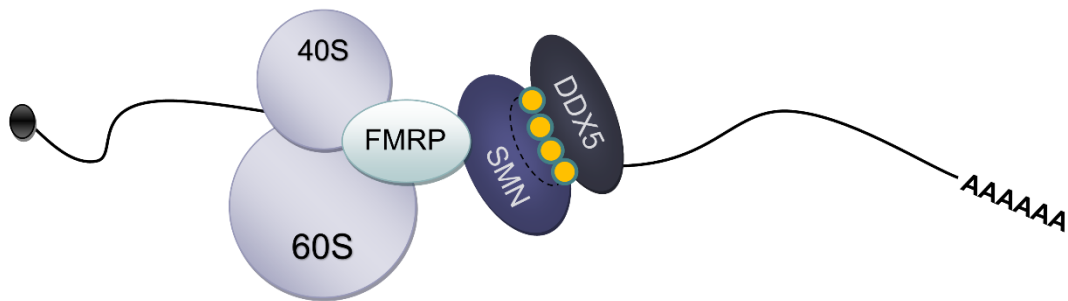


Figure 10: Proposed and revised molecular model in which SMN regulates mRNA translation with FMRP and DDX5.

7. Future directions

7.1. Determine whether SMN's Tudor domain interacts with DDX5's RGG motif

We reported here that SMN's Tudor domain is required for the interaction between SMN and DDX5. The Tudor domain is known to interact with several proteins by sensing methylated arginine residues. It is known that DDX5 contains an RGS-RGG-RGG domain

that could potentially be methylated by a protein arginine methyltransferase. Therefore, we speculate that SMN could interact with DDX5 through arginine methylation; subsequently, DDX5's RGS-RGG-R₅₀₂GG domain could be necessary for this interaction (Ford et al. 1988). To test the hypothesis, we obtained plasmid constructs containing myc-tagged DDX5. The plasmid was used as a template to delete the RGS-RGG-RGG domain within DDX5 by mutagenesis. We also used the same method to mutate one of the most probable arginines to be methylated, residue 502. The arginine (R) was mutated to a lysine (K) in order to maintain the positive charge of the arginine residue. To determine whether SMN's Tudor domain interacts with DDX5's RGG motif, these constructs could be expressed in cells before using them for a co-immunoprecipitation experiment targeting the myc tag. The outcomes of this study will allow us to better understand the interaction of SMN with its novel interacting protein, DDX5.

1.1. Investigate other mRNA targets that are translationally regulated by SMN

SMN seem to have a translational regulation effect on a subset of mRNAs. Using a candidate approach we studied and proposed two mRNA targets that could be regulated by SMN, CARM1 and Anxa2. Nonetheless, it still remains to identify other potential mRNA targets regulated by this translational mechanism. mRNAs associated with polyribosomes could be identified using POL-Seq technique with SMN-deficient cells compared to control cells. This approach will allow us to identify the various mRNAs that are misregulated in SMA conditions. Subsequently, we will understand the effects of impaired translation in SMA on the individual mRNAs.

Appendix I

SwissProt	Description
SMN core complex	
sp Q9CQQ4 GEMI2_MOUSE	Survival of motor neuron protein-interacting protein 1 OS=Mus musculus GN=Sip1
Ribosomal Proteins	
sp Q9CXW4 RL11_MOUSE	60S ribosomal protein L11 OS=Mus musculus GN=Rpl11
sp P53026 RL10A_MOUSE	60S ribosomal protein L10a OS=Mus musculus GN=Rpl10a
sp P14869 RLA0_MOUSE	60S acidic ribosomal protein P0 OS=Mus musculus GN=Rplp0
sp P99027 RLA2_MOUSE	60S acidic ribosomal protein P2 OS=Mus musculus GN=Rplp2
sp P14206 RSSA_MOUSE	40S ribosomal protein SA OS=Mus musculus GN=Rpsa
sp P62908 RS3_MOUSE	40S ribosomal protein S3 OS=Mus musculus GN=Rps3
sp P62270 RS18_MOUSE	40S ribosomal protein S18 OS=Mus musculus GN=Rps18
Translation Factors/Regulators	
sp P62960 YBOX1_MOUSE	Nuclease-sensitive element-binding protein 1 OS=Mus musculus GN=Ybx1
sp P10126 EF1A1_MOUSE	Elongation factor 1-alpha 1 OS=Mus musculus GN=Eef1a1
sp P29341 PABP1_MOUSE	Polyadenylate-binding protein 1 OS=Mus musculus GN=Pabpc1
sp Q61937 NPM_MOUSE	Nucleophosmin OS=Mus musculus GN=Npm1
sp Q9ESJ0 XPO4_MOUSE	Exportin-4 OS=Mus musculus GN=Xpo4
Microprocessor Complex	
sp Q9EQM6 DGCR8_MOUSE	Microprocessor complex subunit DGCR8 OS=Mus musculus GN=Dgcr8
sp Q61656 DDX5_MOUSE	Probable ATP-dependent RNA helicase DDX5 OS=Mus musculus GN=Ddx5
Other	
sp Q9CWL8 CTBL1_MOUSE	Beta-catenin-like protein 1 OS=Mus musculus GN=Ctnnb1
sp Q99N48 SYTL3_MOUSE	Synaptotagmin-like protein 3 OS=Mus musculus GN=Sytl3
sp P97460 NPAS2_MOUSE	Neuronal PAS domain-containing protein 2 OS=Mus musculus GN=Npas2
sp B2RUP2 UN13D_MOUSE	Protein unc-13 homolog D OS=Mus musculus GN=Unc13d

Table 1: Potential SMN's interacting proteins. A proteomic screen on SMN-interacting proteins within polyribosomes were identified using a pulling down of GFP-SMN and its interacting proteins from polyribosome containing sucrose gradient fractions. Several putative proteins were reveal by the proteomic screen. DDX5 is one of the putative interacting proteins identified (Red box), and investigated in this study. (Experiment done by Dr Gabriel Sanchez, former postdoctoral follow in Dr Jocelyn Côté's lab)

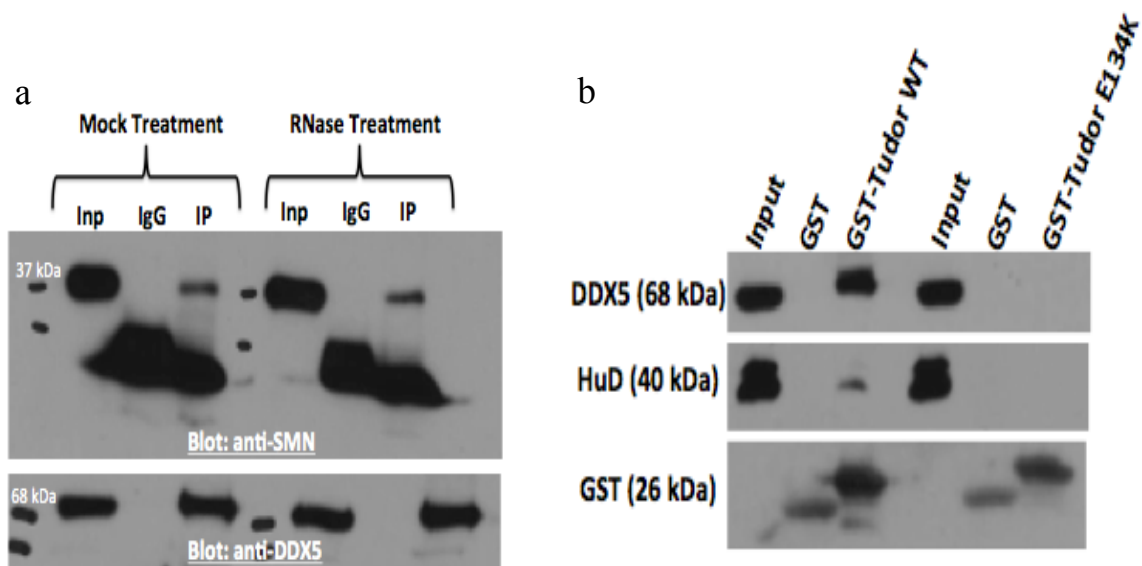


Figure 1: Endogenous interaction of SMN with DDX5 and SMN's Tudor domain interaction with DDX5. (a) Co-immunoprecipitation experiments were used to confirm the interaction between DDX5 and SMN, which is consistent with the proteomic screen. The same experiment was performed using RNase treatment and the interaction remained present. This suggests that the interaction is independent of RNA. (b) Using GST pull downs, the previous student next determined that the interaction is mediated through SMN's Tudor domain. The interaction is abolished using the purified GST-tagged Tudor domain of SMN harboring the severe type I SMA mutation (E134K). (Experiments done by Camille Tremblay Laganière, former summer/honours student in Dr Jocelyn Côté's lab)

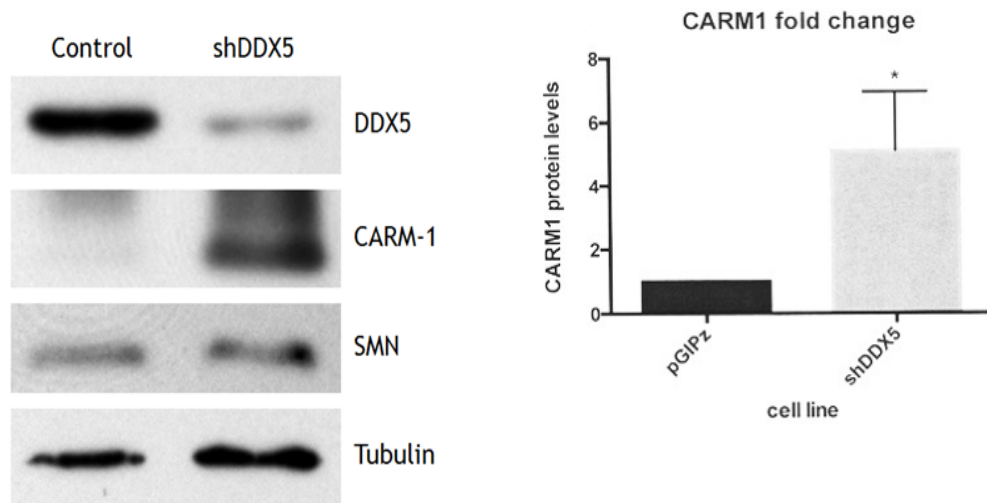


Figure 2: CARM1 proteins levels in DDX5-deficient cells. Reduces CARM1 protein levels DDX5-deficient cells. (Experiments done by Camille Tremblay Laganière, former summer/honours student in Dr Jocelyn Côté's lab)

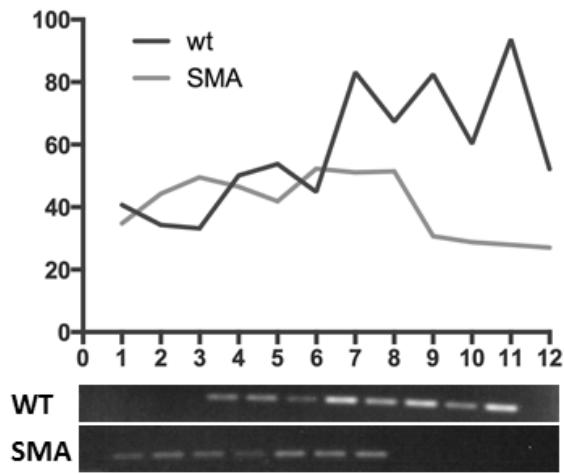


Figure 3: SMN could have a stimulating effect on Anxa2 mRNA expression. we used sucrose gradient sedimentation with SMA mouse brain tissues and with control mice. Anxa2 mRNA levels were examined by reverse-transcription (RT)-PCR using isolated mRNA from each of the gradient fractions. We observe a shift towards the lighter fractions in SMA-like condition compared to control. Preliminary result performed once.

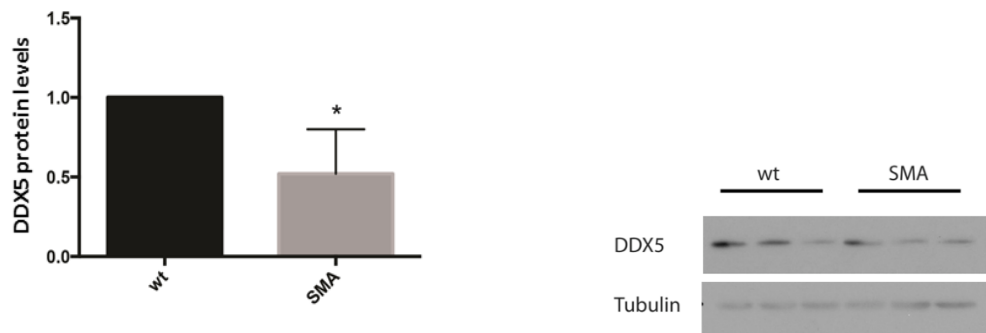
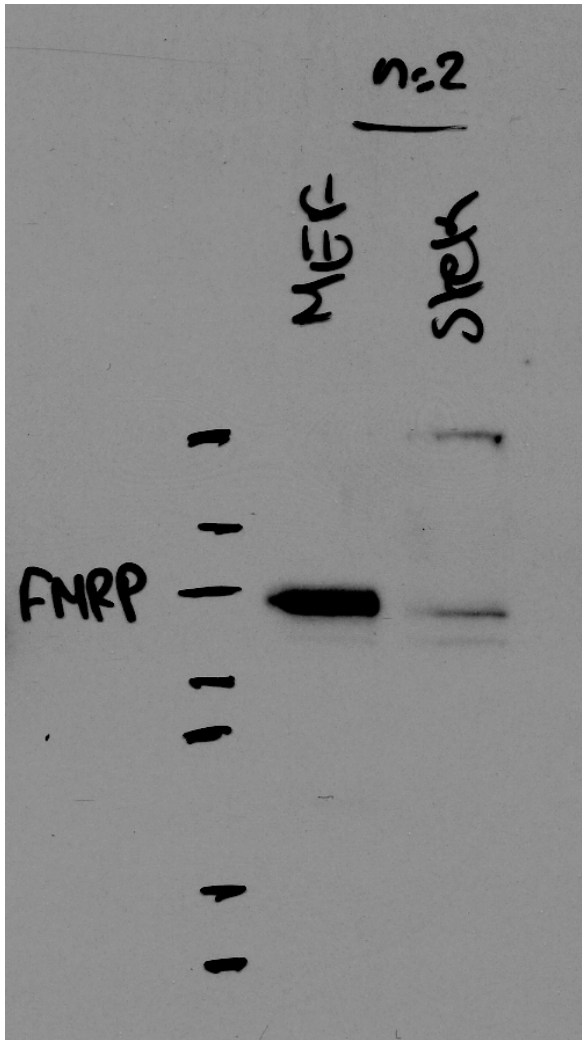


Figure 4: Down-regulation of cytoplasmic DDX5 protein levels in SMA mice brains. Cytoplasmic DDX5 protein expression levels in mouse brain postnatal day 4. Total protein

from mouse brain tissues cytoplasmic lysates, of either wild type mice (WT) or mice with SMA type 1 phenotype, were resolved by SDS-PAGE, and subjected to immunoblotting with the indicated antibodies. DDX5 expression is represented on the left graph (n=3; statistical significance is indicated with asterisks; p=0.0416; t-test).



Raw data for FMRP knockdown in STEK cells compared to MEF. It seems there is residues of FMRP in STEK cells. This could be explained by either a lack of specificity of the antibody, or cross contamination of the STEK cells with MEF cells.

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